Operating Instructions

for ZetaView®

Nanoparticle Tracking Analyzers

PMX-120, PMX-220, PMX-420

Particle Metrix GmbH

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Imprint

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About these Operating Instructions

These Operating Instructions apply to the ZetaView® Nanoparticle Tracking Analyzers PMX-120 (mono-laser instrument), PMX-220 (TWIN) and PMX-420 (QUATT) controlled with ZetaView® **software version 8.05.16_SP2 or later.** However, some functions described in this document only relate to a specific model or equipment variants. This is noted accordingly.

The software functionality described in this document is also valid for ZetaView® x30 series instruments. The hardware related functions of the ZetaView® x30 series are not covered in these Operating Instructions.

ZetaView® instruments with an older software version can also be operated using these Operating Instructions. However, it should be noted that ZetaView® devices with older software versions may not offer the full range of functions described and explained in this document.

The screenshots shown in these Operating Instructions may show the graphical user interface of different ZetaView® software versions. For technical reasons, ZetaView® instruments with different equipment variants (mono-laser), TWIN and QUATT devices were used in the design of these Operating Instructions. Some screenshots therefore do not always show all function buttons or software information. However, each screenshot shows all the information required for the corresponding chapter.

Content

1 Fundamental safety instructions

1.1 Obligations, liability

1.1.1 Observe details stated in the Operating Instructions

These Operating Instructions contain important precautions in order to operate the Particle Metrix ZetaView® Nanoparticle Tracking Analyzer (NTA) instruments models PMX-120 (mono-laser instrument), PMX-220 (TWIN) and PMX-420 (QUATT) in compliance with the pertinent safety regulations. Knowledge of and compliance with these safety precautions and the pertinent safety regulations are basic prerequisites to ensure a safe and trouble-free operation of the PMX instruments.

Moreover, the pertinent regulations and prerequisites applying to accident prevention at the place of installation shall be observed.

1.1.2 Obligations of the ZetaView® owner

The guaranteed properties and warranty shall only be applicable, if the following points are complied with:

- The ZetaView® instrument must be exclusively operated by persons trained by PARTICLE METRIX GmbH or its representative.
- The ZetaView® instrument may only be used in accordance with the regulations.
- All specifications defined by PARTICLE METRIX GmbH with regard to handling the ZetaView® must be observed. This term not only refers to operation, but also to maintenance and storage of the device.
- Maintenance and repairs may only be carried out by persons authorised explicitly by PARTICLE METRIX GmbH or its distributor to perform such tasks.
- If any parts of the ZetaView® instrument are replaced by other than original parts supplied by PARTICLE METRIX GmbH, this shall lead to a direct loss of all claims which can be raised vis-a-vis PARTICLE METRIX GmbH.
- Safety-relevant disturbances must be eliminated immediately and prior to the next operation of the ZetaView® instrument.
- If the ZetaView® instrument reflects any functional disturbances or visible damages, or if any damage is assumed, e.g. in the event of loose parts within the instrument, the device shall be put out of operation.

1.1.3 Warranty and liability

The ZetaView® instrument has been manufactured in compliance with the requirements stipulated in ISO 9001 and CE, tested and shipped in a proper condition with regard to safety engineering requirements. The owner and user are responsible for ensuring that all precautions listed in these Operating Instructions and the regulations applying to the installation site of the equipment are observed. Our Standard Sales and Delivery Terms which shall be available to the Owner at the latest at the point of undersigning the purchase agreement shall also be applicable. Warranty and liability claims shall be excluded in the event of damage to persons or property, if at least one of the following points is applicable:

- Use of the ZetaView® contrary to the regulations
- Non-authorised intervention into the housing, damage to the sealing on the housing screws
- Inappropriate installation, operation, maintenance and repair of the ZetaView® instrument
- Operation of the ZetaView® instrument with defective safety equipment or featuring damages which may affect safety
- Operation of the ZetaView® instrument without the prescribed or with non-functional safety installations
- Non-compliance with precautions stated in these Operating Instructions with regard to operation, storage, transport and maintenance of the ZetaView® instrument
- Any modification of the equipment is against the delivery conditions
- Damage as a result of intervention by a foreign substance, acts of God or catastrophes
- Faulty maintenance

If the equipment is sent to PARTICLE METRIX GmbH for repairs, always ensure that the appropriate measuring Cell assembly is enclosed in the shipment. The warranty period is 1 year from the date of delivery. PARTICLE METRIX GmbH shall not warrant under any circumstances for warranty damage exceeding the purchase price of the equipment. PARTICLE METRIX GmbH is only obliged within the scope of warranty to replace or repair parts classified as defective within the scope of the examination performed by PARTICLE METRIX GmbH at its own discretion, if these parts were returned within the given time schedule. Parts which are modified or damaged as a result of improper handling without written approval by PARTICLE METRIX GmbH, shall be exempt from warranty. Furthermore, PARTICLE METRIX shall not assume any further obligations in connection with selling the ZetaView® instrument.

1.1.4 Exemption from liability

The contents of these Operating Instructions have been verified. It is nevertheless impossible to exclude deviations to the device or errors. PARTICLE METRIX GmbH therefore does not assume any warranty for the correctness of the information provided in these Operating Instructions. If appropriate, modifications will be integrated into a following version.

1.2 Use according to regulations

The ZetaView® instrument was developed to determine the electrophoretic mobility, zeta potential and particle size distribution in aqueous solutions. Operation shall only be permitted in interior rooms. There is no protection against dust and spray water. Any deviating use or noncompliance with the Operating Instructions shall be deemed to be a use which is contrary to the agreed terms and shall lead to non-liability on the part of PARTICLE METRIX GmbH.

1.3 Use contrary to regulations

The following ZetaView® instrument shall be deemed to be use of the equipment contrary to the regulations (misuse):

- Each and any application deviating from the use of the equipment in accordance with the above-mentioned terms
- Non-compliance with instructions and safety precautions stated in these Operating Instructions
- Non-compliance with safety regulations
- Operating the equipment despite safety-relevant disturbances
- Operating the equipment despite functional disorder or visible damage
- Any modification of the equipment as against the delivery status

1.4 Qualification of staff

Persons who have been trained by PARTICLE METRIX GmbH or by an authorized representative shall exclusively operate the ZetaView® instrument.

1.5 Safety instructions

In handling the ZetaView® instrument, the following points must be observed:

- Only trained staff may put the device into operation or operate the equipment.
- Before performing any repair within the device, ensure that it is disconnected from the power supply.
- Only original parts supplied by PARTICLE METRIX GmbH may be used for maintenance or repairs.
- Only trained staff may carry out any maintenance operations, in so far as these are described in these Operating Instructions.
- PARTICLE METRIX GmbH or a service company, which has been explicitly authorised to perform such operations, may only execute any maintenance or repair operations, which are not described in these Operating Instructions.

1.6 Provision of the safety instructions

These Operating Instructions, as well as any regulations applicable at the place of installation shall be stored in direct vicinity of the equipment and must be observed. If the documents mentioned become illegible for example due to damage, they must be replaced.

1.7 Packaging & Transport

In the case the ZetaView® should be transported and installed at alternating locations, the following must be observed:

For truck, train, sea and air freight or shipment by mail an appropriate sturdy outer packaging is required.

- If the device temperature after transportation is more than 10°C below the ambient temperature at the place of installation, it is necessary to ensure that the device reaches room temperature before putting it into operation.
- For transportation it is necessary to clean the measuring cell and fluid connectors (both ends) before packing into the extra box.
- Some smaller parts may be packed with the ZetaView®, but have to be properly wrapped and stowed in a way to avoid shifting around during transport and by this damaging the ZetaView®
- In order to prevent harm to service personnel, remove all residual fluids from the fluid containers and the ZetaView®. If the fluids are considered to be dangerous, e.g. biohazard, the parts in contact with the liquid have to be sterilized properly and rinsed with sufficient amount of distilled water afterwards

- In case of shipping the ZetaView® instrument, eject the Cell assembly and store it in the designated box to prevent damage to the instrument.
- Before shipping, take 3 (or 4, depending on model) M6x25 mm screws and fix at the bottom of ZetaView® instrument (hand tight). Take a size 5 Allen wrench to carefully tighten the screws. Do not apply force.

2 Product description

2.1 Basic principles

The Particle Metrix ZetaView® instrument is a Nanoparticle Tracking Analyzer (NTA) that captures the Brownian motion of each individual particle in a video. Based on the different diffusion movements of large and small particles in the surrounding liquid, the hydrodynamic diameter of the particles is determined. The NTA principle is based on the Stokes-Einstein relationship with which a diffusion coefficient is calculated for each individual particle.

$$Dt = \frac{< \overline{x,y}^2 >}{4} \qquad D = \frac{k_B T}{6\pi\eta r}$$

D = Diffusion coefficient; k_B = Boltzmann constant; T = Temperature;
η = Viscosity of medium; $< \overline{x,y}^2 >$ = Mean square displacement; r = radius

Figure 2-1: Stokes-Einstein relationship

In addition to technical nanoparticles, biological nanoparticles such as extracellular vesicles (EVs), exosomes, viruses, or virus-like particles are a rapidly growing area of research in the life science and nanomedical field. The rapid in vitro measurement of multiple physical parameters such as size, concentration, phenotype characteristics, and surface charge is the special feature of the Particle Metrix ZetaView®. The charge state of the particle surface (zeta potential) can be measured via the movement of the particles in an applied electric field (electrophoresis).

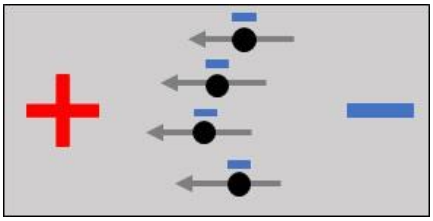
Electrophoresis

As the schematics below shows, in a micro-electrophoresis configuration, the particles in a suspension move within an applied electrical field. Depending on the polarity of the particles they either move to the anode or cathode. The electrophoretical mobility of particles reflects the repulsive force of the ionic charge on the surface of particles. The Smoluchowski equation is one of the formulas relating measured electrophoretic mobility μ_e [µm/s/V/cm] to the zeta potential ZP [mV]. Smoluchowski applies for aqueous media with conductivity < 1 mS/cm and particle sizes around 1µm.

The zeta potential of a sample is determined in the ZetaView® instrument by measuring the electrophoretic mobility (Mb) of particle migration in an electric field. The electrical conductivity of the buffer in which the particles are suspended plays a decisive role as well. The electrophoretic mobility μ_e of each individual particle is calculated via the particle migration v in the applied electric field *E*. The zeta potential, that is considered to be the surface charge of each individual particle, is then calculated using the Helmholtz-Smoluchowski equation.

$$\mu_e = \frac{v}{E}$$

$$\zeta = \frac{4\pi\eta}{\varepsilon} f(\kappa a) \cdot \mu_e$$



μ_e=Electrophoretic mobility; v = Velocity of particle in E-field; E = Electrical field
ζ = Zeta potential; η = Viscosity of medium; ε = Dielectric constant; $f(\kappa a)$ = Debye function

Figure 2-2: Electrophoretic mobility (left) and Helmholtz-Smoluchowski equation (middle).

To capture the movement of particles in the ZetaView®, a laser scattering microscope with video camera is used. From the video data a velocity distribution of the particles is derived. By knowing the electric field strength, the electrophoretic mobility and zeta potential can be calculated. Laser light is directed into the focal point of a microscope lens. The intensity of this is collected under 90 degrees. The scattered light intensity is such, that 50 nm sized polystyrene latex particles or 20 nm gold nanoparticles are still visible. Upper and lower measurement limits depend on the material being measured, the upper limit being migration, the lower limit the scattering efficiency of the particles. The uppermost limit is 50 µm (fibers staying suspended).

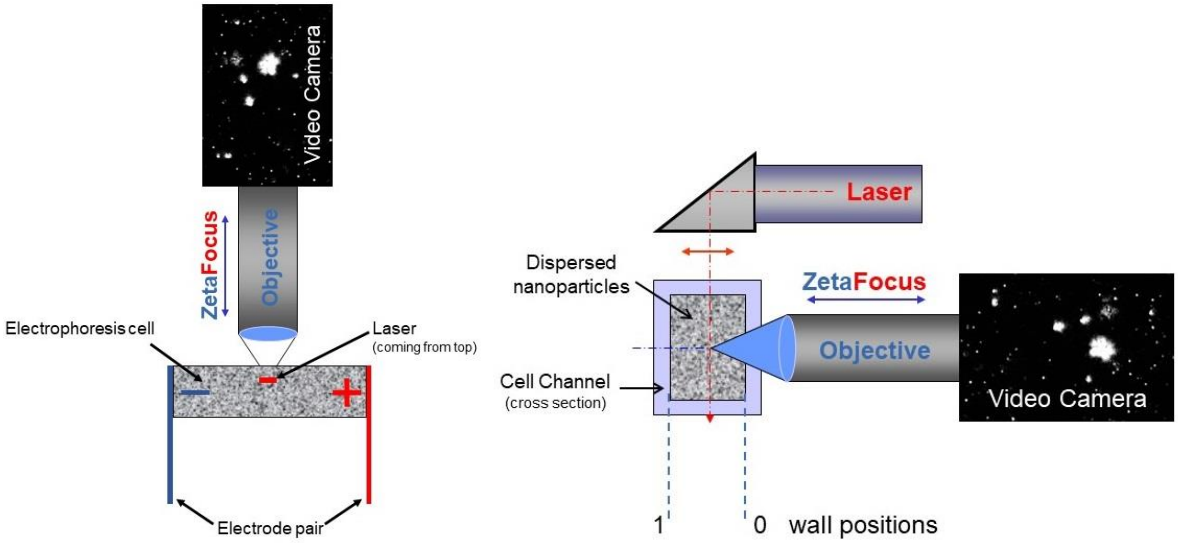


Figure 2-3: Left: Electrophoretic setup; Right: basic optical setup of the ZetaView® instrument in the vertical plane.

Another cross section in a vertical plane shows the lay-out inside the ZetaView®. The microscope axis is horizontal and focused into the cell channel. The laser beam is horizontal in the beginning, then bent into a vertical direction. Both, laser and microscope focus are brought into coincidence by moving their translation stage support in forward and backward direction. The laser light curtain serves as illumination of the focal plane of the microscope. The particles suspended in this laser curtain scatter light, which can be observed by the microscope at 90 degrees. With this set-up particle sizes below the diffraction limit of conventional microscopes (1µm) can be observed. This can be compared to the observation of stars. Most of the stars could not be observed directly.

But as they emit much light, we can see them. However, when there is too much background from other scattering sources like water vapor, the visibility of the stellar objects is limited. In a cell channel, background scattering may emanate from dirty cell walls. In a clean cell situation, the lower size limit is at 50 nm (polystyrene latex particles). It is important to understand, that laser and microscope can be moved simultaneously through the cell in the direction of the microscope axis whilst staying in a coincident autofocus position. This controlled movement is the key for being able to automatically measure the inner thickness of the cell channel from wall 0 (near to the microscope) to the remote inner wall 1. Through this "Zetafocus" mode the competitive electro-osmosis effect can be kept under control without demanding the operator to do any alignment.

Simultaneous electro-osmosis and electrophoresis: The negatively charged cell wall causes an electro-osmosis effect. It occurs, when an electric field is applied causing the liquid to flow into one direction at the cell walls and into the opposite direction in the middle of the cell carrying the particles with it. Between the "negative" and the "positive" flow domains the electro-osmosis velocity is zero. It is therefore advantageous to measure the electrophoretic mobility at this "stationary layer". In the ZetaView® this position is controlled automatically.

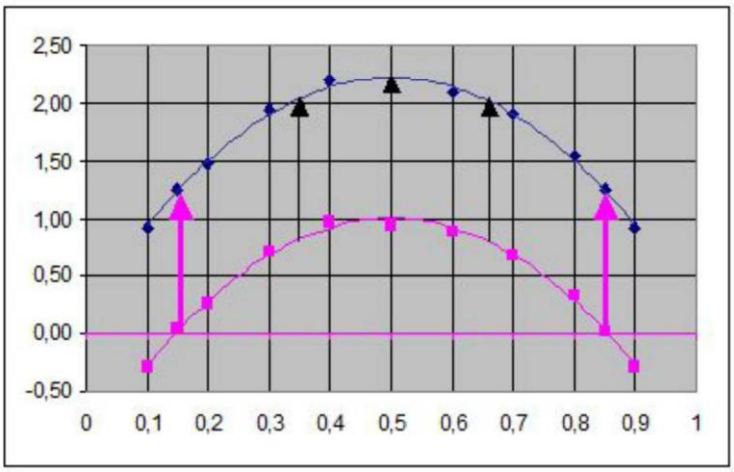


Figure 2-4: Velocity profile inside the cell with applied field. X-axis: 0 = inner wall next to the microscope objective; 1 = opposite inner wall, Pink curve = uncharged particles carried by the liquid revealing the plain electro-osmosis effect; Blue curve = charged particles with electrophoretic mobility of 1.25 µm/sec/V/cm. The curve shows the addition of both effects.

With uncharged particles the pure electro-osmosis effect is revealed (purple curve above). For charged particles the electrophoretic movement adds to electro-osmosis over the whole profile (blue curve). To researchers the flow profile and its curvature can provide valuable additional information on the ionic coating of the walls. The ZetaView® software simply uses the profile for the automatic alignment control.

2.2 Short description of the ZetaView® instrument

The ZetaView® instrument is a Nanoparticle Analyzer that consists of cell unit, laser, microscope imaging unit with video camera and two motorized carriages, one for the microscope and one for the illuminating laser. An optional external conductivity and pH measurement device, mod. pH/cond 340i from WTW, a brand from Xylem analytics, can be connected to the rear of the instrument via the P1 RS232 interface.

The measurement cell unit consists of a fused silica channel. Wetted surfaces within the instrument are made of PEEK, stainless steel, Polypropylene, gold and silicone. The approved pH - range is between 1 and 13. The applied voltage to the electrodes of the cell is +/- 24 Volt. An external transformer delivers the 24 Volt supply through a special connector in the back side of the instrument.

For operation, the Cell assembly must be engaged in proper position, otherwise no measurement is possible. Laser and voltage are switched off for safety reasons. Conductivities >1mS/cm may cause convection within the cell affecting the measurement. Through the video camera, convection or any other disturbing effects

like drifts or bubbles are detected. This "seeing is believing" - principle is a major advantage over other nanoparticle techniques.

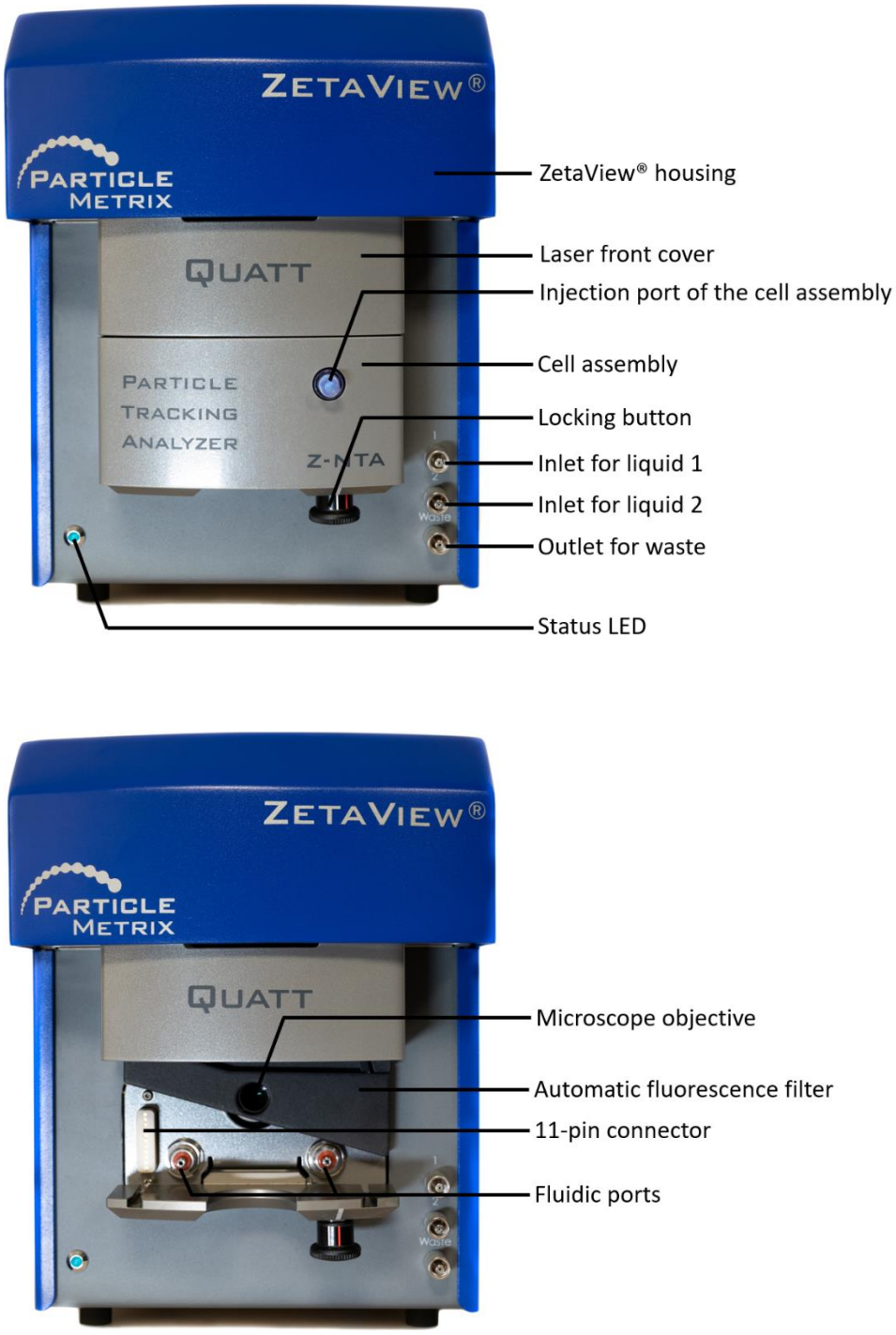


Figure 2-5: Description of the front parts of a ZetaView® multilaser instrument with and without cell assembly attached.

Five different operating programs are available:

4 video programs:

- Mobility profile measurement (11 positions) to control alignment symmetry and to calculate mobility and zeta potential.
- "Stationary" measurement at the 2 stationary layers SL1 and SL2 to determine high resolution mobility and zeta potential spectra.
- "Single" position measurement of the mobility at one of the pre-determined positions, for checking purposes.
- "Size" distribution measurement based on the Brownian motion analysis in 11 positions, 2 positions (SL-positions) or in one single position.

Manual acquisition program

- This program allows the user to pick out individual particles to measure their mobility / zeta potential by start / stop action.

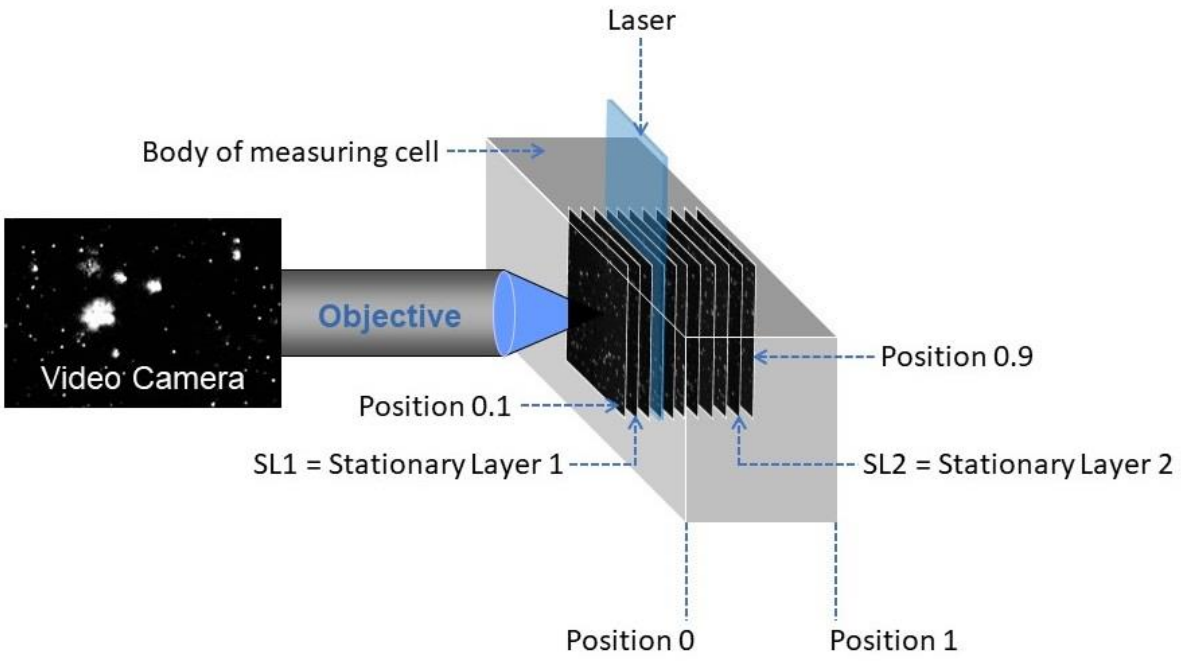


Figure 2-6: Sketch of the location of the measuring positions. Laser and microscope optics are perpendicular to each other and can be moved automatically from position to position.

The ZetaView® is a reference instrument

All physical parameters are calibrated and obvious by video observation. The calibration parameters are shown in the calibration menu which is accessible in the administrator mode. This instrument therefore serves as a reference instrument for electrophoretic, electro-osmotic and Brownian movement measurements. It can also be used as an absolute tool for zeta potential determinations, provided that the formulas used are valid. The various theories for zeta potential calculation depend on sample circumstances. The Smoluchowski formula is offered here in the ZetaView®. This formula is valid for aqueous samples with particle size distribution around 1µm.

2.3 CFR 21 Part 11

Title 21 CFR Part 11 represents a part of Title 21 of the Code of Federal Regulations. It defines the regulations of the US Food and Drug Administration (FDA) for electronic records and electronic signatures. CFR 21 Part 11 defines the criteria for electronic records and electronic signatures to be considered reliable, trustworthy, and equivalent to paper records.

The Particle Metrix ZetaView® instrument can be equipped with a CFR 21 Part 11 software package. CFR 21 Part 11 represents an extension to the already installed ZetaView® software and is available as a separate software package.

For CFR 21 Part 11, a separate user manual is available.

3 Unpacking and Installation

3.1 Packaging list

Quantity	Description
1	ZetaView® instrument
1	24 Volt power supply
1	Cell assembly (Z-NTA or NTA, depending on zeta potential option)
1	Measuring cell
1	Kit with spare parts, tools and cleaning material
1	Bottle kit with 2x 250ml, 1x500ml (zeta potential option) Bottle kit with 1x250ml (w/o zeta potential option)
1	Alignment suspension (aqueous), ca. 100 nm, for AutoAlignment and testing, must be diluted 1:250,000
1	Zeta potential standard suspension (if Z-NTA Cell assembly is provided)
1	Fluorescence standard beads (depending on laser wavelengths and fluorescence option)
1	Computer (NUC desktop PC or laptop) incl. power supply
1	Ethernet cable (cross over)
1	Software license from National Instruments
1	Operation Manual with factory test report and ISO 9001 certificate

3.2 Connecting the ZetaView® instrument

The ZetaView® instrument is connected with a laptop or NUC computer according to the following sketch.

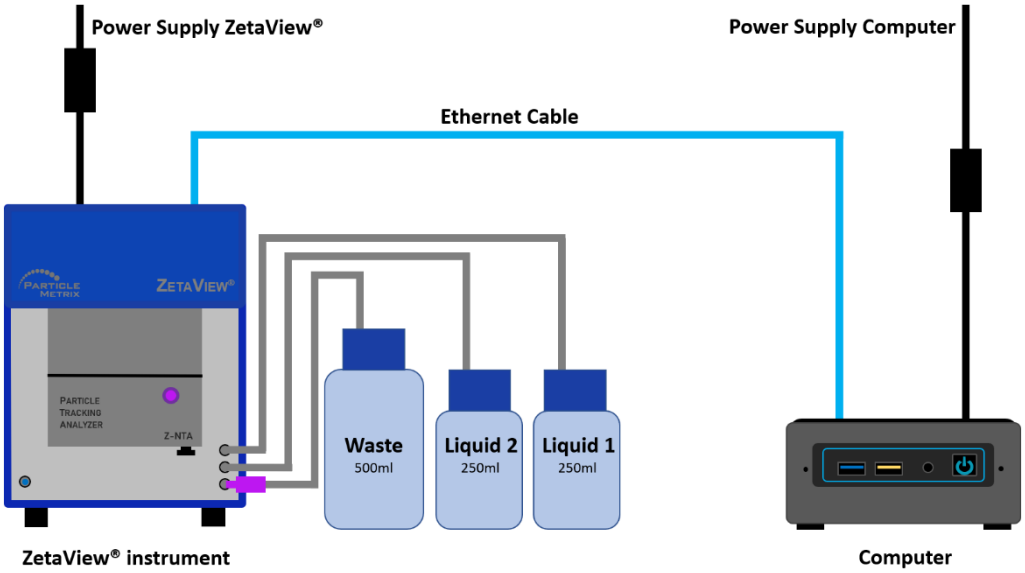


Figure 3-1: Electrical and fluidic connection of the ZetaView® instrument and computer.

There are 2 bottles (250ml) for liquid 1 and 2 and one big bottle (500ml) for the waste. The waste bottle (500ml) is equipped with a check valve (one-direction-valve) with a Luer plug, the bottles for “liquid 1” and “liquid 2” are not equipped with a check valve but with Luer plugs as well.

For connecting the bottles with the ZetaView® instrument, please screw the plugs into the threads located at the front of the ZetaView® instrument.

Figure 3-2: Fluidic connectors of the ZetaView® instrument located at the front side.

Connect the power supply into the “Voltage Supply 24V DC” plug and the ethernet cable into the “PC” plug.

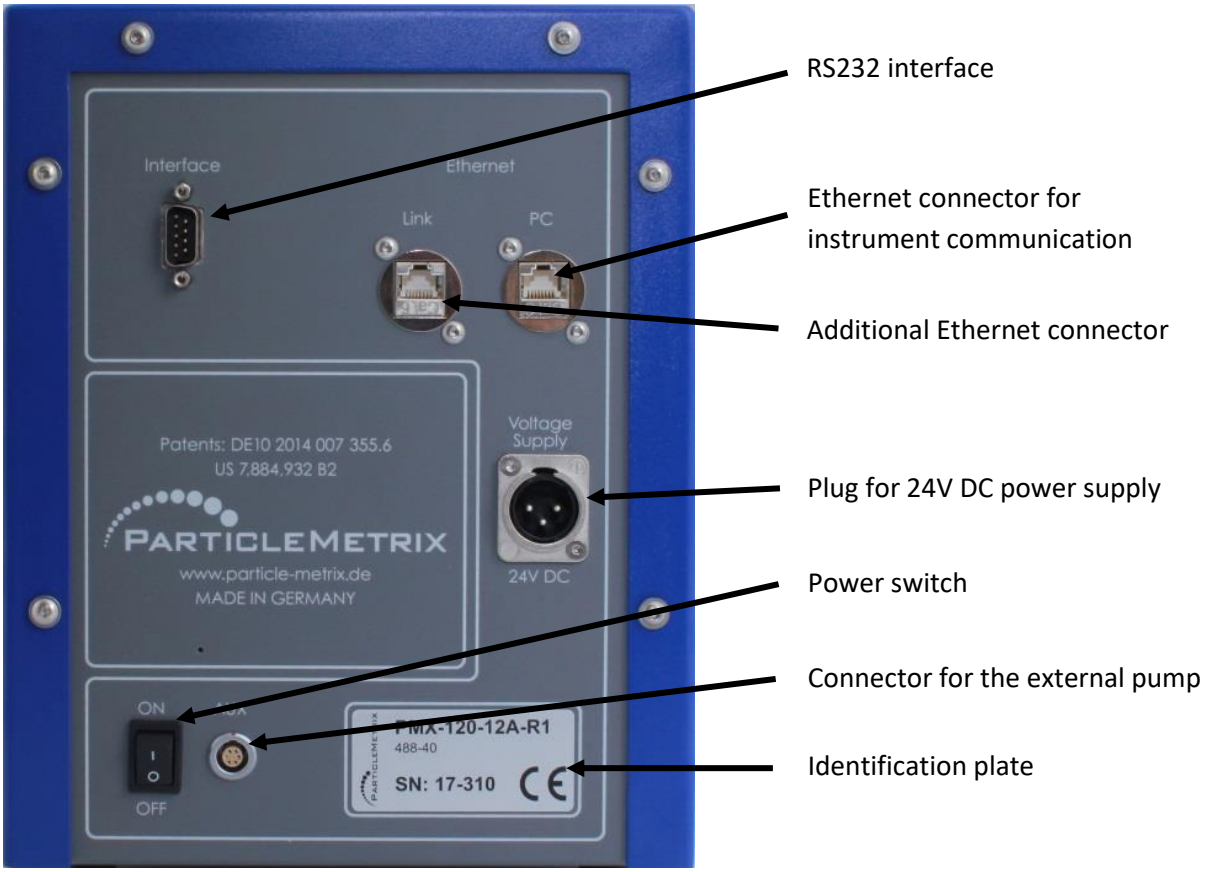


Figure 3-3: Rear panel and connectors of the ZetaView® instrument.

3.3 Mounting the fluorescence filter

This section only applies for PMX-120 mono-laser instruments equipped with a manual fluorescence filter mechanism.

For mounting the fluorescence filter, place the filter into the slot of the base plate that carries the Cell assembly until it clicks into the first position (underneath the microscope objective).

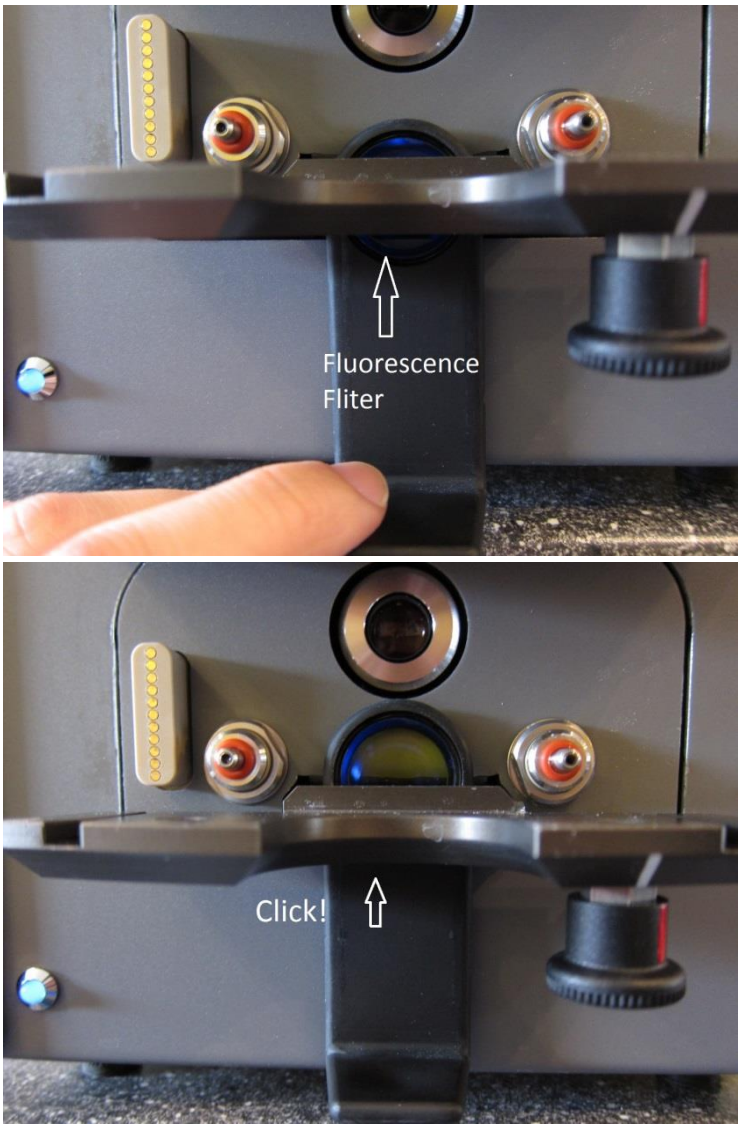


Figure 3-4: Mounting the manual fluorescence filter. The filter snaps in by a "click" and remains under the microscope optics.

4 Mounting and mechanical handling of the Cell assembly

4.1 Mounting of the Cell assembly

This section applies to the Z-NTA Cell assembly and NTA Cell assembly

For attaching the Cell assembly make sure that the red marking of the round black knob is in line with the white marking painted on the base plate. For that, pull the knob downwards, turn it into the red position as indicated below and let the knob release. The red position is the “unlock” position.

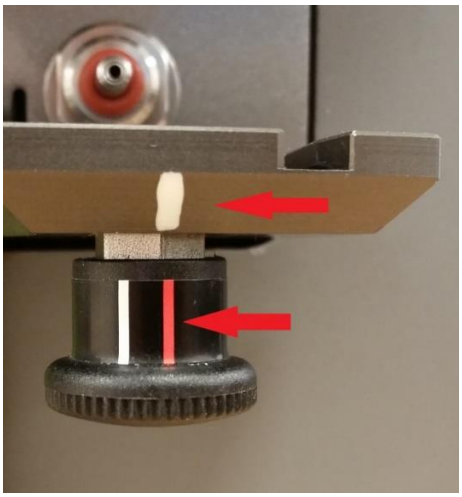


Figure 4-1: Unlock position.

Slide the Cell assembly towards the microscope objective until the Cell assembly touches the front panel of the machine. Do not apply force.

Figure 4-2: Push the Cell assembly carefully using both hands. Do not apply force while pushing it towards the front of the ZetaView® instrument.

Here, the Cell assembly has already been slid in, but it is not locked yet. This is indicated by the red marking on the black knob that is still in line with the white marking on the base plate.

Figure 4-3: Still unlocked position.

To lock the Cell assembly, change the position of the knob from red to white by pulling the knob downwards, turning it into the white position and release the knob afterwards. Both white markings should now be in line with each other.

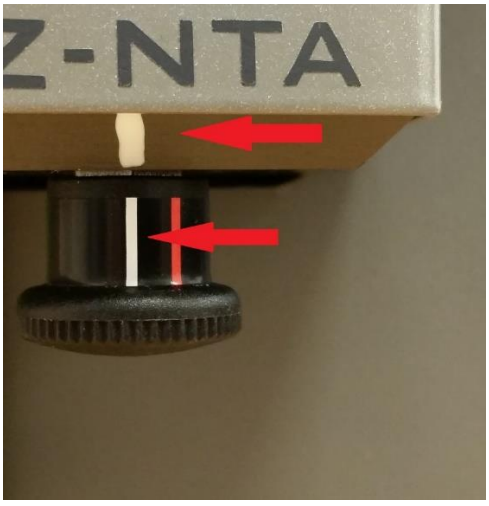


Figure 4-4: Locked position.

Now lock the Cell assembly into position by squeezing your thumbs of both hands carefully against the lower part of the Cell assembly while holding the base plate with your forefinger and middle finger in the grooves of the base plate as shown below.

Figure 4-5: While locking the Cell assembly (white markings are in line with each other), hold the base plate at both sides with your fingers.

You should hear a “Click” sound of the knob when the Cell assembly locks into position. Note that you do not push the Cell assembly into place; you squeeze it with your wrists.

Figure 4-6: The Cell assembly clicks in when locking it on the ZetaView® instrument.

4.2 Z-NTA Cell assembly and measuring cell

The front and upper side of the Z-NTA Cell assembly consists of the cell carrier, cell (not visible here) and the injection port for injecting the sample. The injection port is a check valve (one-direction-valve), that lets the sample pass in one direction. It can easily be replaced by un-screwing it from the Cell assembly.

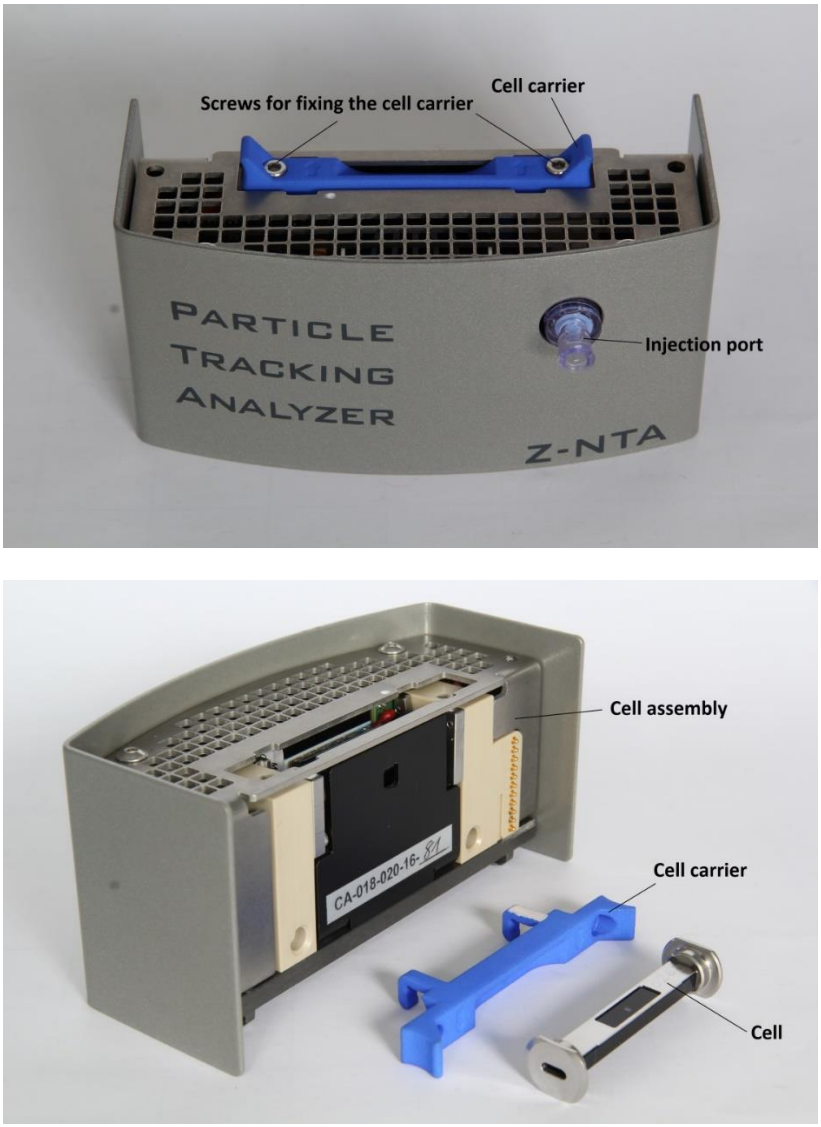


Figure 4-7: Components of Z-NTA Cell assembly and measuring cell.

The back side of the Cell assembly consists of the cell carrier, measuring cell (not visible here) electric connections to the cell, gold pins for the electric connection to the ZetaView® instrument, fluidic connectors and the serial number.

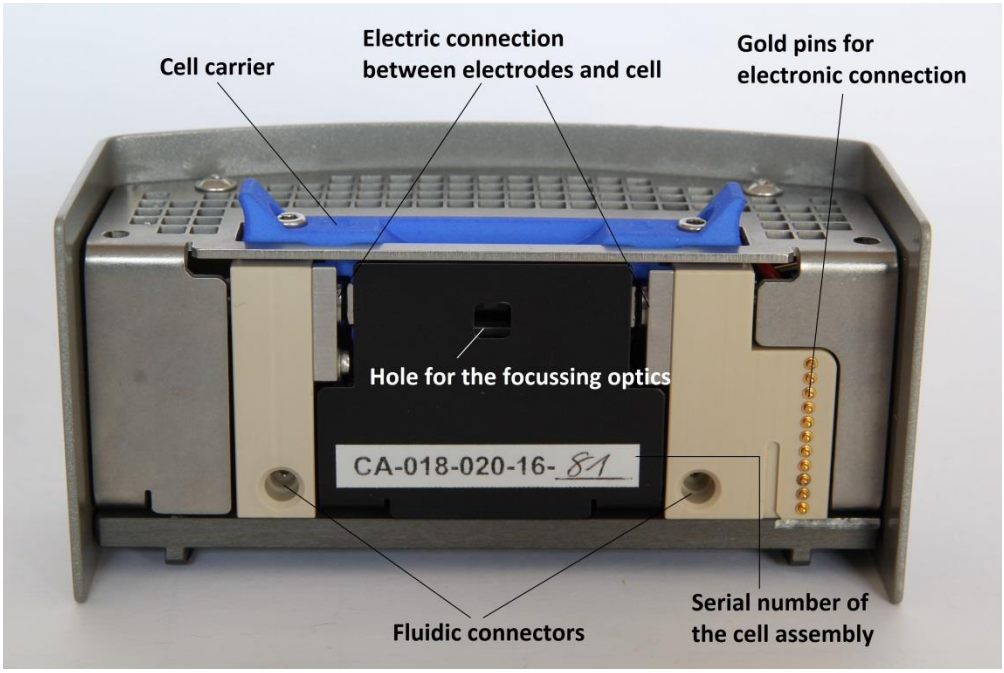


Figure 4-8: Back side of the Z-NTA Cell assembly.

4.3 Inserting, removing and handling of the measuring cell from the Z-NTA Cell assembly

The cell is usually mounted in a black or blue colored cell carrier. The cell carrier features two holes that are located on the left and right side for fixing it by two screws at the Cell assembly. The following description shows how to remove the cell carrier from the Cell assembly.

Remove both screws that fix the cell carrier on the Cell assembly by using the Allen wrench provided in the toolkit.

Figure 4-9: For removing the cell carrier, unscrew the screws on the left and right side.

After removing the screws hold the protruding parts of the cell carrier with your thumb and fore finger and pull the cell carrier slowly and evenly upwards out of the Cell assembly by applying some counter pressure with your middle fingers.

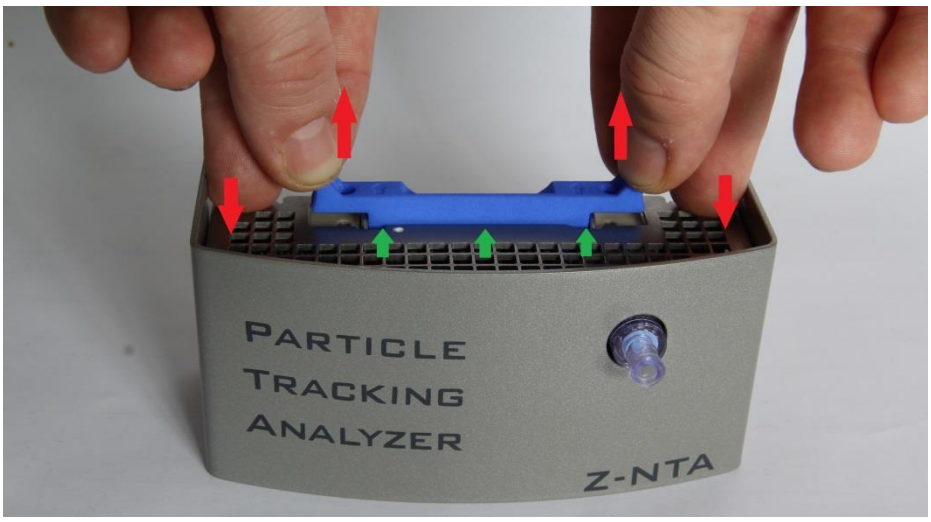


Figure 4-10: Raise the cell carrier (green arrows) by holding the protruding parts (red arrows pointed upwards) and creating some counter pressure with your middle fingers (red arrows pointed downwards).

To remove the cell from the cell carrier, open the first bracket slightly with your thumb or thumb nail and push the top part of the cell slightly out of the bracket by using your forefinger. Repeat this procedure with the lower bracket of the cell carrier and the lower part of the cell as shown below.

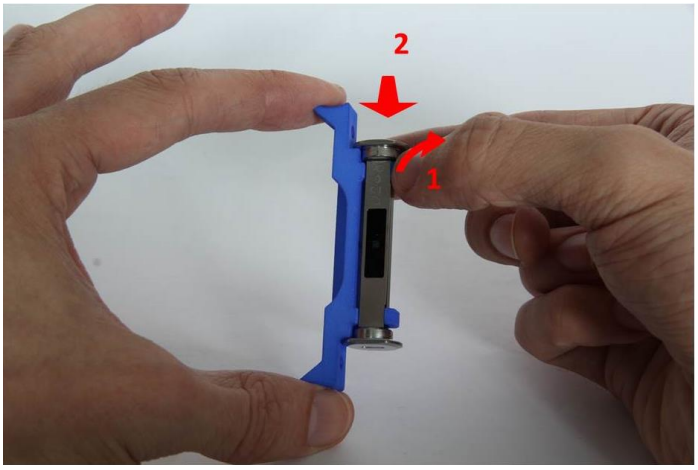


Figure 4-11: Hold the cell carrier in your left hand and open the upper bracket slightly by using the thumb of your right hand (1). Push the top part of the cell slightly and carefully out of the bracket by using your forefinger (2).

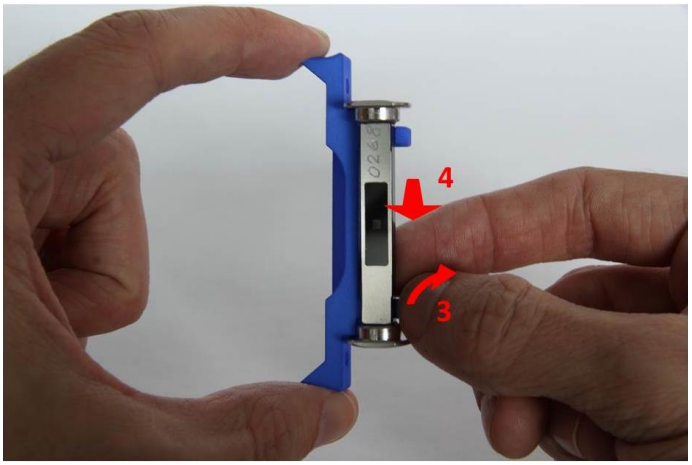


Figure 4-12: Repeat the procedure described above with the lower bracket (3) of the cell carrier and the lower part of the cell (4).

While re-attaching the cell to the cell carrier, both must be mounted to each other in one specific orientation. When the cell is mounted on the cell carrier, the serial number must be visible at the open part of the cell carrier´s right bracket (from this point of view as shown below).

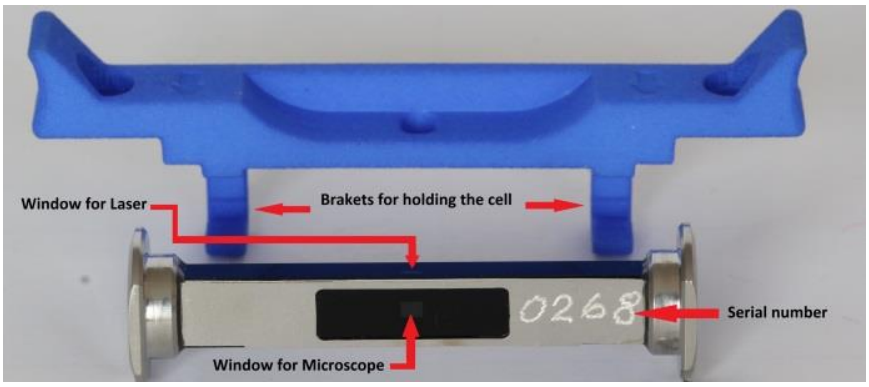


Figure 4-13: Cell is not incorporated in the cell carrier but in the correct orientation to each other.

Figure 4-14: Cell and cell carrier are attached to each other. Please note that the cell is attached such that the serial number is still visible at the open part of the cell carrier´s right bracket.

Re-inserting the cell carrier (including the cell) must be done in one specific orientation too. Please make sure that the white painting on the cell carrier faces the dot located on top of the Cell assembly. Please also note that the serial number of the cell fixed in the cell carrier faces towards the back side of the Cell assembly (see below).

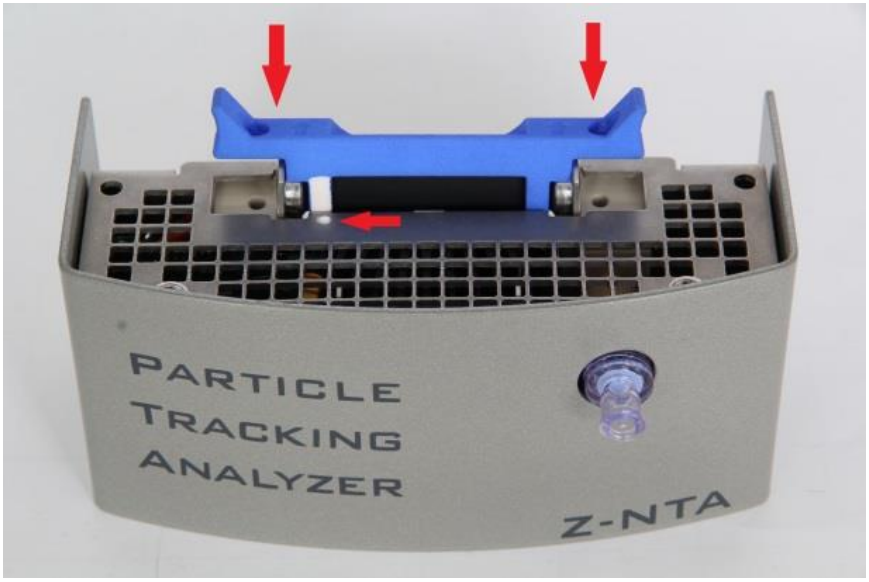


Figure 4-15: Z-NTA Cell assembly shown from the front side. White painting of the cell carrier must align with the white dot on top of the Cell assembly.

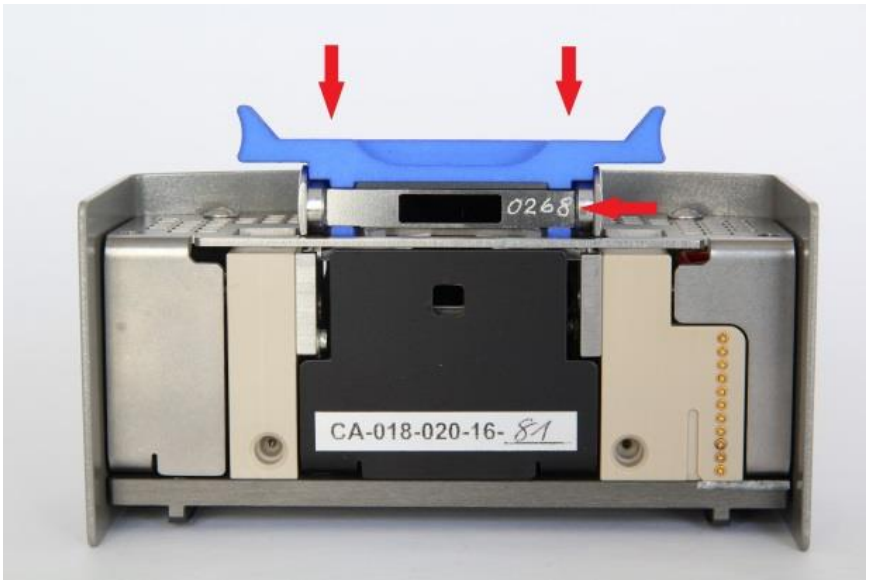


Figure 4-16: Z-NTA Cell assembly shown from the back side. The serial number of the cell must be facing towards the back side of the Cell assembly.

4.4 Inserting, removing, and handling of the cell from NTA Cell assembly

The NTA Cell assembly consists of the measuring cell, usually covered by a blue or red holder and the inlet on the right side and outlet on the left side. Inlet and outlet each carry a check valve.

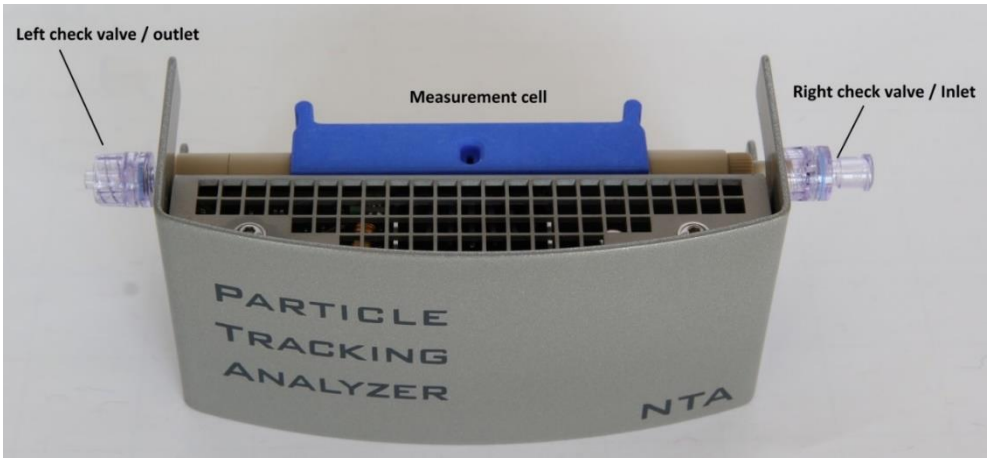


Figure 4-17: NTA Cell assembly shown from the upper front side.

The back side of the NTA Cell assembly consists of the measuring cell, gold pins for the electronic connection to the instrument and the serial number. The blue plastic part

covers the cell and features an aperture for the laser and a small window for the microscope.

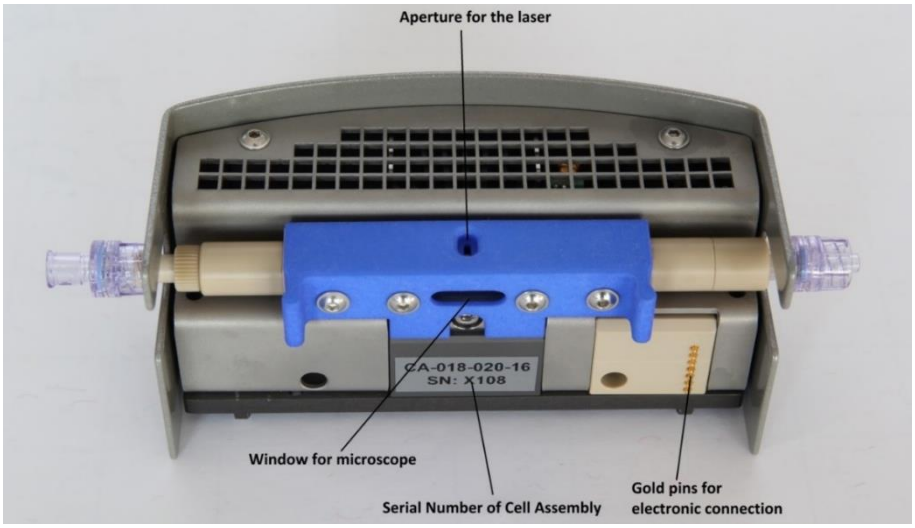


Figure 4-18: NTA Cell assembly shown from the upper rear side.

Usually it is not required to remove the cell from the NTA Cell assembly. Even for cleaning purposes the cell can usually remain attached to the Cell assembly. However, in the unlikely situations that the cell needs to be replaced for some reason (e.g. for thorough inspections) it can be easily removed from the Cell assembly. The procedure of how to remove the cell from the Cell assembly is described below.

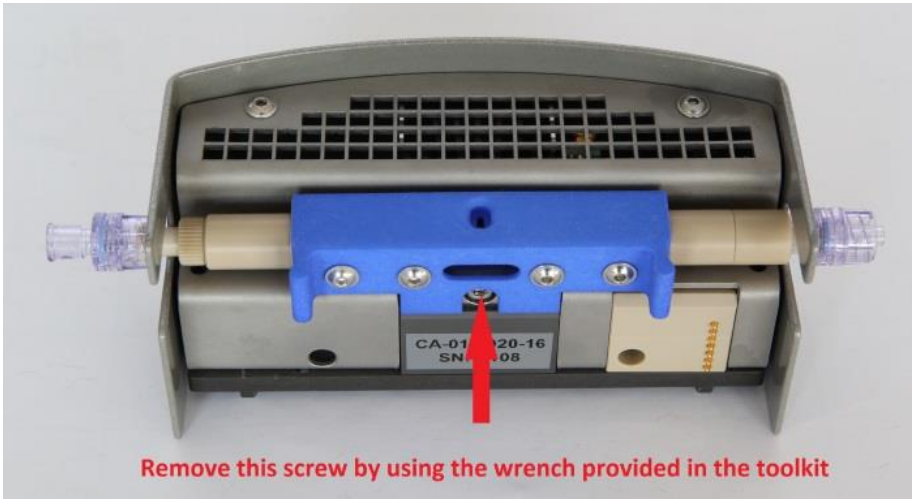


Figure 4-19: NTA-Cell assembly shown from the back side. The red arrow indicates the screw that must be removed in case you need to remove the cell, for example for cleaning or inspection.

Figure 4-20: NTA Cell assembly shown from the back side. The cell has been removed.

5 Start-up routine

The daily start-up routine must always be carried out when the ZetaView® device is switched on and / or after the Cell assembly has been mounted on the instrument again (for example after cleaning the cell). The daily start-up routine has 3 main tasks:

1. The fluidic system inside the ZetaView® and the measuring cell are filled with water in order to help avoid disturbing air bubbles for subsequent measurements.
2. It serves to focus the detection optics on the particles. This is guaranteed by the implemented auto alignment function which is optimized for 100 nm polystyrene (PS) standard beads ("alignment suspension").
3. Ensures symmetry of the measuring cell with respect to the x-axis by measuring the electrophoretic mobility of the particles in the alignment suspension in all 11 positions. Any asymmetry will be corrected immediately after this test measurement.

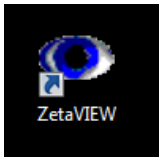
Please note: Since an NTA Cell assembly does not provide zeta potential capability, a symmetry routine will not be performed if the instrument is equipped with an NTA Cell assembly.

The following procedure describes how to perform the daily start-up routine:

1. Turn on the ZetaView® machine.

2. Turn on the computer and enter the password of the computer.

3. Start the ZetaView® software by double clicking the ZetaView® icon on the desktop.



4. If the ZetaView® instrument is equipped with an automatic changer for fluorescence filters (PMX-220 TWIN and PMX-420 QUATT), you can hear the noise of the mechanism when the changer moves to the first and to the last filter position. After that, the ZetaView® initializes, which is visualized by the progress bar at the lower right of the software interface as well as in the window that normally shows the measuring positions. While this procedure is in progress, laser and microscope move along their precision sliding system until they recognize the electronic end-switches.

   By default, only the 405 nm laser is shown during initialization, regardless of whether this laser is available, or how many lasers are installed in the device. After initialization, all available lasers are shown in the software.

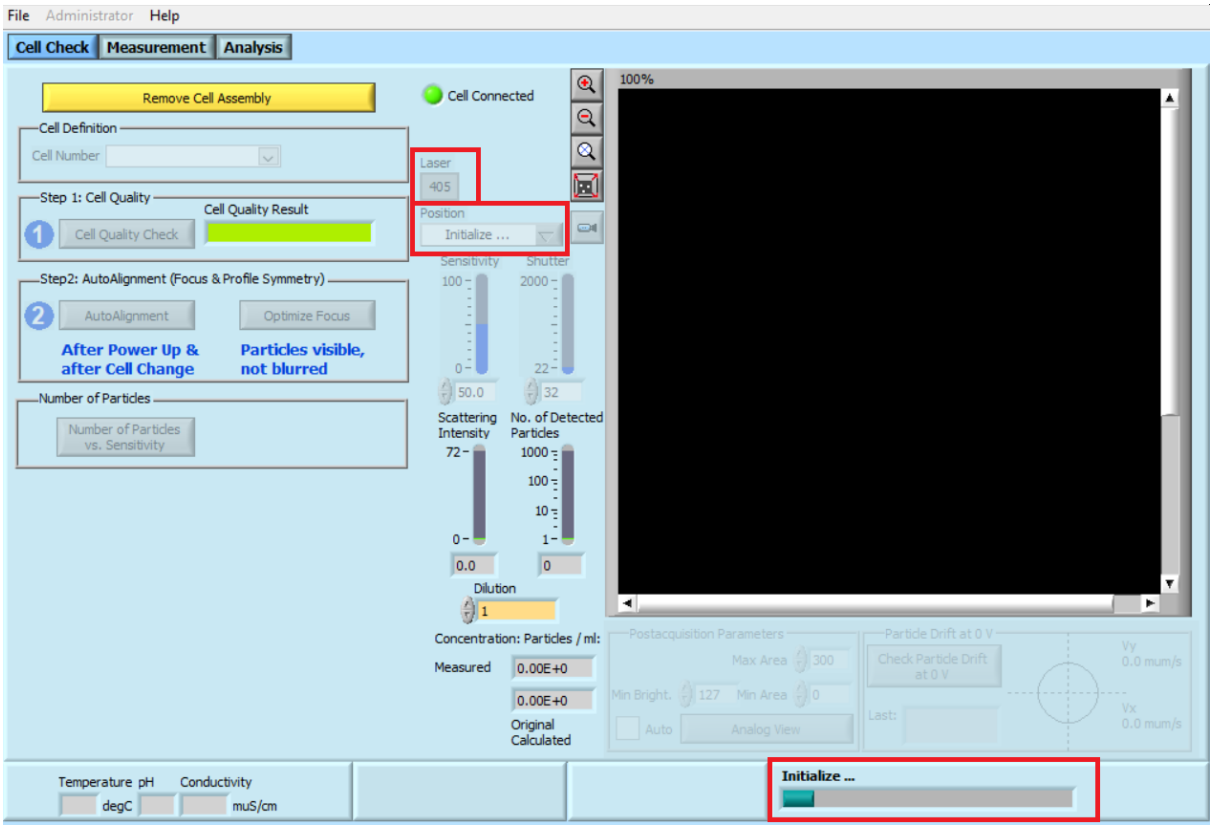


Figure 5-1: Initialization of the ZetaView® instrument can be observed by the progress bar at the lower right and the window that normally shows the measuring positions.

5. After initialization, select the appropriate pump for rinsing the fluidic system of the ZetaView® with water. The corresponding water reservoir should be filled with particle-free water such as milliQ-water or similar to avoid excessive number of particles during the Cell Quality Check.

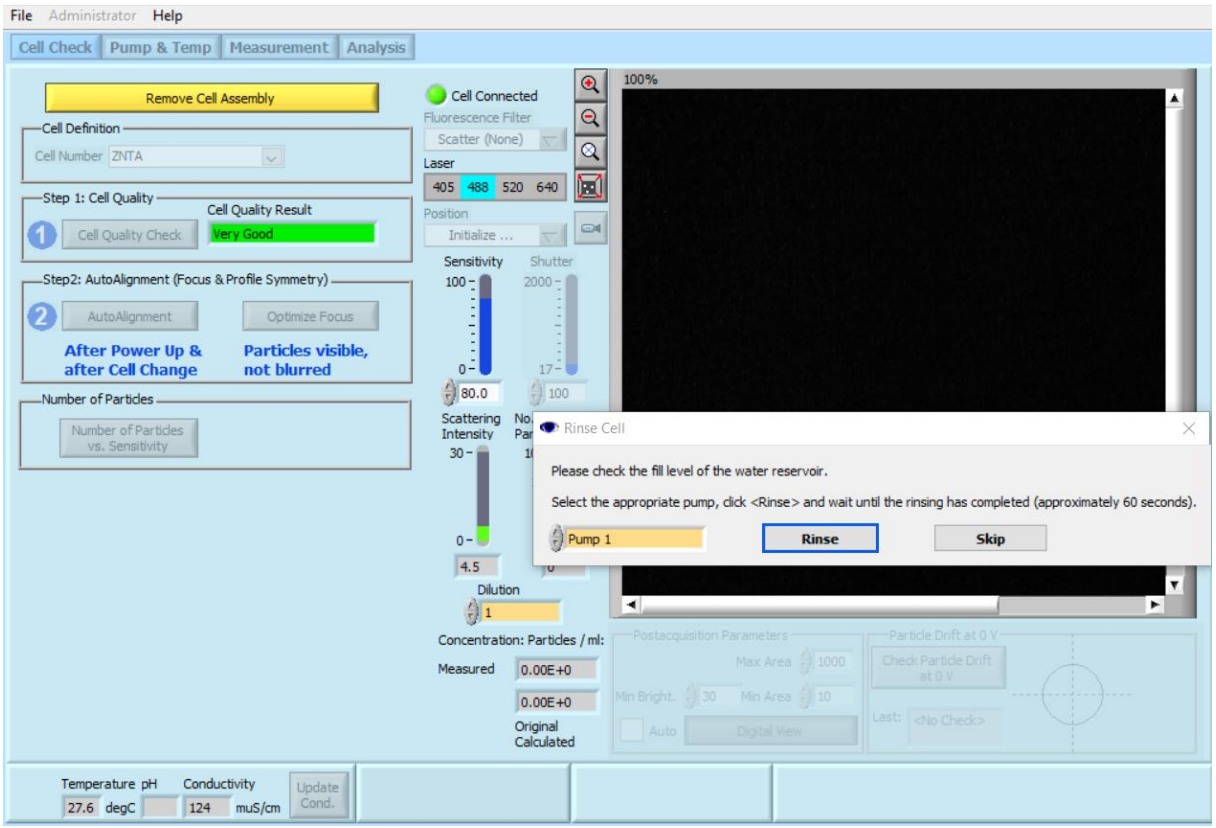


Figure 5-2: Message for rinsing the measuring cell by selecting the appropriate pump.

6. While rinsing, it is recommended to inject 10-20 ml of water into the injection port located in front of the instrument using an appropriate syringe. This is important because the “dead volume” between injection port and measuring cell should also be filled with water in order to avoid air bubbles.

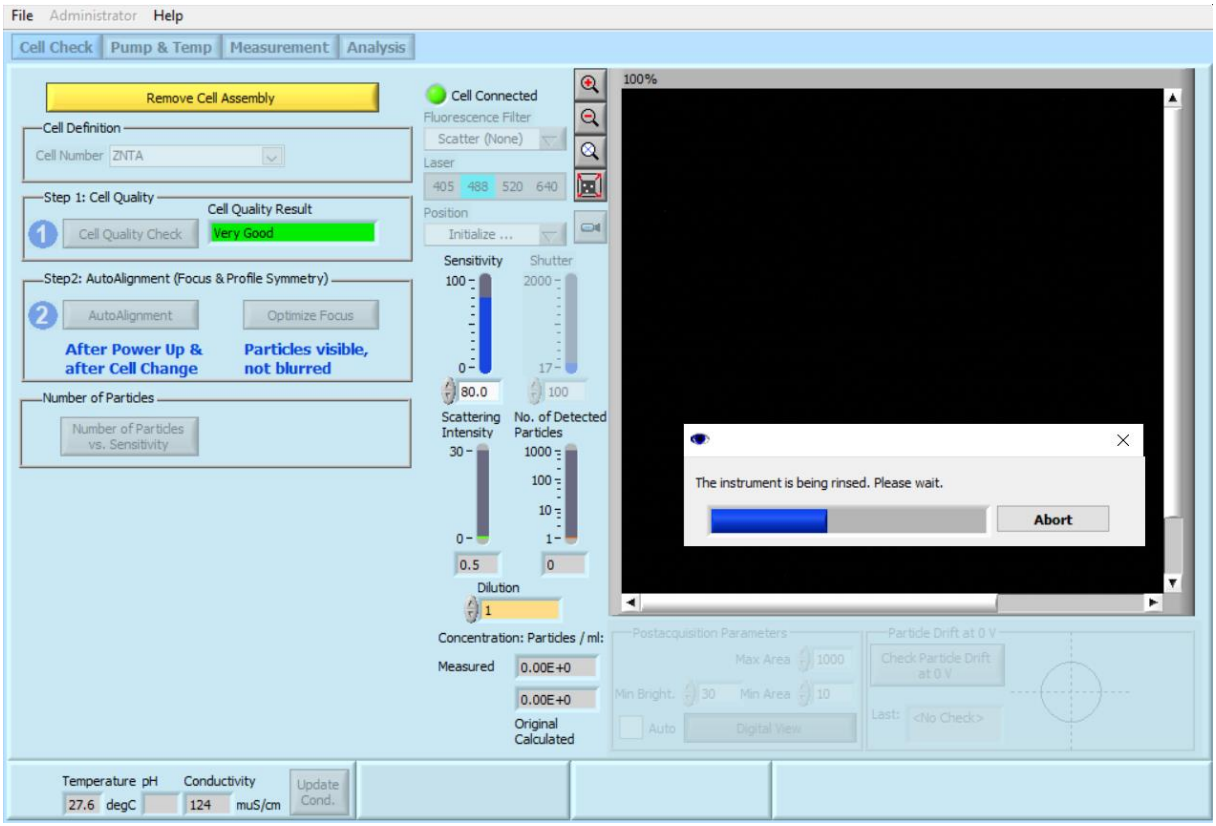


Figure 5-3: The instrument is being rinsed. To remove air bubbles between the injection port and the measuring cell, it is recommended to inject water from the front as well while rinsing the instrument.

7. After rinsing the instrument, the Cell Quality Check can be performed. Since the system is already filled with water, the Cell Quality Check can be confirmed by clicking “OK”.

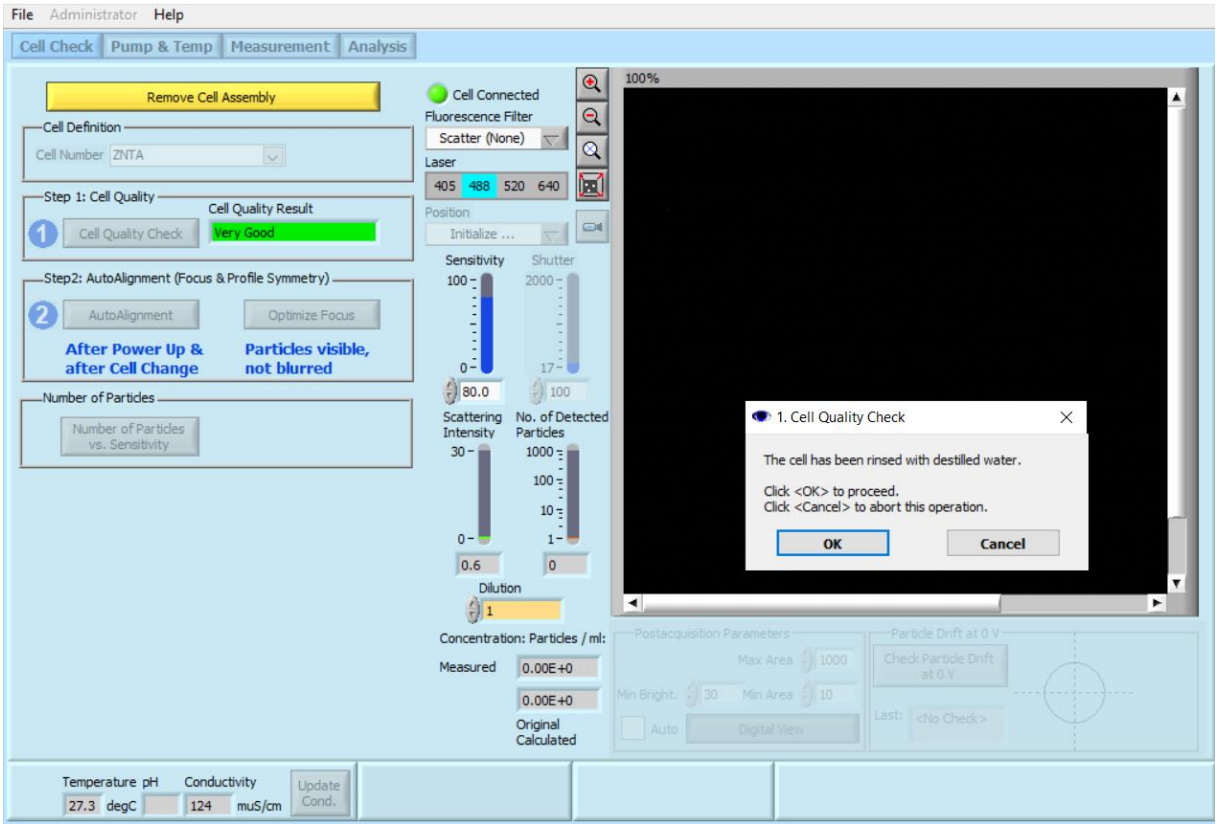


Figure 5-4: Once the instrument and the cell is filled with water, the Cell Quality Check can be executed.

8. Cell Quality Check in progress

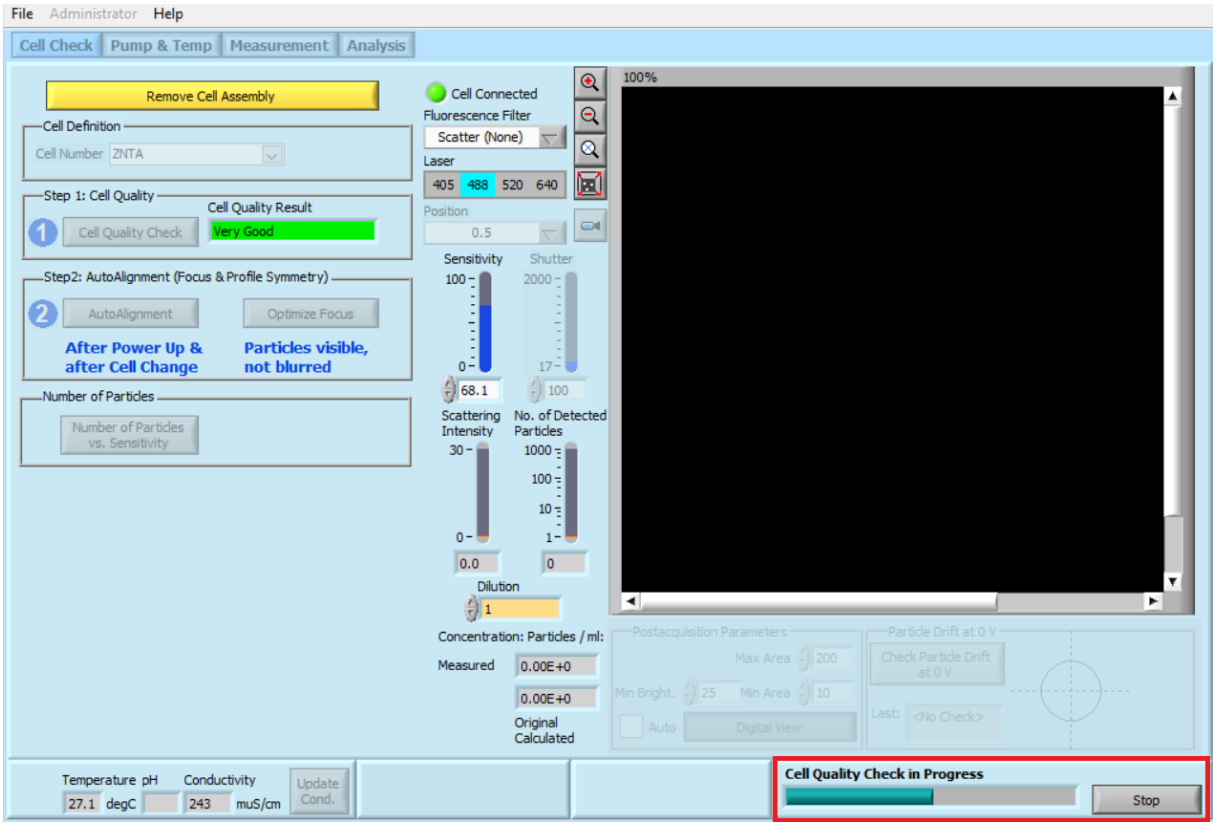


Figure 5-5: Cell Quality Check can be stopped any time if necessary.

9. After Cell Quality Check has passed successfully, the Autoalignment function is started by showing the message “Please fill the cell with 100 nm alignment suspension (dilution 1:250,000)”. It is highly recommended to inject freshly prepared 100 nm standard beads (ref. section 5.1). For more information about the AutoAlignment please refer to section 5.2.

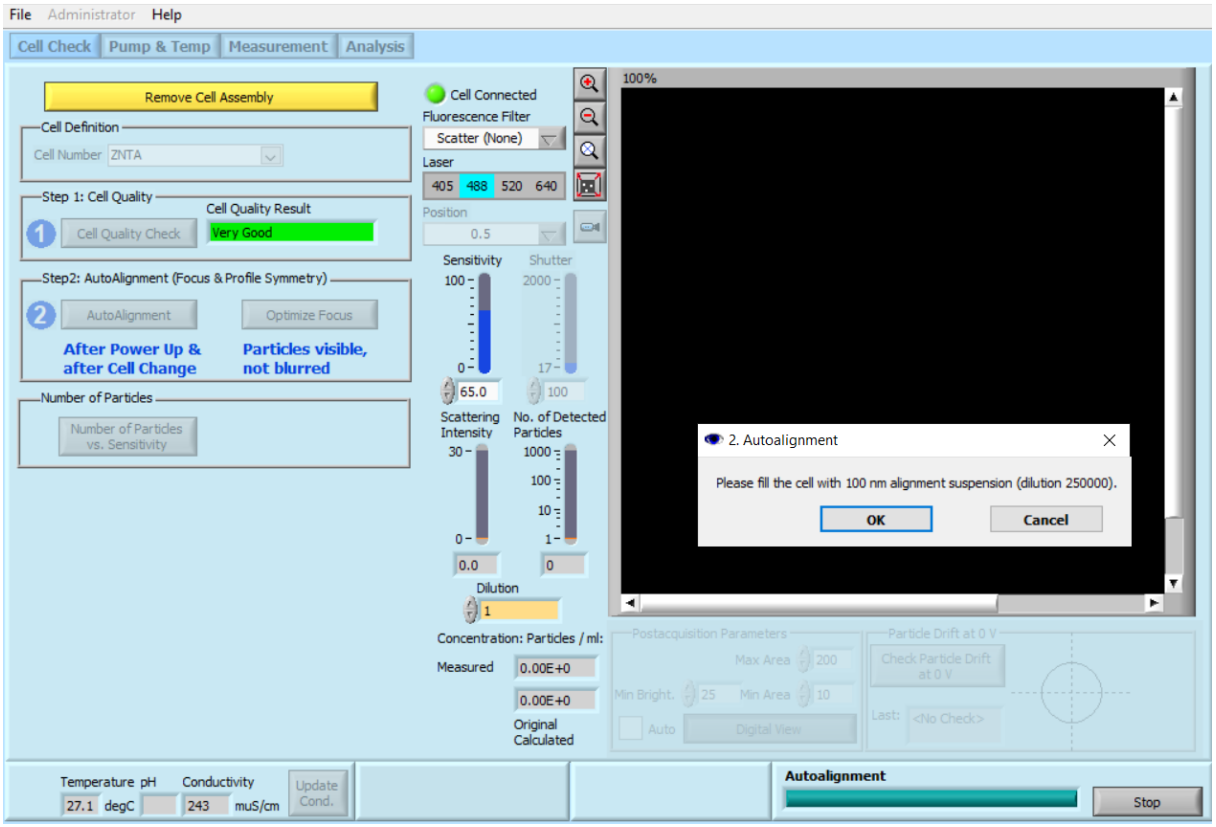


Figure 5-6: For subsequent AutoAlignment, 100 nm alignment suspension must be injected.

10. Please inject at least 2 ml alignment suspension (for preparation of alignment suspension see section 5.1) and click OK. Please avoid generating air bubbles during injection (ref. section 5.2).

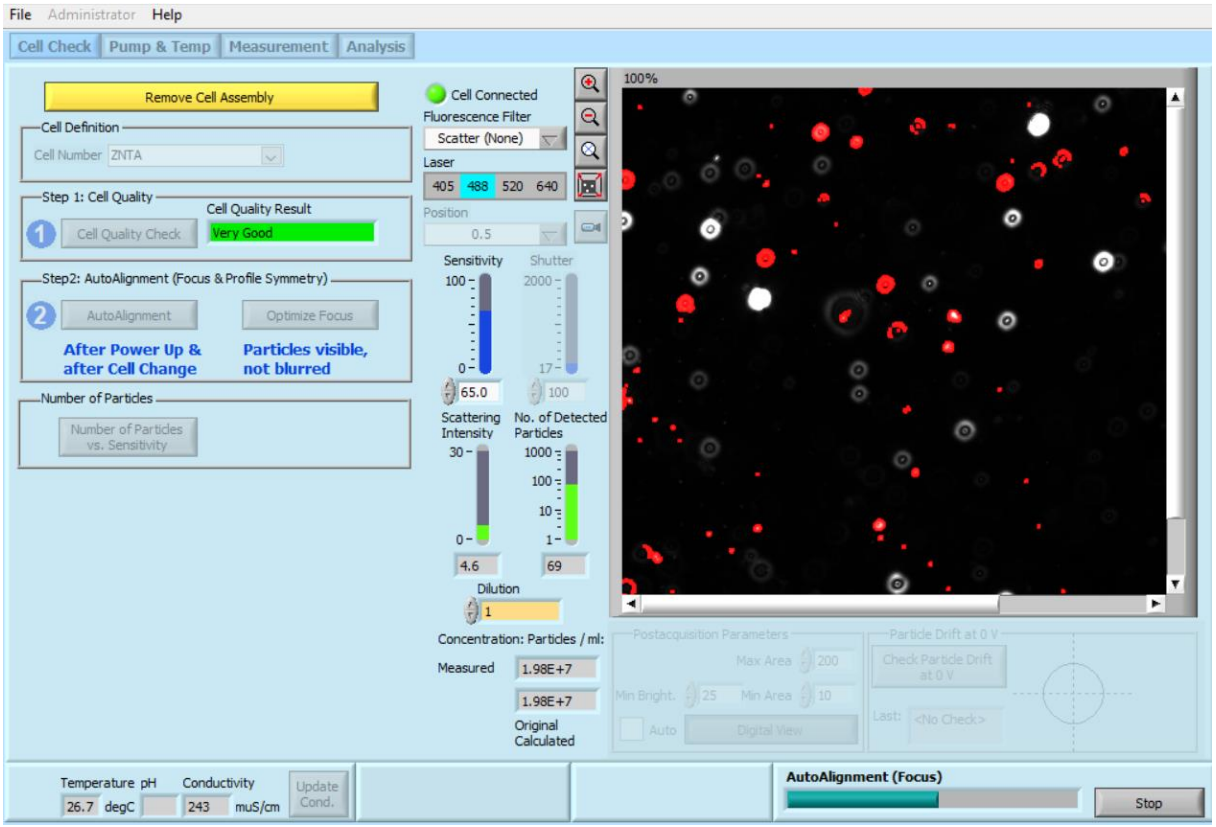


Figure 5-7:Autoalignment in progress, indicated by the progress bar in the lower right. AutoAlignment can be stopped at any time if required.

11. The ZetaView® instrument performs focus optimization

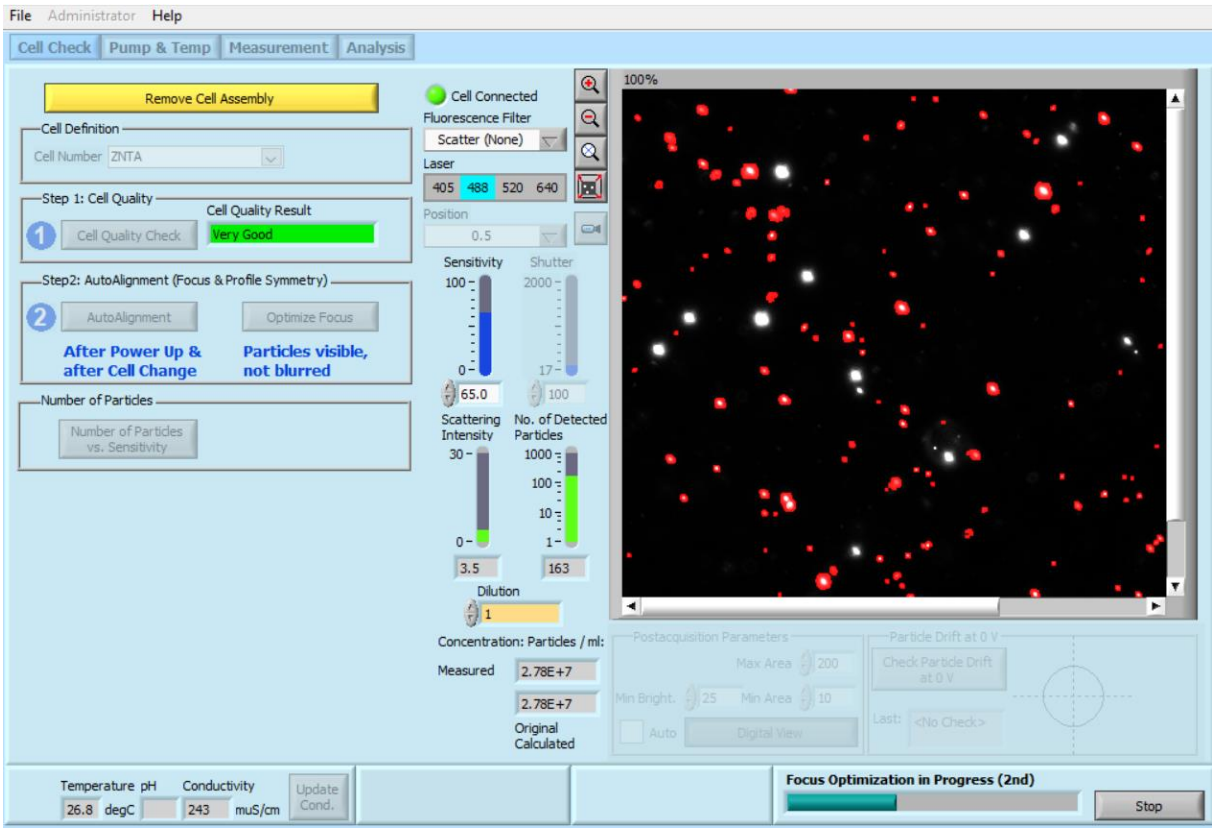


Figure 5-8: Focus Optimization in progress indicated by the progress bar in the lower right. Focus Optimization can be stopped at any time if required.

12. When a Z-NTA Cell assembly is used on the instrument, a profile symmetry routine is performed which results in establishment of a parabola. The parabola shows up automatically in the “Analysis” tab and indicates the cleanliness and symmetry of the measuring cell.

Figure 5-9: Parabola indicating cleanliness and symmetry of the measuring cell.

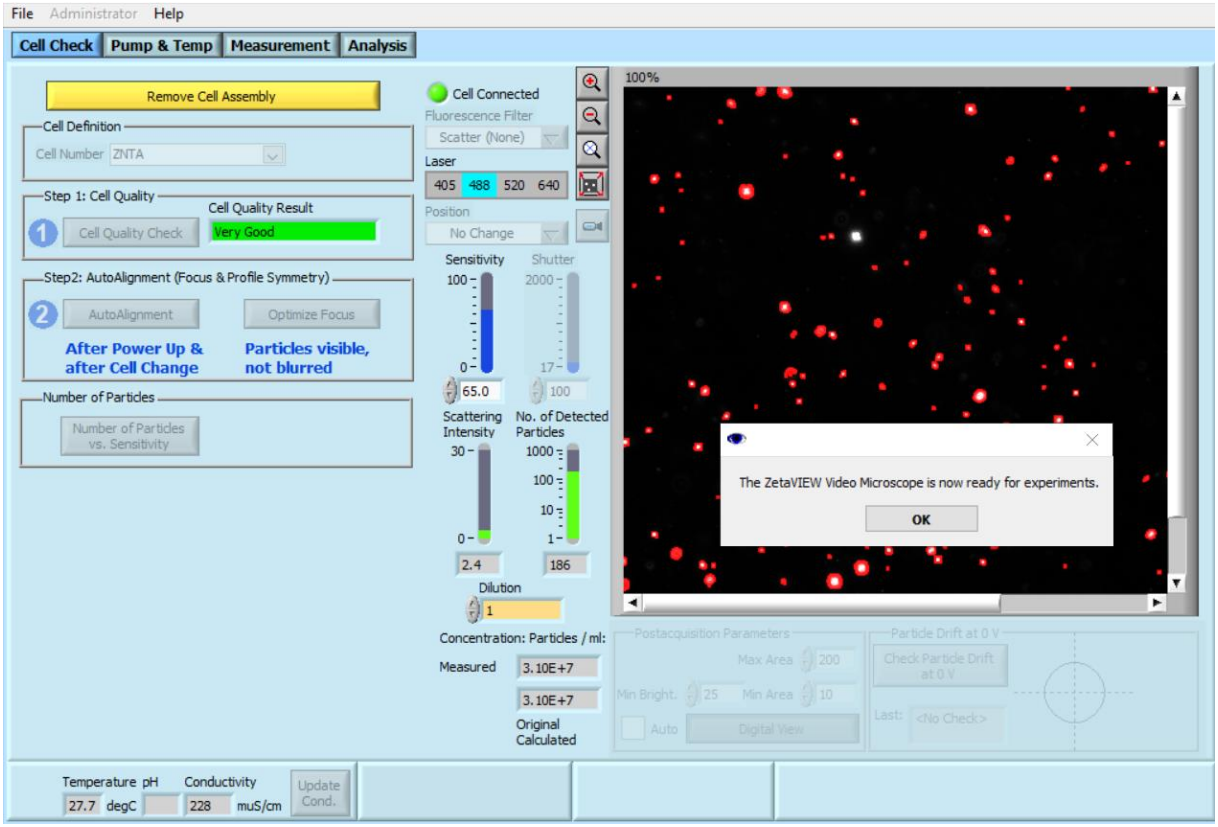


Figure 5-10: The start-up routine successfully ends with the message "The ZetaVIEW Video Microscope is now ready for experiments".

5.1 Preparation of the polystyrene (PS) 100 nm alignment suspension

Requirements:

- Appropriate beakers, tubes or vials
- Appropriate pipets (e.g. 1-10 µl and 20-200 µl or 100-1000 µl pipets)
- Particle-free water
- 100 nm polystyrene standard
- 0.2 µm syringe filter if necessary

To prepare the alignment suspension, two consecutive dilution steps are recommended.

1. Recommendation for preparation of **dilution 1**: Add 10 µl of 100 nm polystyrene beads to 10 ml of particle-free water. This yields to a **1:1,000 dilution.**
   The shelf life of the solution is about 2-3 days when stored at 4°C.

2. Recommendation for preparation of **dilution 2**: Add 80 µl of dilution 1 to 20 ml of particle-free water. Scale up or down if necessary. This results in a **1:250,000 alignment suspension.**
   This suspension is stable for around 30-60 minutes, before it starts to agglomerate.
   For the AutoAlignment script it is therefore highly recommended to prepare a fresh dilution of 2.

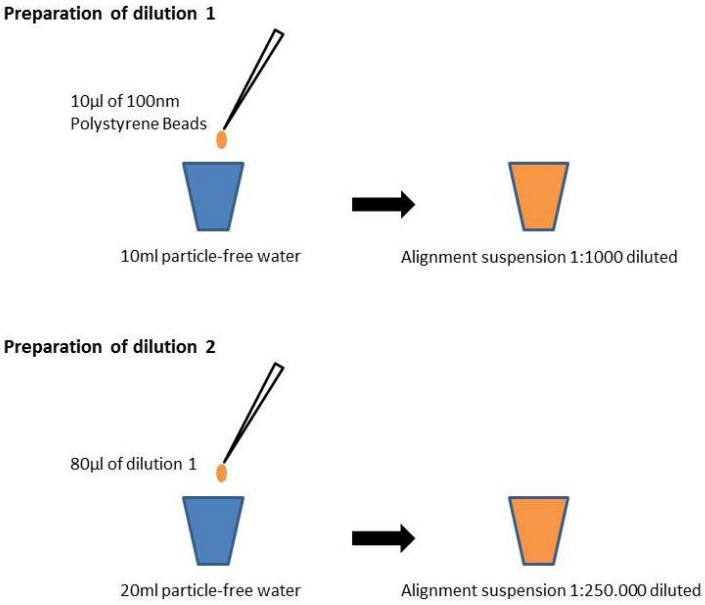
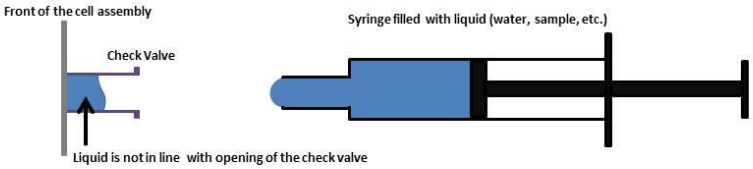
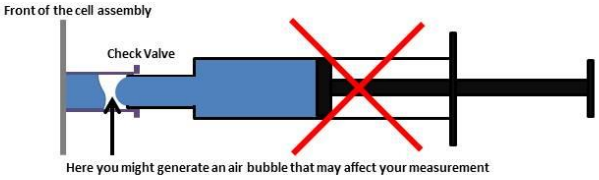


Figure 5-11: Recommendation of a pipetting scheme for the preparation of the alignment suspension. The 1:250,000 alignment suspension (dilution 2) is required for the daily start-up routine.

5.2 How to inject without introducing air bubbles

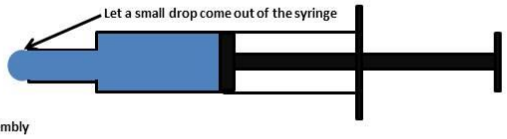
Please avoid injecting your sample in this way because you might create air bubbles!



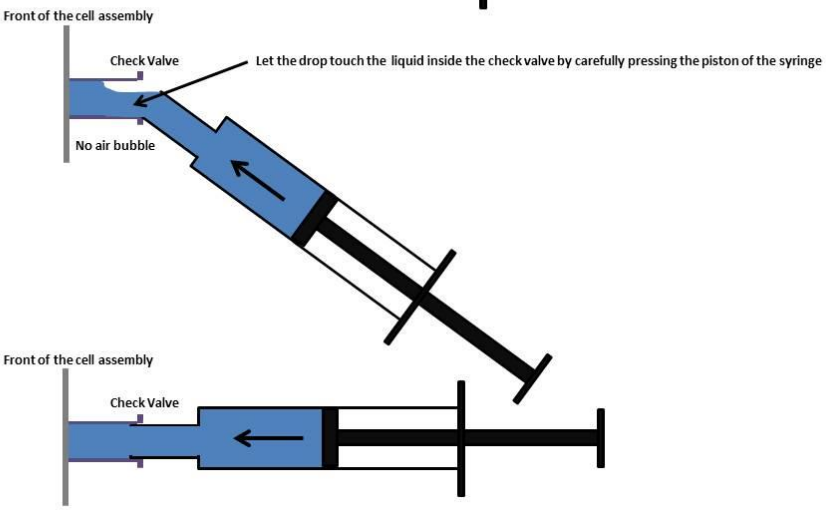


Try to inject your sample in this way, here you are not creating air bubbles!

1.



2.



3.

The procedure described here applies not only to the PS 100 nm alignment suspension, but also whenever samples are injected and measured. In this way, air bubbles in the system can be largely avoided.

6 Graphical user interface and general software operation

The graphical user interface (GUI) of the software is divided into 4 main parts. These main parts comprise the tabs *"Cell Check"*, *"Pump & Temp"*, *"Measurement"* and *"Analysis"*. The first three mentioned tabs (*Cell Check, Pump & Temp, Measurement*) always differ in the left part of the graphical user interface, whereas the right part is always identical. The Analysis menu, on the other hand, has a completely different layout.

6.1 Common layout of the tabs "Cell Check", "Pump&Temp" and "Measurement"

The right part of the tabs *"Cell Check"*, *"Pump & Temp"*, *"Measurement"*, is always identical, and described below.

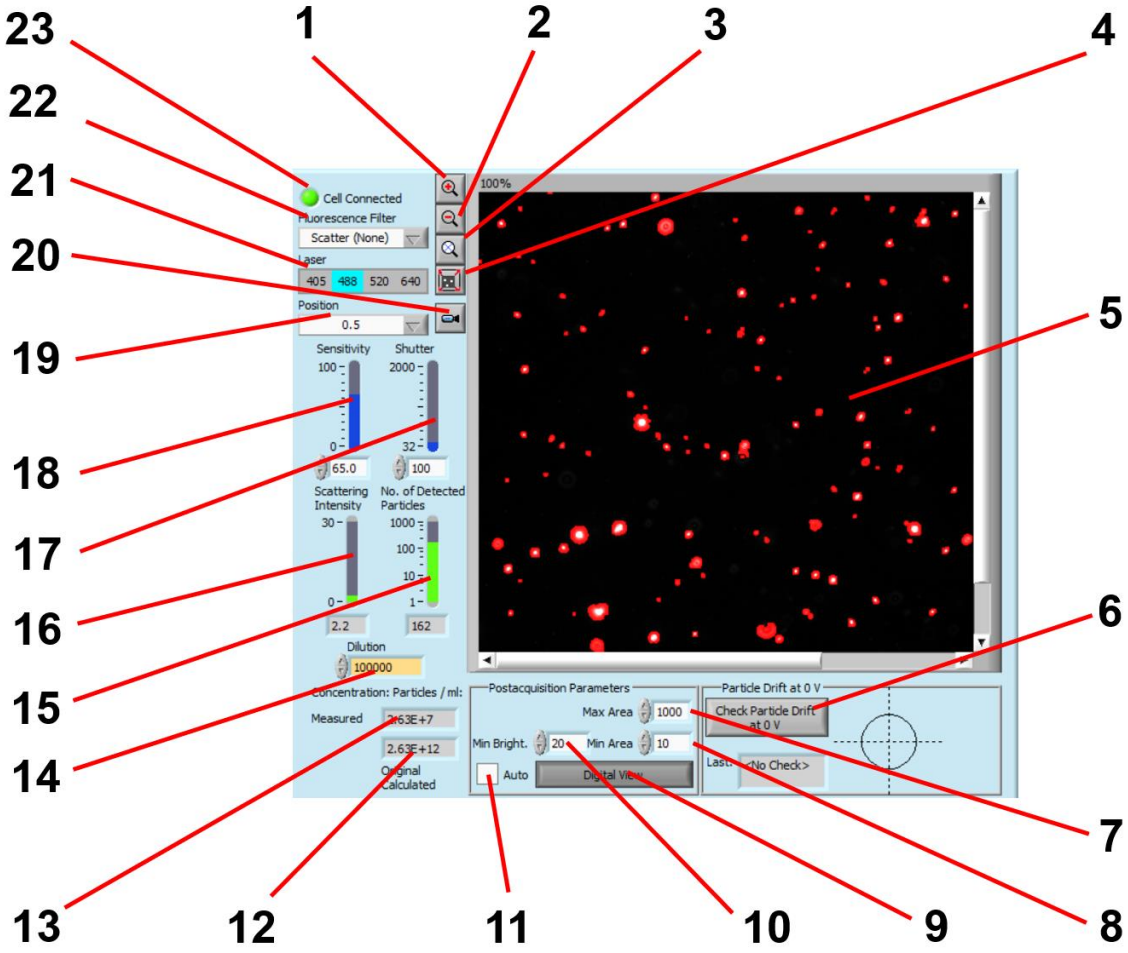


Table 6.1: Description of parameters and functions that are common in the “Cell Check” tab, “Pump&Temp” tab and “Measurement” tab.

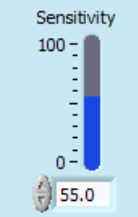
Number	Function	Remarks
1	Zooms the live view in	Provides a zoomed image of the live view. Since it is a digital zoom the function is of limited use.
2	Zooms the live view out	Provides a zoomed-out image. Since it is a digital zoom the function is of limited use.
3	Resets the zoom to normal (100%)	100% represents the default value.
4	Enlarge live view window	Generates a separate window with a larger size.
5	Live view of the particles	The live view shows the scattered light of every particle in the field of view in the current measurement position.
6	Checks the speed of movement of the particles in X-direction and Y-direction	Too much drift results in incorrect measurements.
7	Maximum Area	You can adjust the maximum number of pixels a particle must contain for being included in the analysis.
8	Minimum Area	You can adjust the minimum number of pixels a particle must contain for being included in the analysis.
9	Switches between digital and analog view	Helps to find particles that are not visible by eye in the analog view, however, the camera detects them. The digital view shows what the system will track with current settings.
10	Minimum Brightness	Considers the adjusted minimum digital grey-value (brightness) of each particle.
11	Automatic Brightness	Selects automatic brightness threshold. This function is recommended for particles larger than 200 nm showing high scattering intensity.
12	Calculated particle concentration considering the dilution factor	Indicates the real particle concentration if a dilution factor has been entered before the measurement.

Number	Function	Remarks
13	Live read-out of the particle concentration	Indicates the measured particle concentration without considering the dilution factor.
14	Field for entering the dilution factor of the sample	If a diluted sample is measured, the dilution factor corrects the particle concentration result.
15	Shows the current number of the particles detected in the live view. The optimum range is between 50 and 200 particles.	Number of detected particles is affected by pre- and post-acquisition parameters.
16	Scattering intensity of the particles in the live view	Shows the scattering intensity of the particles in arbitrary units in the field of view. Scattering intensity should stay in the green range.
17	Shutter of the camera	Adjust the duration of the exposure time of the camera. The indicated value is reciprocal in seconds (e.g. 1/70 sec). High values mean short exposure time, low values represent longer exposure times. The shutter is a pre-acquisition parameter.
18	Sensitivity of the camera sensor. Depending on the scattering behavior of the sample the sensitivity should be adjusted accordingly	The sensitivity directly affects the detected number of the particles. The sensitivity is a pre-acquisition parameter.
19	Shows the measuring position during measurement; Change position manually	Provides you with information about the current measurement position. Via drop down menu you can choose and change the measurement position.
20	Gives access to camera controls such as gain, width and height of field of view, laser timing, synchronization with the laser and frame rate	Except frame rate no other parameters should be changed if you are not asked for that (e.g. during trouble shooting).

Number	Function	Remarks
21	Buttons for switching between the laser wavelengths and turning the lasers on and off	Buttons may differ dependent on numbers and wavelengths of the lasers built into the ZetaView® instrument.
22	Drop down menu for switching between the scatter mode and fluorescence mode	If the ZetaView® instrument is not equipped with fluorescence option, no buttons are available here.
23	Status LED for cell connection	Indicates that the measuring cell is connected and detected.

6.1.1 Sensitivity

The sensitivity of the camera sensor can be changed and adjusted on the blue sensitivity bar that is accessible in the tabs “Cell check”, “Pump&Temp” and “Measurement”.



The sensitivity values can be adjusted in three different ways:

1. By entering the desired value in the window
2. By clicking on the arrows located on the left side of the sensitivity value up and down
3. By moving the blue bar up and down with the mouse

The sensitivity setting ranges from 0 to 100 and is given in arbitrary units. In terms of operation, the sensitivity of the camera sensor in the ZetaView® instrument is comparable to the sensitivity setting of a camera used in digital photography (ISO value).
Sensitivity must be adjusted accordingly, depending on the scattered light of the particles in the sample and their refractive index. It is true that very small particles (20-60 nm) and those which have a low refractive index require a higher sensitivity setting than particles which are very large (300-1000 nm) and/or have a high refractive index.

Note that the number of detected particles in the field of view is associated with the sensitivity setting of the camera. This means that as the sensitivity increases, so does the number of particles detected in each sample. This can be seen in the field of view as well as on the bar "No. of Detected Particles" (ref. sections 6.1.3 and 6.1.4).

The following figure shows the same sample observed with three different sensitivity settings. In the field of view and in the bar "No. of Detected Particles "different numbers of particles can be recognized.

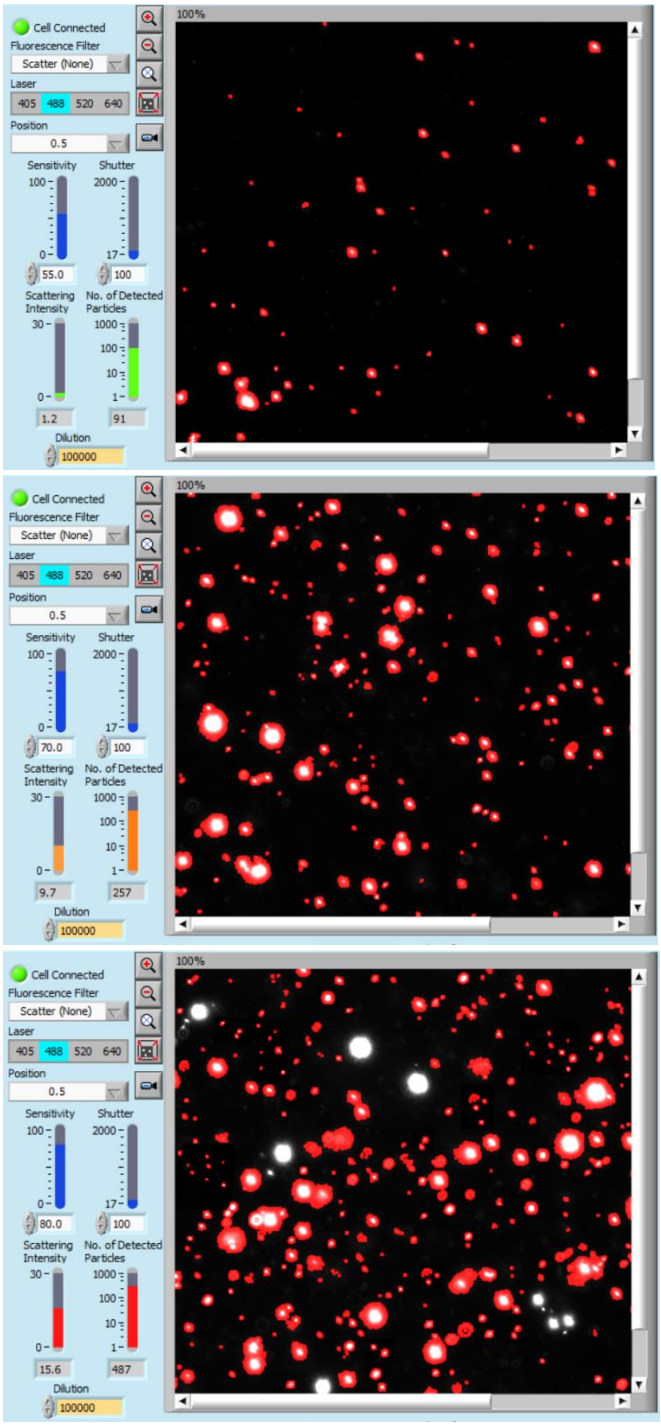


Figure 6-1: Dependence of the "Number of Detected Particles" on the sensitivity setting.

Depending on the sensitivity setting, a different number of particles will be detected. Basically, as the sensitivity increases, so does the number of particles detected. At the same time, the particles appear larger and slightly blurry when the sensitivity is increased. This is because the scattered light of the particles appears brighter due to the fact that the camera sensor can catch more scattered light.

This is also reflected in the "Scattering Intensity" bar. The sensitivity of a given sample in the ZetaView® should always be adjusted so that the “Scattering Intensity” bar is in the optimal green range, or just turning orange (below a value of 8, ref. section 6.1.3 and 6.1.4). When the scattering intensity bar is red, many of the counted particles are actually noise. This can be seen by toggling the analog/digital button. The maximum number of particles allowed in each viewing area is determined by the max track radius squared parameter.

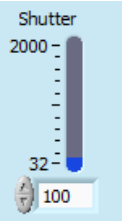
It should be noted that the ZetaView® device is a camera-based device, which means that it (like digital photography) detects different numbers of particles depending on the sensitivity settings. In order to be able to compare all samples within a test series, they must be measured with the same sensitivity settings. This also applies to the camera shutter (see section 6.1.2).

6.1.2 Shutter

The shutter of the ZetaView® camera allows light to pass for a determined period, exposing the photosensitive digital sensor to light in order to capture a permanent image of the scene. The shutter features variable speed and is used to control the exposure time of the movie.

The shutter value can be adjusted manually from 32 to 2000.

The shutter can be changed and adjusted on the blue shutter bar that is accessible in the tabs “Cell Check”, “Pump&Temp” and “Measurement”.



The shutter values can be adjusted in three different ways:

1. By entering the desired value in the window
2. By clicking on the arrows located on the left side of the shutter value up and down
3. By moving the blue bar up and down with the mouse

The set shutter value corresponds to the reciprocal of one second. For example, a shutter value of 50 thus corresponds to an exposure time of 1/50 second. In comparison to that, a shutter value of 400 indicates an exposure time at 1/400 second and is therefore much shorter.
Like the sensitivity (see section 6.1.1), the setting of the shutter affects the number of particles in the field of view, but to a much lesser extent.

The following figure shows an example of how the “No. of Detected Particles” and the scattering intensity of the shutter values (50-400) append at a laser wavelength of 640 nm. However, it applies for other wavelengths available for the ZetaView®.

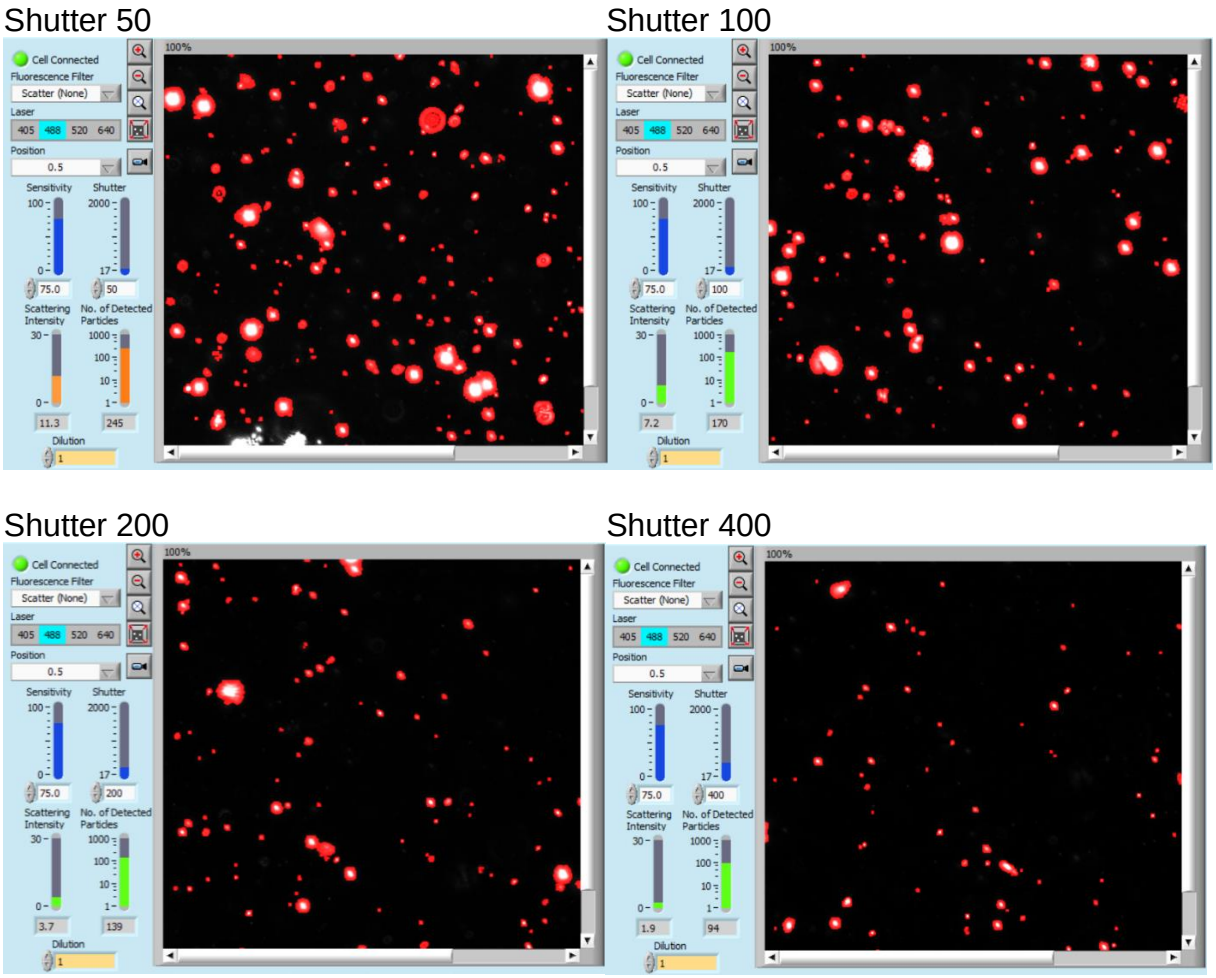


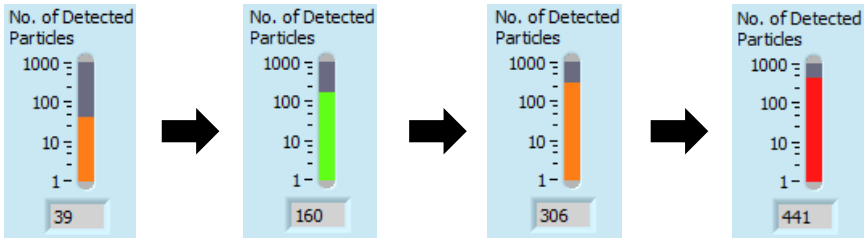
Figure 6-2:Changes of the "Scattering Intensity" and the "No. of Detected particles" depending on different shutter values.

The figure above shows that the number of particles detected, and the scattering intensity depend on the shutter value. The larger the shutter value, the smaller and darker the particles appear in the live view. This is also reflected in the "Scattering Intensity" bar and is due to the fact that less scattered light falls on the camera sensor due to a shorter exposure time. At the same time, due to the short exposure times, small particles are no longer detected in the live view, with the result that the number of particles detected decreases at high shutter values.

The optimum setting of the shutter is to keep the scattering intensity of the particles at a level in the green range. This is particularly important if the particles in a sample requires high sensitivity or if a whole set of samples is to be measured and the samples are to be compared.

6.1.3 No. of detected Particles

The information bar "No. of Detected Particles" displays the current number of particles in the field of view in the selected measurement position. Since the particles move due to the Brownian motion and particles move out of or into the field of view of the camera, the number fluctuates constantly. The number of detected particles is marked with a color code that matches that of the function “Number of Particles vs. Sensitivity" (see section 6.2.4).



1-49 particles | 50-200 particles | 201-400 particles | >400 particles

Figure 6-3: Color code and corresponding particle numbers.

The optimal number of particles for a robust statistic measurement should be between 50 and 200 particles. The upper orange area represents a particle count of 201-400, in which a reliable measurement also works. With more than 400 particles the red area starts. If you start a measurement with this number of particles, the instrument will allow this, but it is not recommended. The reason for this is that too many particles can interact with each other in the field of view and cover each other, which can lead to incorrect measurement results. Therefore, a dilution of the sample or a reduction of sensitivity (see section 6.1.1) is recommended.

6.1.4 Interplay of Sensitivity, Shutter, Scattering Intensity and No. of Detected Particles

The parameters “Sensitivity”, “Shutter”, “Scattering Intensity” and “No. of Detected Particles” are directly related. On the device side, the sensitivity and shutter can be actively changed. No. of detected particles can be changed by the operator by modifying the concentration of the particles in the sample. Only the “Scattering Intensity” is detected by the ZetaView® and cannot be changed directly on the device.

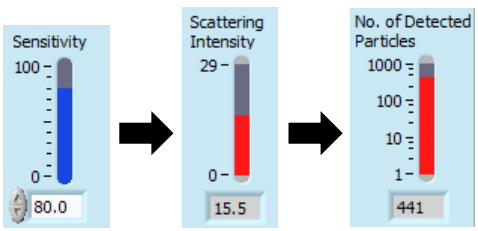
Changing the sensitivity:

If a sample is present in the measuring cell of the ZetaView® and the sensitivity of the camera sensor is changed with otherwise constant parameters, the scattering intensity of the particles in the field of view and the “No. of detected particles” changes.

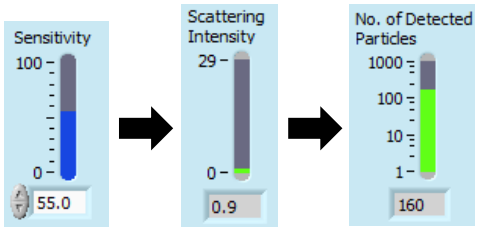
Increasing the sensitivity leads to the camera sensor being more sensitive to the scattered light of the particles. As a result, the scattering intensity detected by the device increases and in the field of view each individual particle is displayed brighter and larger. At the same time, increasing the sensitivity causes an increase in the number of particles detected.

The reason is that particles that show low scattered light behavior (e.g. particles are quite dark and were not visible before increasing the sensitivity), can only be detected by increasing the sensitivity. This mainly affects small particles. Therefore, it is important to increase the sensitivity so that even smaller particles can be detected in the sample.

If the sensitivity is lowered, the camera sensor detects less scattered light of the particles in the sample. As a result, the scattering intensity of the particles as well as the number of particles in the field of view are lowered. In addition, the particles appear smaller due to the smaller volume of scattered light. A low sensitivity is therefore associated with a low number of particles, regardless of whether there are many particles in the measuring cell of the ZetaView® instrument.



Increasing the sensitivity results in higher scattering intensity and larger No. of Detected Particles



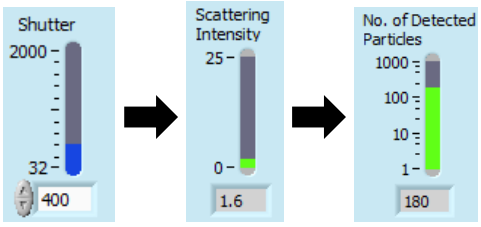
Decreasing the sensitivity results in lower scattering intensity and lower No. of Detected Particles

Figure 6-4: Dependence of “No. of detected Particles” and “Scattering Intensity” on high and low sensitivity.

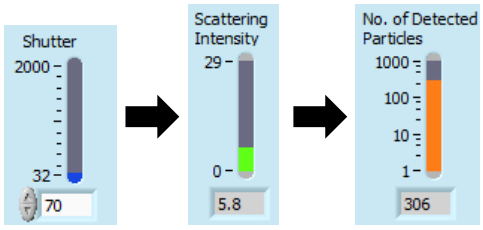
Changing the shutter:

If a sample is located in the measuring cell of the ZetaView® and the shutter is then changed with otherwise constant parameters, the scattering intensity of the particles in the field of view and the “No. of detected particles” changes.

Increasing the shutter value causes the exposure time at the camera sensor to be shortened. As a result, the scattering intensity detected by the device decreases and in the field of view each individual particle is displayed darker and smaller.
At the same time, increasing the shutter value causes a decrease in the number of detected particles. However, this decrease is far less significant than lowering the sensitivity. The reason for the decreased number of detected particles at high shutter values is short exposure time, resulting in less light scattered light on the camera sensor.
Small particles, that already show weaker scattering intensity, are then no longer detected. Therefore, it is important to adjust the shutter value in a way, that even smaller particles in the sample can be detected.
If the shutter value is lowered, the exposure time is longer, and the camera sensor can detect more scattered light from the particles in the sample. As a result, the scattering intensity of the particles, as well as the number of particles in the field of view increases. In addition, the particles appear larger due to the higher volume of scattered light. A low shutter value is therefore associated with a higher number of particles.



Increasing the shutter results in lower scattering intensity and lower No. of Detected Particles



Decreasing the shutter results in higher scattering intensity and higher No. of Detected Particles

Figure 6-5: Dependence of “No. of detected Particles” and “Scattering Intensity” on high and low shutter value.

The following figure illustrates the dependence of scattering intensity and “No. of Detected Particles” on various sensitivities and shutter values by showing a matrix of images.

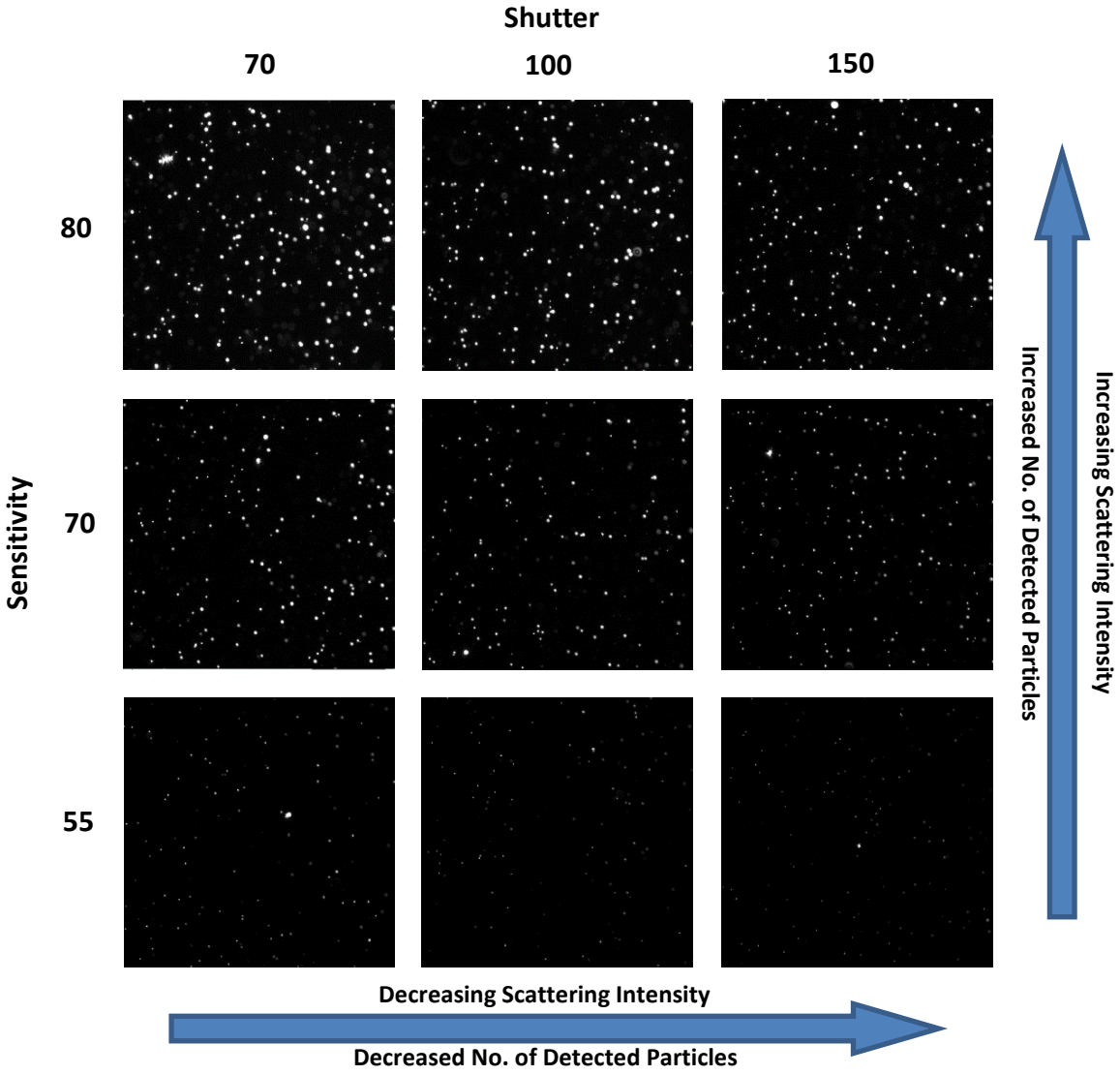


Figure 6-6: “Scattering Intensity” and “No. of Detected Particles” are dependent on sensitivity and shutter settings.

With increasing sensitivity and low shutter value, the “Scattering intensity” and the “Number of detected particles” increase. By lowering the sensitivity and increasing the shutter value, the “Scattering Intensity” and the “Number of Detected Particles” get lower.

Change in the concentration of the particles:

If the camera parameters remain the same, the scattering intensity of the particles in the field of view changes depending on the number of particles detected. A high concentration of particles (high “No. of Detected Particles”) in the sample thus leads to a higher scattering intensity than a low concentration of particles of the same sample with constant camera parameters.

It is therefore recommended to adjust the concentration of the particles in the sample set of a series of measurements by dilution or by increasing the particle concentration in the sample (if possible) so that all samples can be measured with a specified sensitivity. Only then is it possible to directly compare all samples after the measurement.

Changing the Scattering Intensity:

Changing the Scattering Intensity is an indirect process that results from changing the camera's sensitivity, the shutter value, and / or the concentration and size variance of the particles in the sample. It can therefore not be actively set by the user on the device itself.

6.1.5 Dilution, dilution factor and concentration

As already mentioned, the ZetaView® instrument is very sensitive for low particle concentrations. A minimum particle concentration of $5x10^6$ x cm^{-3} can thus be measured reliably. Therefore, in most cases, depending on the origin and the type of purification of the particles and the size of the particles, the sample must often be diluted prior the measurement.
As a rule of thumb, assuming the same mass concentrations, the smaller the particles, the larger the dilution required.

The ZetaView® instrument has a window in which the dilution factor of the sample can be entered. For a 100 nm polystyrene alignment suspension, the dilution factor is typically 1:250,000.

In the "Measured" window, the currently measured particle concentration in "Particles / ml" is displayed live in the sample, regardless of whether a dilution factor was entered or not. The measured number of the particles in the field of view is indicated on the display “No. of Detected Particles”.

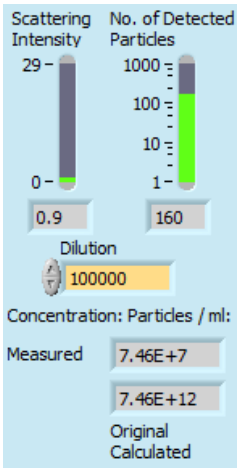


Figure 6-7: The measured ("Measured") concentration of the particles is directly linked with the display "No. of Detected Particles”. It is not influenced by the dilution factor.

The number of particles in the field of view is therefore converted into the particle concentration, considering internally adjusted concentration calibration factors. Because the particles move due to the Brownian motion and some particles move out of or into the field of view of the camera, the concentration and the “No. of Detected Particles” fluctuates constantly.

If a dilution factor has been entered, the "Original Calculated" window shows the original concentration of the particles in the sample from which the diluted sample derives. If no dilution factor is entered, this corresponds to the value 1 and the "Original Calculated" window shows the same value as in the "Measured" field.

Dilution factor, measured concentration and the calculated original concentration are displayed in the pdf report (ref. section 7.3).
It should be noted that the dilution factor can no longer be changed after a measurement.

6.1.6 Post-Acquisition parameters

The post-acquisition parameters are digital filters applied to images. The camera produces a grey scale image. By clicking on the button “Digital/Analog View” either the grey scale image (analog view) or the black and white image (digital view) is shown. For better contrast, the particles in the digital image are colored in red. Parameters are optimum if the background is perfectly black with no noise visible. Use the “Digital/Analog View” button to toggle between analog and digital view.

The digital view directly visualizes the result of the post-acquisition parameter settings. All particles visible in the digital view are picked up for analysis.

For comparison, digital and analog view from the same image are shown in the figure below.

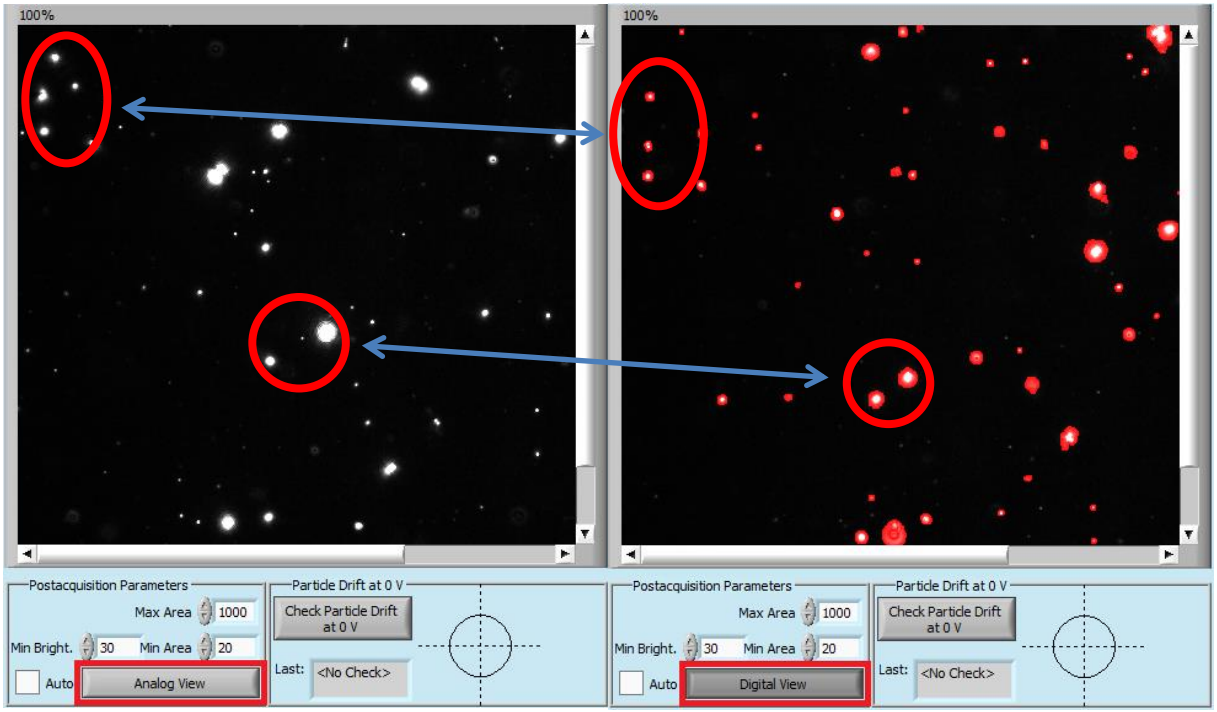


Figure 6-8: Analog and Digital View.

The outcome of the digital view is directly influenced by the post-acquisition parameters (“Minimum Brightness”, “Max Area”, “Min Area”) while the analog image is determined by the pre-acquisition parameters (“sensitivity” and “shutter”). This is an enormous advantage as the user directly sees in a live image what will be fed into the algorithm.

6.1.6.1 Minimum Brightness

The minimum brightness is the threshold of when a grey value is recognized as a white pixel. When the grey value is lower than the threshold, the pixel will be black (background). All the information for the subsequent image processing of tracking and analysing is in the white pixels; therefore, the value for decision between background noise and information needs to be chosen carefully. Typical values are ranging between 15 (weak scatterer) and 150 (strong scatterer). Default values for biological particles such as extracellular vesicles are ranging between 20 and 30.

6.1.6.2 Maximum Area

The particles in the field of view appear as spots. This means that each red spot in the digital image represents a particle and has a certain area. The area is measured in pixels, which are the sum of red pixels of the corresponding spot. When the spot size is rather small (for example below 5 pixels) the spot can be regarded as noise.

With “Max Area”, the maximum number of pixels per spot (particle) allowed can be adjusted.
The following image shows a comparison between the “Max Area“ settings of 75 and 1000.

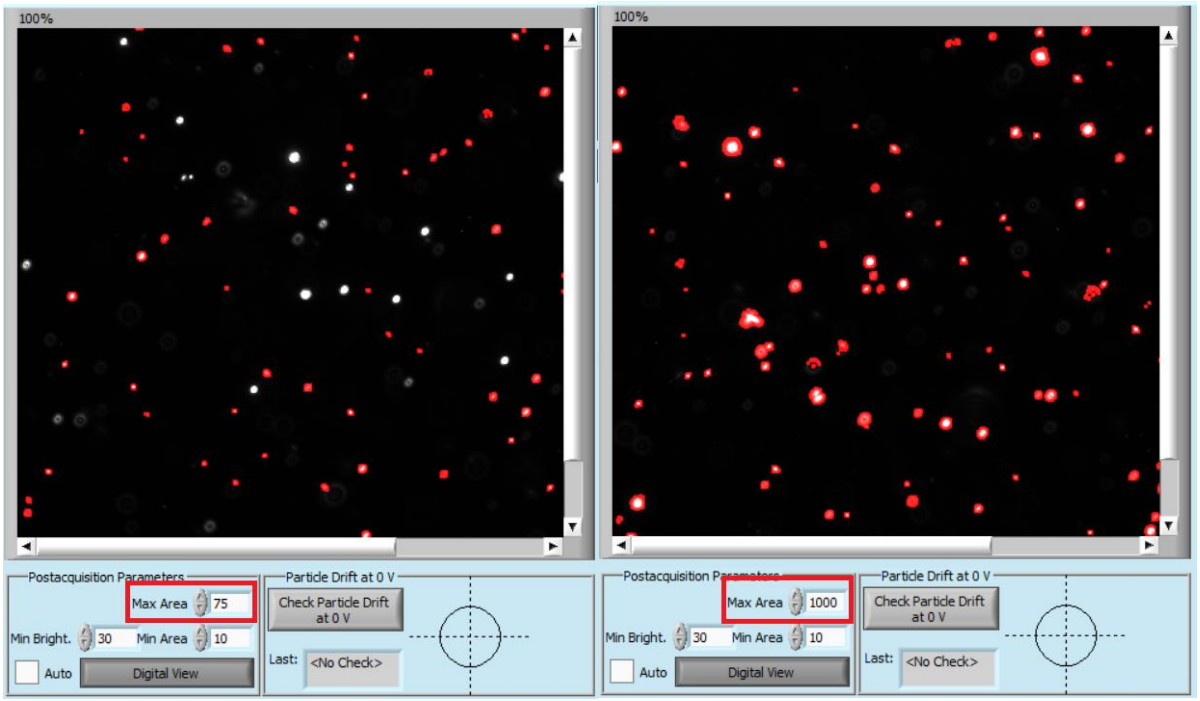


Figure 6-9: Comparison between “Max Area“ adjusted to 75 and 1000.

On the left side, white particles have a larger pixel number than 75; therefore, they will not be picked up in the analysis. Red colored particles comprise 75 pixels or less and

will be included in the analysis. On the right side “Max Area“ is adjusted to 1000. All particles are colored in red and will be picked up for subsequent analysis.
For the above example, please note that the final result of the particle concentration is lower for “Max Area“ 75 than for “Max Area“ 1000. The reason is that white particles do not fulfill the adjusted “Max Area“ and will therefore not be analysed.

For biological particles such as extracellular vesicles or similar particles in a size range between 70 and 400 nm, “Max Area“ setting of 1000 is adequate for including all particles into the analysis.

When measuring small particles (e.g. below 100 nm), if you see spots of quite large sizes (e.g. above 500 pixels), in most cases these spots are large agglomerates or contaminations. Such strong scatterers may hamper image quality and result in poor particle size distribution. By setting “Max Area“, these spots can be eliminated from further processing.
When measuring large particles (>500 nm), please verify if the large particles are still present (colored in red) in digital view. You may increase the “Max Area“ setting to 2000 or 10000.

Please note that the size of the spots is significantly dependent on the pre-acquisition parameters (sensitivity and shutter). With a high sensitivity or with a low shutter value, the spots (and thus the particles) appear larger because the particles show a higher volume of scattered light. Conversely, the spots appear smaller if the sensitivity is low and the shutter value high. It is therefore recommended to keep an eye on the scattering intensity when setting the post-acquisition parameters, which should ideally not exceed a value of eight.

6.1.6.3 Minimum Area

With the “Min Area” setting, noise can be filtered out and only spots larger than the “Min Area” are preserved. As an example “Min Area” = 50 means that only those spots (=particles) with an area of 50 pixels or larger are accepted. Objects with an area smaller than that are omitted in further analysis. Especially when measuring larger particles, the suppression of small objects may increase stability of the analysis and result.

The following image shows a comparison between the “Min Area” settings of 5 and 50.

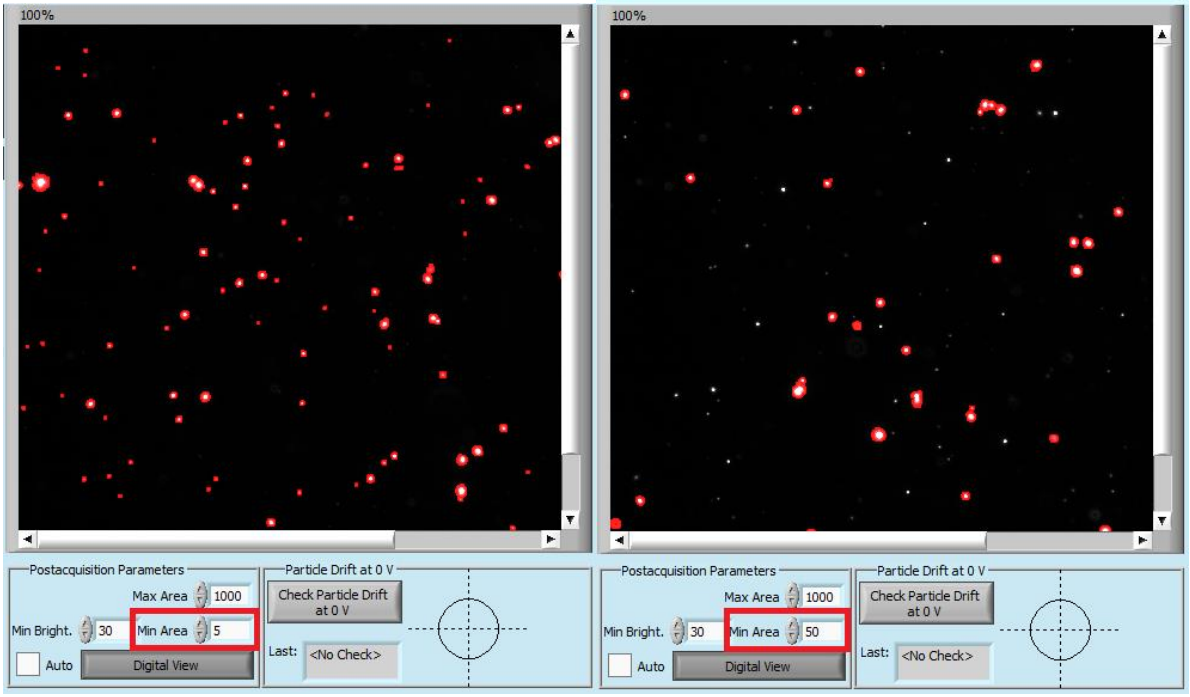


Figure 6-10: Comparison between "Min Area" adjusted to 5 and 50.

On the left side all particles are colored in red and will be picked up for subsequent analysis. On the right side "Min Area" is adjusted to 50. White particles have a lower pixel number than 50 and will not picked up in the analysis. Red colored particles comprise 50 pixels or more and will be included in the analysis.
It is important to consider that the number of detected particles and consequently the concentration output is strongly affected by the post-acquisition parameters.

Please also note that all particles will always be recorded and included in the video file regardless of the post-acquisition parameter settings. The post-acquisition parameters determine which particles will be picked up for the subsequent analysis once the video has been recorded.

6.1.7 Check particle Drift

Drift is the movement of the particles within the measuring cell that goes beyond the normal Brownian movement. There are limits to how much drift the software can handle before size, concentration and zeta potential measurements are compromised. It is still possible to make a measurement, but not recommended because the drift will then adversely impact Brownian motion (size) or electrophoretic mobility (zeta potential) measurements, resulting in incorrect data.

The speed of the particle drift in the field of view can be checked with the "Check Particle Drift" button. The software switches to digital mode so that the operator can observe the drift of all particles. The figure below shows an example in which the drift of the particles is ok and not ok.

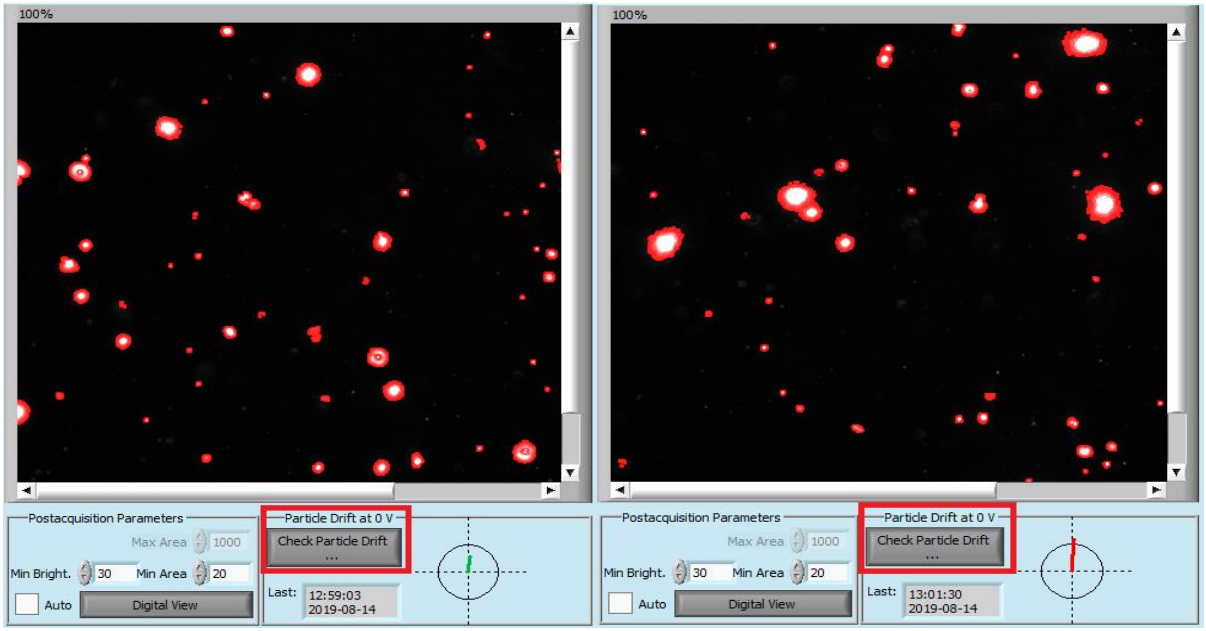


Figure 6-11: In the left picture the drift is in the tolerable range (green line), in the right picture the drift is too high for a measurement (red line).

After clicking the button "Check Particle Drift" you can observe the cross located to the right of this button. As long as the green line has not yet crossed the circle, the speed of the drift is ok, and a measurement can be carried out. If the drift is too high, the line crosses the circle and turns from green to red. A measurement is still possible, but not recommended. The length and orientation of the line reflects the speed and direction of the particle drift.

6.2 Cell Check tab

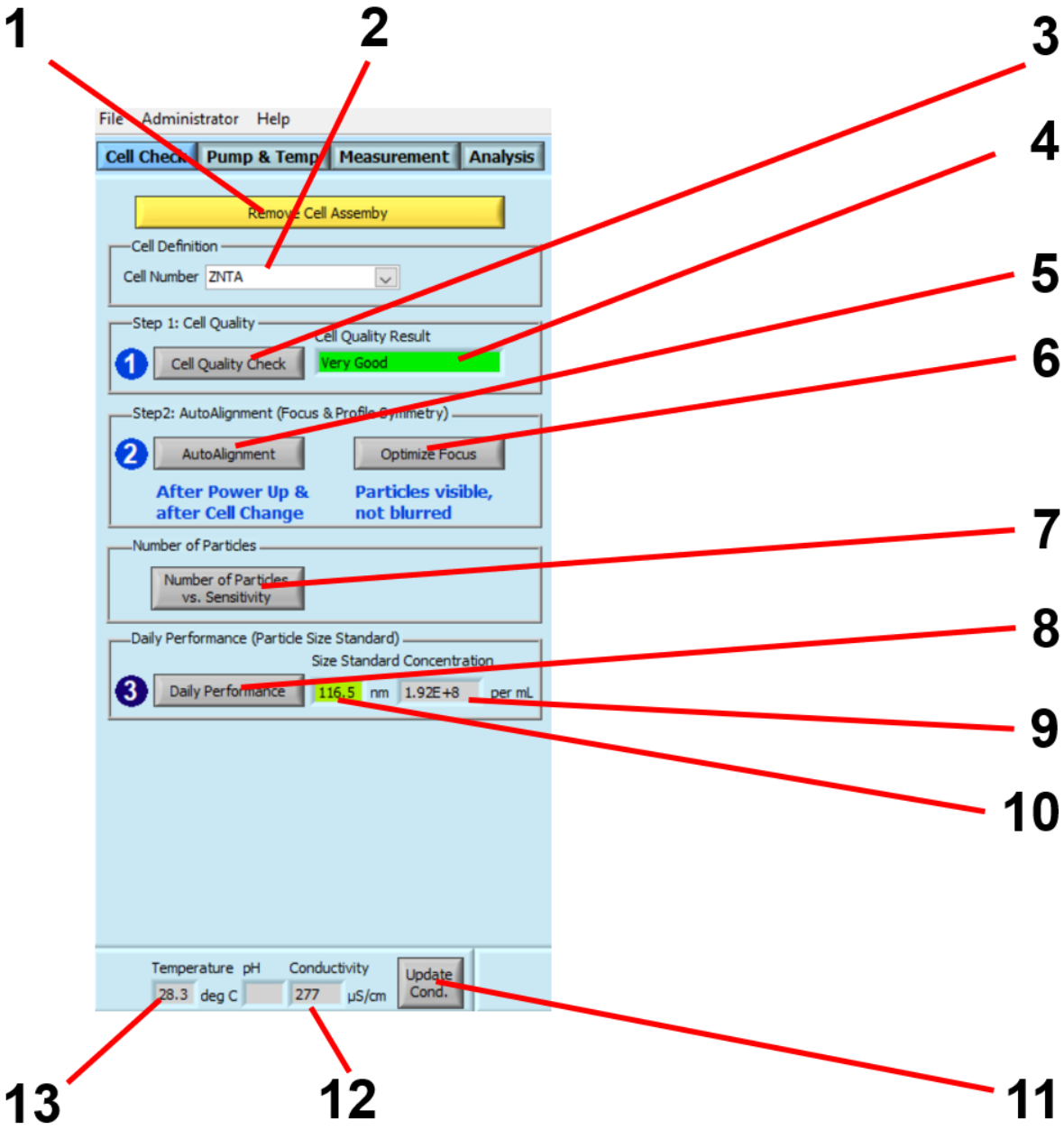
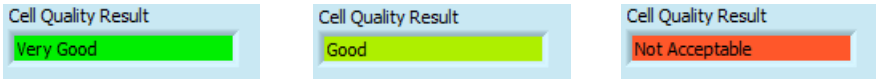


Table 6.2: Description of parameters and functions in the Cell Check Menu.

Number	Function	Remarks
1	Button for removing the Cell assembly	After pressing this button, you are asked to empty the instrument by rinsing it with air.
2	Cell calibration file	Provides the calibration for Z-NTA (with) or NTA (without zeta potential option). Only important for PMX110 instruments.
3	For checking if the cell is free of excessive background scatter	This function is part of the daily start-up routine but can be executed manually at any time.
4	Result of the Cell Quality Check	Colors green, bright green and red highlight the quality result.
5	Important for adjusting the optics (microscope & laser) of the instrument in order to get all particles in focus. However, this is not considered as a calibration. AutoAlignment must be performed with 100 nm polystyrene beads.	This function is part of the daily start-up routine but can be executed manually at any time.
6	For doing a focus adjustment in fine steps.	This function is part of the daily start-up routine. It can be executed manually at any time with 100 nm polystyrene beads as well as with particles of the injected sample.
7	Helps to determine the optimal sensitivity for measuring a specific sample at a given concentration	Provides a rough estimation if the particles in the sample are in the optimum concentration for measurement.
8	Measures “trueness” and “precision” of a freshly prepared 100 nm standard suspension	Most important for GMP purposes.
9	Shows the particle concentration after a daily performance measurement	
10	Shows the percentage of deviation of trueness based on size measurement (Peak diameter) after a daily performance measurement	
11	Button for checking the conductivity of the liquid inside the measuring cell	Only available with zeta potential capability.
12	Result of the latest conductivity measurement	Only available with zeta potential capability.
13	Current temperature of the measuring cell	

6.2.1 Cell Quality Check

With the "Cell Quality Check" function, the optical windows of the measuring cell and the liquid in the measuring cell can be checked for cleanliness and absence from particles. In this case, with steadily increasing sensitivity of the camera sensor, the scattered light intensity in the liquid and the particles possibly contained therein are measured. Depending on internal threshold limits set in the ZetaView® software, the software rates the cell quality as “Very Good”, “Good” or “Not Acceptable”.



Depending on the liquid used (commercially available ultrapure water, Milli-Q water, tap water, etc.), the presence of particles or air bubbles that have been injected with the water, and the cleanliness of the optical window of the measuring cell, the result may vary greatly.

If the software shows "Very Good" or "Good", the system releases the measuring cell for measurement, but for the result "Not acceptable" a measurement is not possible. In this case, the cell should be rinsed again. This can be done either by using one of the internal pumps (Pump1 or Pump2) or, if an NTA Cell assembly is used by pump 3 (see section 6.3.1), connected to a water reservoir bottle, and / or by re-injecting 10-20ml of water from a syringe into the injection port. To remove coarse dirt, the cell can also be cleaned manually using the supplied brushes (see chapter 14).

The “Cell Quality Check” feature is part of the daily automatic start-up routine, which also includes the “AutoAlignment” function. However, the “Cell Quality Check” can be started manually at any time.

6.2.2 AutoAlignment

The "AutoAlignment" script is accessible in the Cell Check menu and should be performed after each restart of the ZetaView® and after the Cell assembly has been removed and put back on. The “AutoAlignment” is not for calibrating the instrument for a specific size. Rather it serves to focus the detection optics of the ZetaView® instrument on the particles. It is optimized for 100 nm polystyrene standard beads ("alignment suspension") but not for particles usually measured as a sample. Therefore, it is highly recommended to use only 100 nm polystyrene alignment suspension. After injection of the alignment suspension and starting the “AutoAlignment”, the laser and video microscope are adjusted to one another in a way that the 100 nm polystyrene particles in the alignment suspension are sharply displayed in all 11 measuring positions.

For “AutoAlignment”, between 100 and 300 particles should be detected in the field of view. A 1:250,000 standard dilution of 100 nm polystyrene particles results in approximately 200 detected particles in the field of view. With this number, an “AutoAlignment” is reliably possible.

The AutoAlignment comprises of 3 main steps:

1. The first step involves finding and focusing the 100 nm polystyrene particles in the alignment suspension. In most cases, the 100 nm PS particles are already visible after injection before starting the “AutoAlignment”. However, they can appear either sharp or defocused. If they appear defocused, please ensure that the Cell assembly is engaged properly, and no bubbles are present before continuing.

The step of “AutoAlignment” scans a large focus plane around the particles and adjusts the focus of the optics quite accurately.

The figure below shows this first step of auto-aligning.

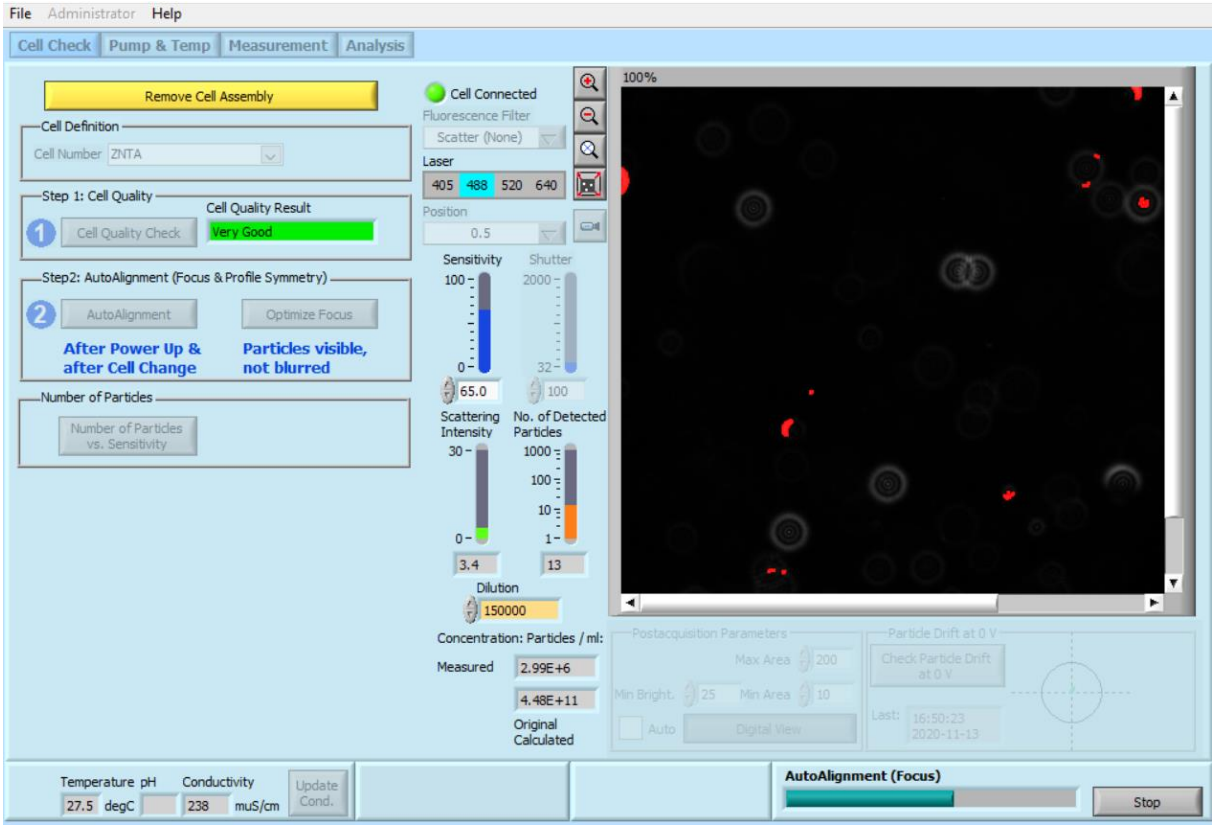


Figure 6-12: The first step of “AutoAlignment” involves finding particles and focusing on them.

The laser and the video microscope will move at the same time during this step, and the particles may appear alternately focused and defocused. The first step of the “AutoAlignment” is also marked with a status bar and the corresponding message "AutoAlignment (Focus)".

2. The second step of the “AutoAlignment” script represents the focus optimization. The sharpness adjustment takes place in fine steps around the focus point already found in the first step. Depending on the quality of the water in which the particles are suspended (larger disruptive foreign particles) or on the age of the alignment suspension, this step will be carried out several times reducing the sensitivity settings for each iteration.

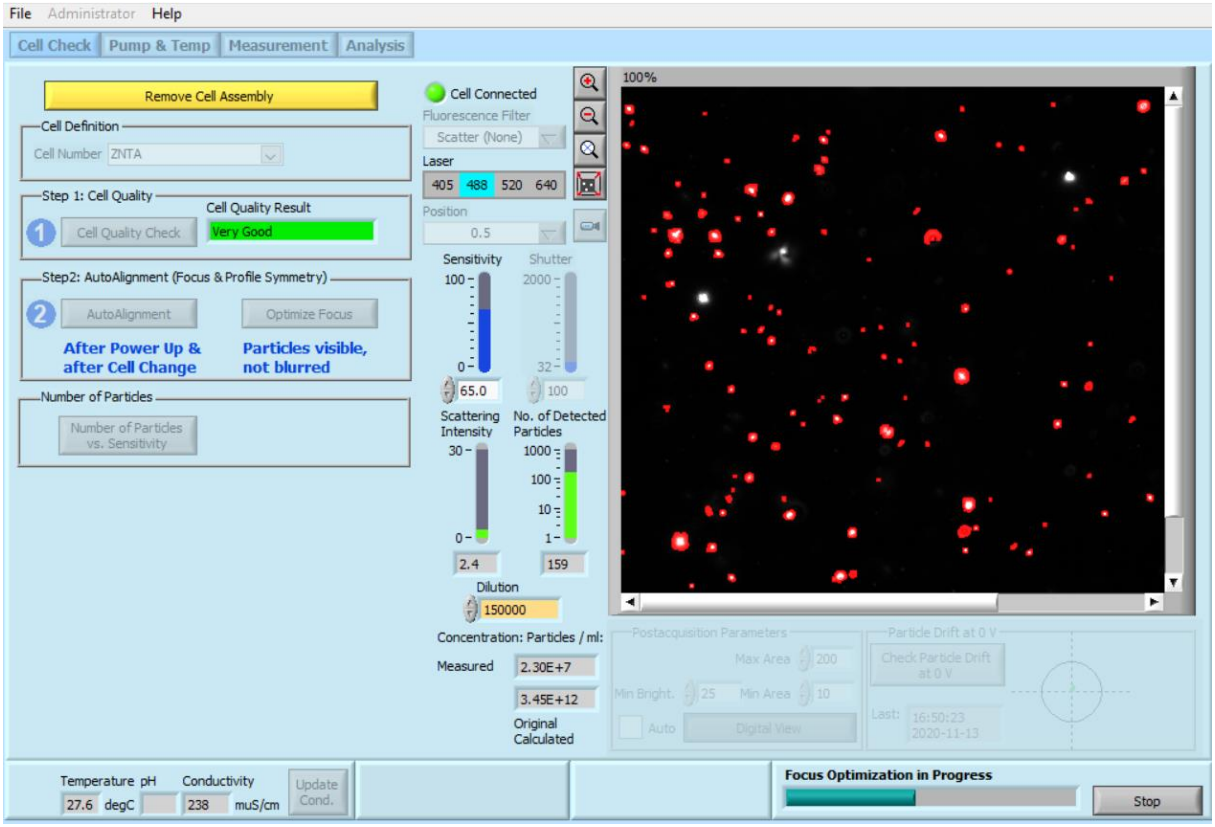


Figure 6-13: In the second step of “AutoAlignment”, the focus is optimized.

The second step of the “AutoAlignment” is marked with a status bar and the corresponding message "Focus Optimization in Progress".

3. The third step includes a zeta potential profile measurement / 11-position measurement (applies only for Z-NTA Cell assembly).

During the zeta potential profile measurement, the electrophoretic mobility of the 100 nm polystyrene particles is determined at all 11 measurement positions. This step is also indicated by a status bar and the associated message “Profile (Autosymmetry) in Progress”.

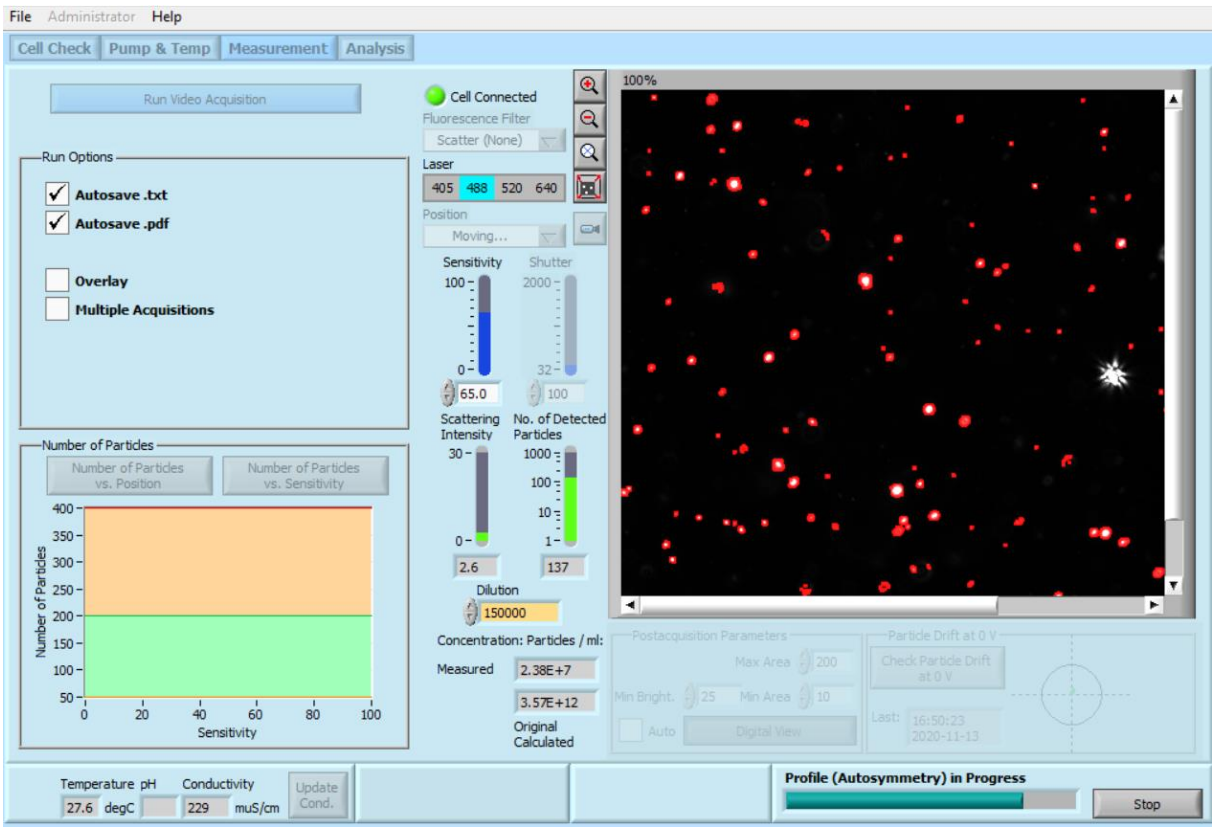


Figure 6-14: In the third step, a profile measurement is carried out.

As a result, a parabola is displayed.

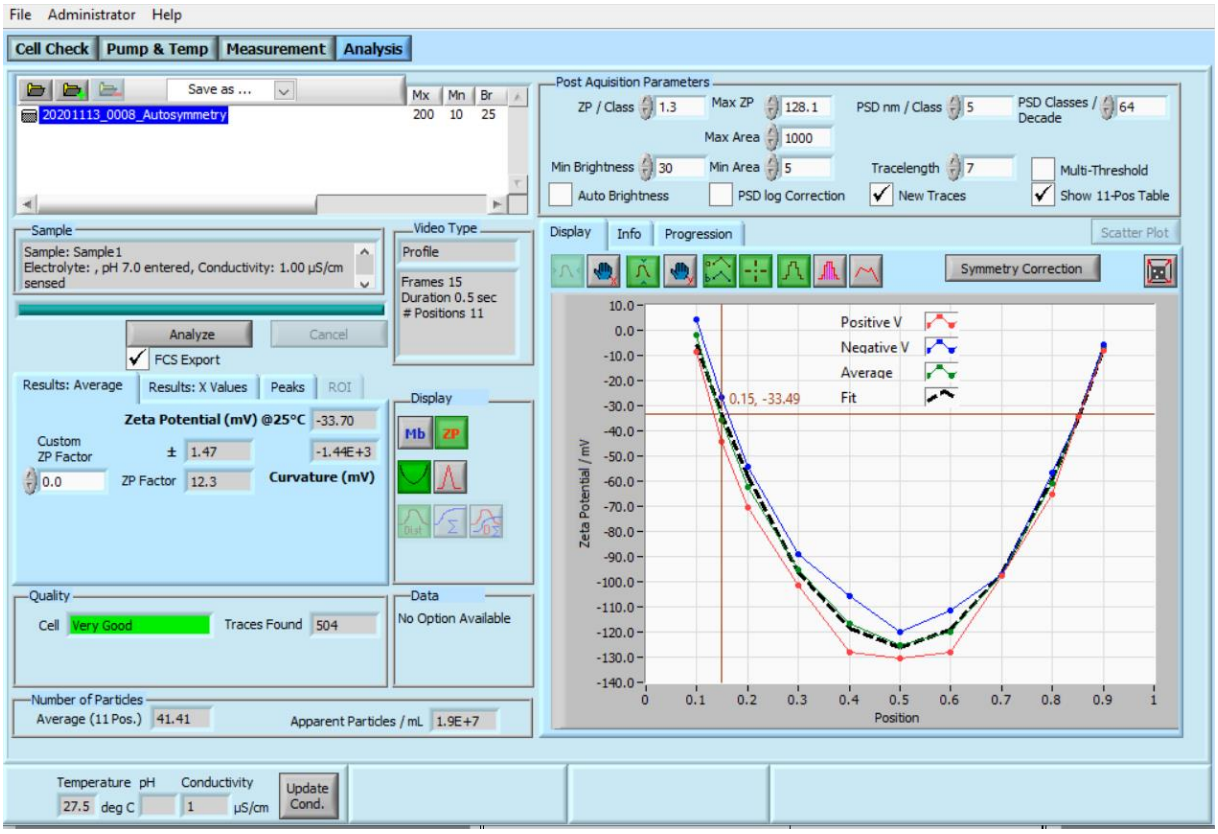


Figure 6-15: Resulting parabola from a profile measurement in the last step of the "AutoAlignment".

The quality of the parabola gives two statements:

1. First, the symmetry of the profile with respect to the x-axis shows whether the optics has been aligned symmetrically. Any asymmetry will be corrected automatically by the software. When repeating the "AutoAlignment" script, the parabola will be symmetrical.

   Each position of the profile (all 11 positions) is based on 4 points marked in RED, BLUE, GREEN and BLACK.

   The mobility resulting from positive current polarity is represented in RED, the mobility resulting from negative current polarity is displayed in BLUE.

   The mean value of RED and BLUE is displayed in GREEN and the resulting value, the parabola fit algorithm is highlighted in BLACK.

2. Second, closely positioned points and a smooth parabola indicate a clean measuring cell without bubbles adhering to the surfaces. On the Analysis tab under

Results:Average, the software states a curvature value in mV indicating the quality of the parabola. A negative curvature value indicates that the parabola opens upwards (opening on the top). Values of |curvature| > 500mV (absolute numbers) indicate a clean measuring cell. Bubbles can be removed from the cell surfaces either by pushing more sample into the measurement cell or, if this is not successful, by cleaning the cell using a soft brush (see chapter 14).

An inverted parabola, with the tip pointing up rather than down, or a zig-zag curve after a profile measurement are a clear indication of cationic contaminants on the cell walls or, more generally, a soiled cell.

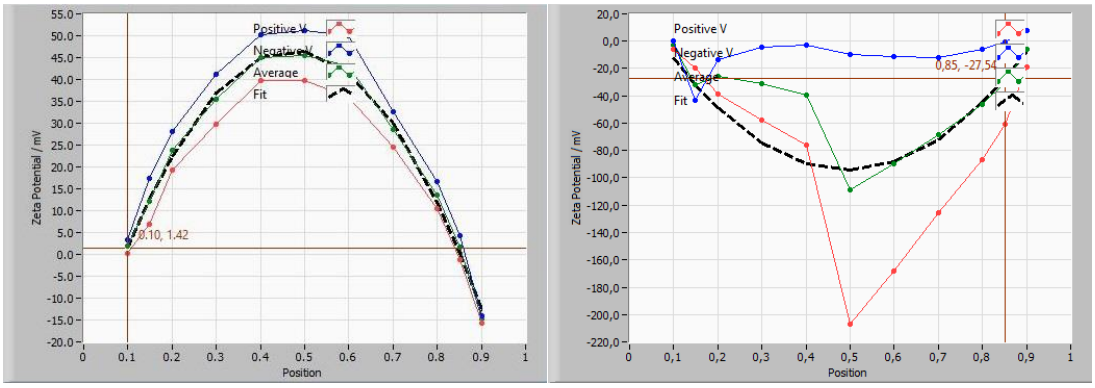


Figure 6-16: Inverted parabola (left) and "zig zag" curve (right) after a profile run. These results derive from cationic contaminations on the cell walls or a dirty measuring cell.

In that case, size and concentration measurements are still possible without any restrictions. Although zeta potential measurements are possible, they will result in incorrect values due to cationic contaminations. It is therefore strongly recommended to first clean the cell and only perform a zeta potential measurement if a normal parabola is shown after a profile measurement.

The skewness of the profile does not indicate that one wall of the measuring cell is filled with more particles than the other. Sedimentation or flotation only affects the upper and lower walls of the measuring cell. The probability of covering the vertical surfaces of the measuring cell with particles or molecules is the same for both surfaces. Therefore, the ZetaView® instrument can be used to coat the cells by e.g. dirt particles and the polarity of their charge are automatically monitored with a profile measurement. The obliquity of the parabola only indicates an asymmetrical alignment of the optics. The reason for this is the vertical orientation of the measuring cell with respect to the axis of movement of the microscope. The orientation of the measuring cell with respect to gravity or optical axes is a means of distinguishing between asymmetric orientation and particle migration. A crooked profile therefore always indicates a bad orientation.

This is corrected by “AutoAlignment” without additional effort of the operator. After successful completion of the symmetry test, measurements can then be started.

The AutoAlignment function is part of the daily start-up routine. However, like the Cell Quality Check function, it can be started manually at any time.

6.2.3 Optimize Focus

The “Optimize Focus” function is accessible in the Cell Check Menu. It represents the second step of the “AutoAlignment”; however, it can be activated separately.

As described before (see section **Fehler! Verweisquelle konnte nicht gefunden werden.**), the sharpness adjustment takes place in very fine steps around the focus point already found. Unlike the “AutoAlignment”, starting the “Optimize Focus” function does not necessarily require particles of the 100 nm alignment suspension but it is also possible to accomplish focus optimization with already injected particles of a sample. Depending on the quality of solvent in which the particles are suspended (large disruptive foreign particles), focus optimization can be carried out several times by the device.

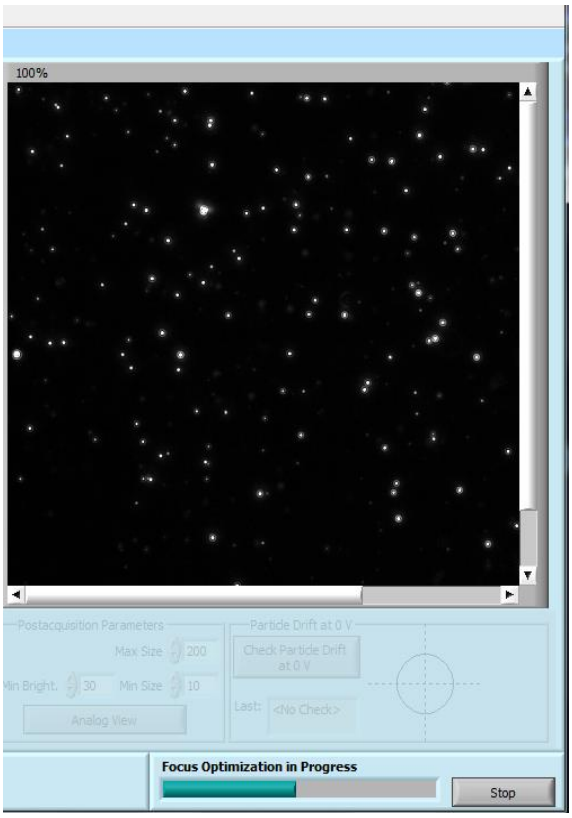


Figure 6-17: Focus optimization can be carried out with already injected particles of a sample, as well as with the alignment suspension.

6.2.4 Number of Particles vs. Sensitivity (NvS)

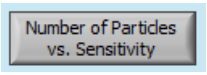
The ZetaView® device is very sensitive to low particle concentrations when compared to other particle analyzers. Since the ZetaView® uses the 90° scattered light of individual particles for the measurements, a minimum particle concentration of $5x10^6$ x cm^{-3} can be reliably measured. Depending on the origin of the particles and the type of purification and their size, the sample typically requires dilution before the measurement.

As a rule of thumb, assuming equal mass concentrations, a larger dilution is required for smaller particles.

To familiarize yourself with the ZetaView® instrument, we recommend starting with 100 nm polystyrene standard particles (alignment suspension), diluted 1:250,000.

The function "Number of Particles vs. Sensitivity" (NvS) helps the user to correctly adjust the concentration of the sample and helps to select the correct sensitivity value for the measurement. Depending on the size, the scattering behavior and the concentration of the particles in an unknown sample, it is sometimes difficult to determine the optimal sensitivity setting for the measurement. The adaptation of the particle concentration is a decisive step. If the particle concentration is too high (e.g. > 400), the particle density in the field of view may be too high for proper measurement. In this case, particle-particle interactions or impact reactions of individual particles can occur. As a result, it may no longer be possible to correctly recognize and trace all the particles since individual particles are covered by others.

The function "Number of Particles vs. Sensitivity "can be accessed both in the Cell Check tab and in the Measurement tab.



When the "Number of Particles vs. Sensitivity" function is started, the ZetaView® scans the entire sensitivity range from 1 to 100 and determines, for each individual sensitivity value, the current number of particles in the sample. This only happens in the measuring position that is currently set. An automated NvS measurement over all 11 measuring positions is not possible. The result is then displayed in a graph in which the sensitivity values of the camera are plotted on the X-axis and the number of detected particles on the Y-axis. In addition, the storage location and the name of the corresponding file is indicated. The storage location is pre-determined, and the file name is automatically assigned. Neither can be changed by the user before saving.

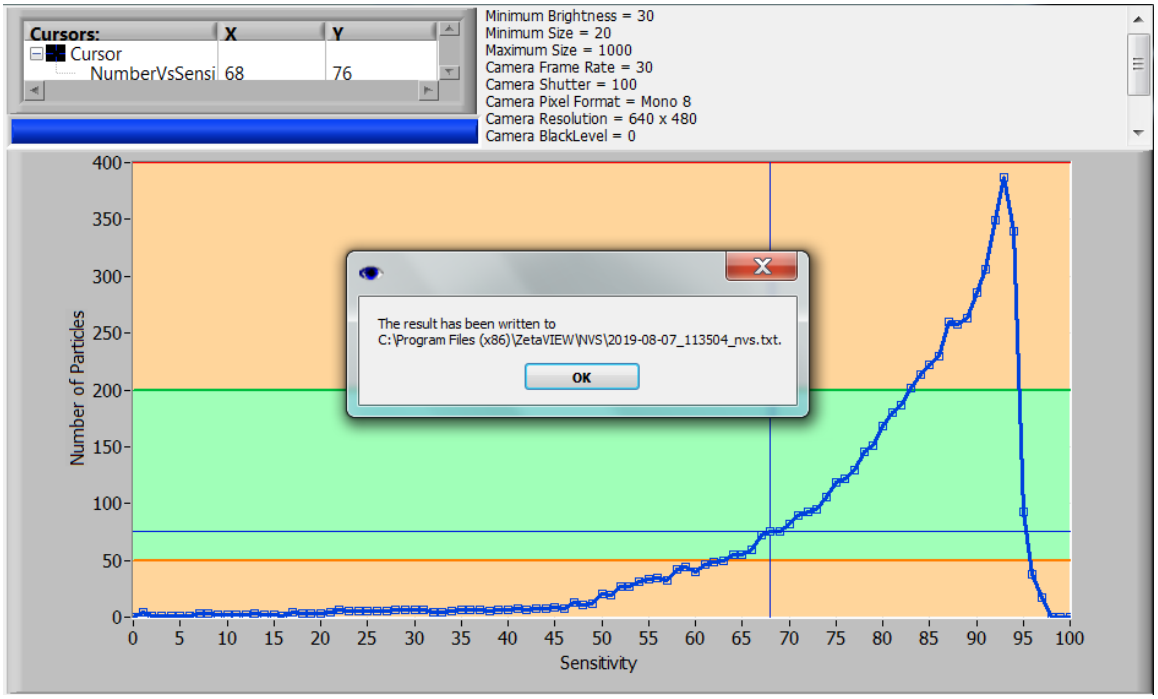


Figure 6-18: Example of a result of an NvS measurement with information about the storage location and file name.

In addition to the diagram shown, a corresponding text file is saved in the ZetaView® folder under NvS in order to subsequently plot the graph in a spreadsheet software (for example, Microsoft Excel, Origin, etc.).

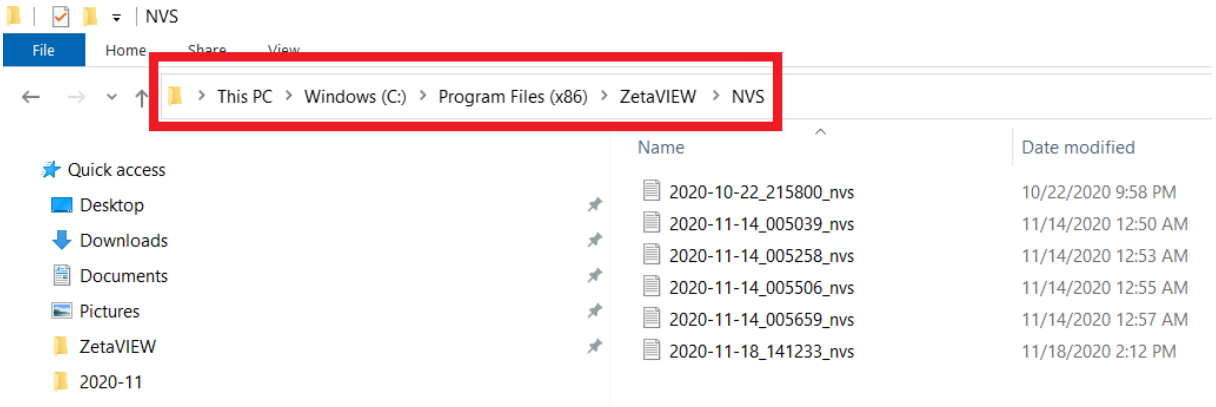


Figure 6-19: Storage location of NvS measurements.

The diagram is provided with the same color code as the indicator bar, in which the “No. of. Detected Particles” (ref. section 6.1.3) can be read. The lower orange area shows 1-49 particles, the green area 50-200, the upper orange area 201-400 and the red area > 400 particles. The red area is shown in the diagram only if the particle concentration in the sample is correspondingly high (>400) at high sensitivities. Otherwise, the red area is not shown.

If you drag the cursor (blue horizontal and vertical line) to a desired sensitivity in the diagram, the window to the left above the diagram shows how many particles were detected at a specific sensitivity value. To the right of this window are shown the post-acquisition parameters as well as the camera settings that were adjusted during the NvS measurement.

In the following figure, four curves of different NvS measurements are summarized by way of example. The image results from 4 individual measurements, which were plotted in MS Excel and colored for better understanding. The ZetaView® software does not offer an overlay over several NvS single measurements.

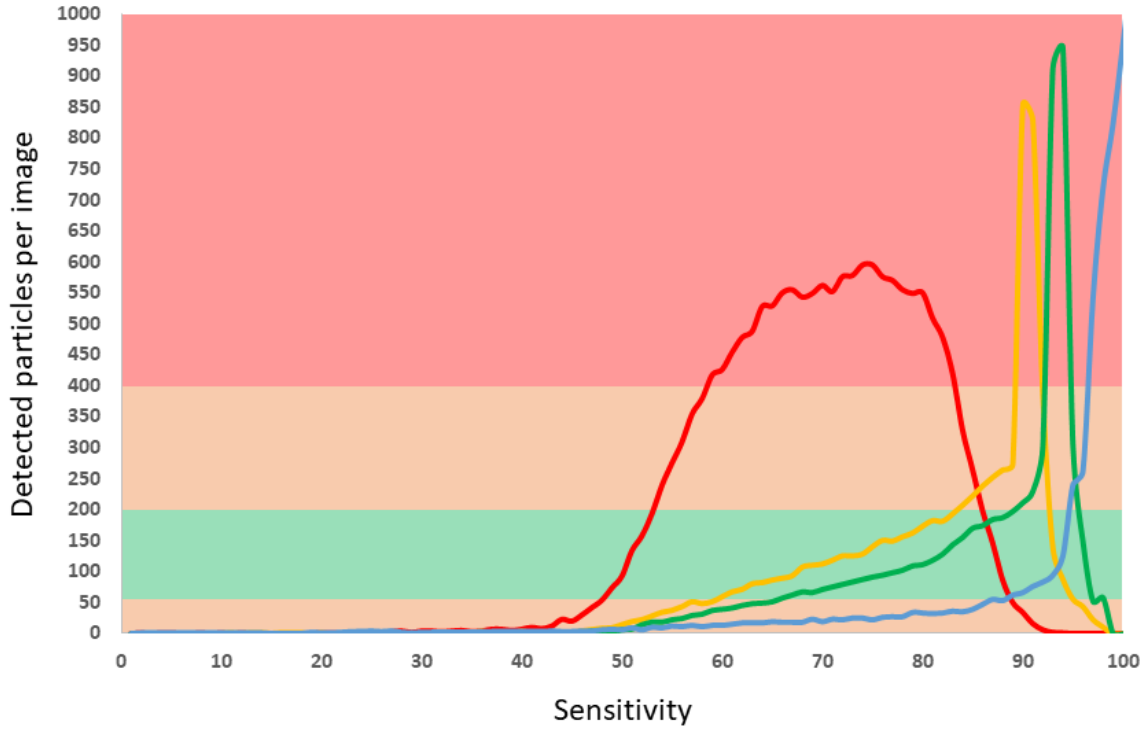


Figure 6-20: Overlay plot of four NvS curves originating from separate NvS measurements. The color code of the diagram matches that one from the bar "No. of Detected Particles".

After dilution (depending on the sample) of a highly concentrated sample (red curve), the maximum shifts to higher sensitivity values (yellow and green curve) or ideally disappears completely (blue curve). In some cases, especially in the presence of very small particles (optimally measured with high sensitivity, low shutter value, and low Minimum Brightness settings, see section 6.1.6.1), the curve shows a sharp increase in high sensitivity settings (yellow, green, and blue curve).

The optimum sensitivity settings are close to the steep slope of the curve, usually at a sensitivity between 75 and 90 for biological samples. The selected sensitivity values should ideally be in the green range that corresponds to a particle count of 50-200 in

the graph. Shutter and Minimum Brightness must be optimized in such a way that curves are created that are analogous to the yellow, green or blue curve.

It is important to note that measurements of high particle concentrations at low sensitivity settings (e.g. at a sensitivity of 60-70) to reduce the number of particles in the field of view to lower values (<1000 red curve) can lead to incorrect particle size distributions.

It should also be noted that an NvS measurement is based on the user-set values for the shutter as well as the post-acquisition parameters “Minimum Brightness”, “Maximum Area” and “Minimum Area” (ref. section 6.1.6). If a new measurement of the same sample is carried out with changed shutter or post-acquisition parameters, a different course of the graph may result.

Since an NvS measurement represents a snapshot of the particles in the field of view of the measuring cell, the result may differ slightly from that of the previous measurement, even with the same camera settings and post-acquisition parameters. This is because the same particles will not be measured in each NvS experiment, as they are constantly in motion due to the Brownian movement. For several NvS measurements, it is therefore advisable to ensure that the particles of the sample are homogeneously distributed in the measuring cell in order to obtain comparative graphs.

As already mentioned above, a sensitivity in the green range (corresponds to a particle number of about 50-200) should be selected for an optimal measurement. If a lower sensitivity is selected for a measurement, particles with weaker scattered light, usually small particles, may not be detected and thus not measured. For small biological particles between 70 nm and 150 nm, sensitivity between 75 and 90 is recommended. However, you may need less sensitivity for metals, Q-dots and other bright particles with a high refractive index.

6.2.4.1 NvS helps to find the optimal measuring concentration

The NvS function can be very helpful for adjusting the optimal measuring concentration of the particles in the sample. There may be samples to be measured with user-specified sensitivity. This is usually the case when it has already been found in previous experiments which sensitivity is most suitable for measuring the concentration and size of the particles.

If the number of particles after an NvS measurement is too high at the sensitivity that one wishes to use for the actual size-, concentration- or zeta potential determination, the sample must be diluted accordingly. This is explained in the following two illustrations:

We assume that a specific sample should be measured for size- and concentration-determination with a specified sensitivity of 70. The sample was diluted 1:100,000. NvS measurement of the sample resulted in a particle count of 504 with a sensitivity of 70.

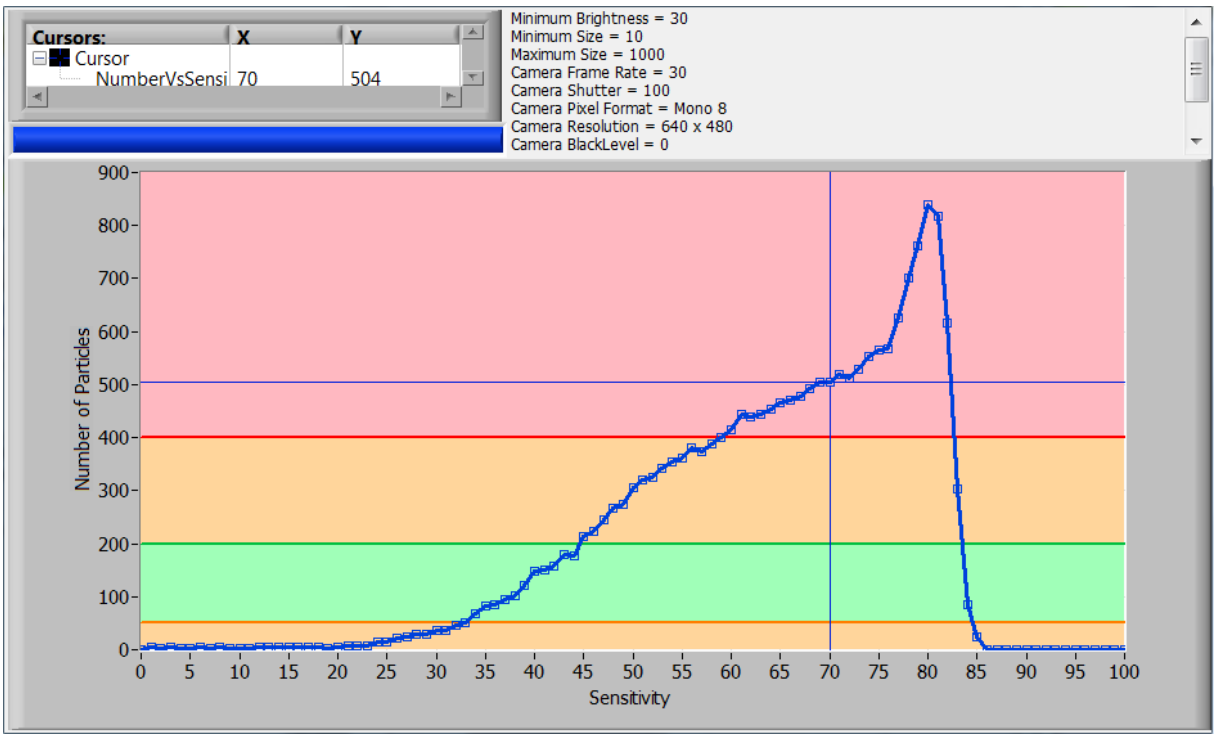


Figure 6-21: The result of the NvS measurement shows a particle count of 504 at the desired sensitivity of 70. The sample was diluted 1:100,000.

Since this number of particles is in the red range and too high to be measured reliably with the desired sensitivity of 70, the sample must be diluted so much that the number of particles at the desired sensitivity is in the green range (50-200). Reducing the sensitivity to between 35 and 45 is not recommended because small and dark particles will not be detected, resulting in an incorrect particle size distribution and incorrect particle concentration data.

In this example, we decide that the already diluted sample should be diluted again by a factor of 5. This will eventually result in a 1:500,000 dilution, taking into account the first dilution of the sample.

An NvS measurement of this highly diluted sample results in a particle count of 111 at the desired sensitivity value.

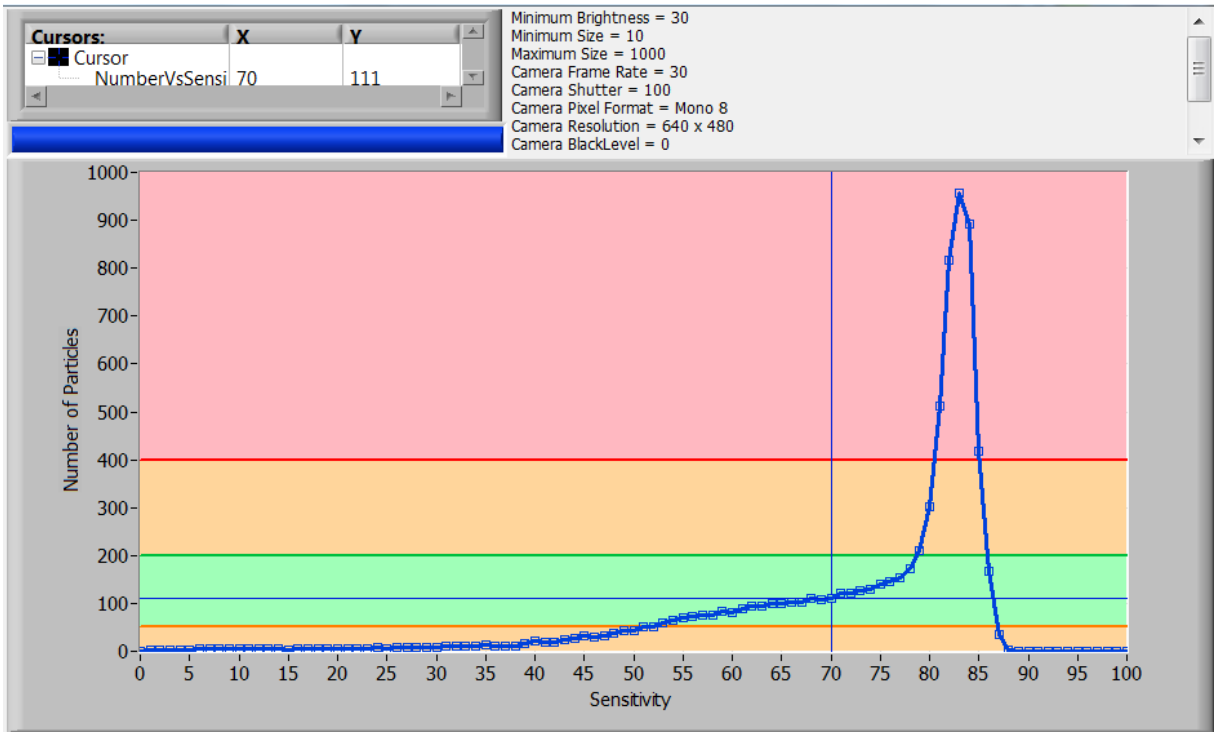


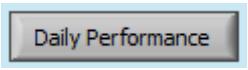
Figure 6-22:This time the result of the NvS measurement shows a particle count of 111 and is in the green range at the desired sensitivity of 70. The sample was diluted 1:500,000.

Consequently, for a subsequent size and concentration determination, the sample should have a dilution of 1:500,000. This example illustrates that with an NvS measurement it is easily possible to find out the optimal measurement concentration of a sample.

In general, sensitivity values between 75 and 90 should be adjusted in order to obtain representative measurement results. If samples in a measurement series are to be compared with each other, it is recommended to keep the sensitivity value for all samples of the test series at a fixed value.

6.2.5 Daily Performance

The "Daily Performance" function is accessible in the Cell Check menu. With the "Daily Performance", the ZetaView® software offers a function for quickly checking whether the device is ready for use.



The function comprises the automatic measurement of a 100 nm polystyrene size standard (Alignment Suspension). The particle size distribution and concentration are measured 5 times at one specific position. Trueness and precision for the particle size are calculated from the 5 consecutive and independent measurements.

The term trueness (also known as accuracy) illustrates the proximity of the measurement results to the reference value saved on the Calibration tab, typically set to 100 nm. If different, enter the actual size of the size reference standard accordingly (see also section 15.1.2).

The precision represents the reproducibility (repeatability) of the measurements.

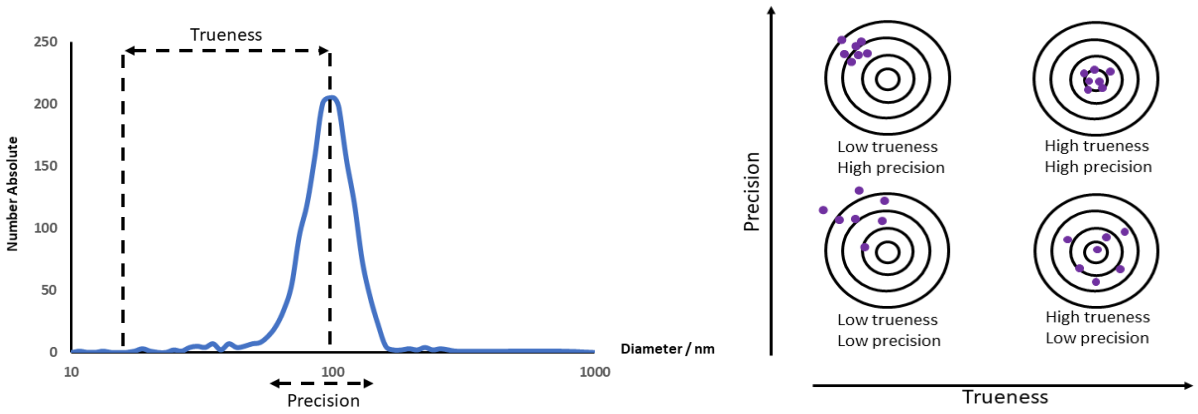


Figure 6-23: Illustration of trueness and precision as examples on a size histogram (left) and target discs (right)

Precision and relative standard deviation are used equally. Both, trueness and precision are shown as percentage to the true value after the “Daily Performance” measurement.

Depending on the measured size of the standard solution, the ZetaView® software displays one of 3 available results for the trueness which is shown in the figure below.

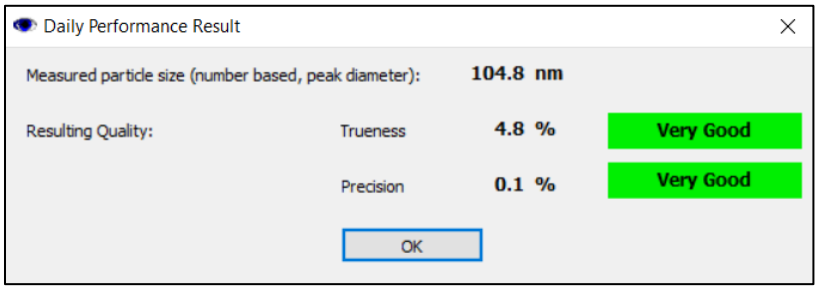


Figure 6-24: Accepted trueness deviation from 100 nm standard 1-10%. The instrument is ready for measurements.

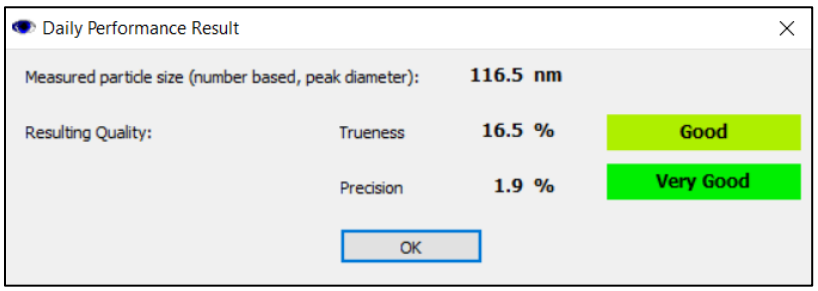


Figure 6-25: Accepted trueness deviation from 100 nm standard 11-18%. The instrument is ready for measurements.

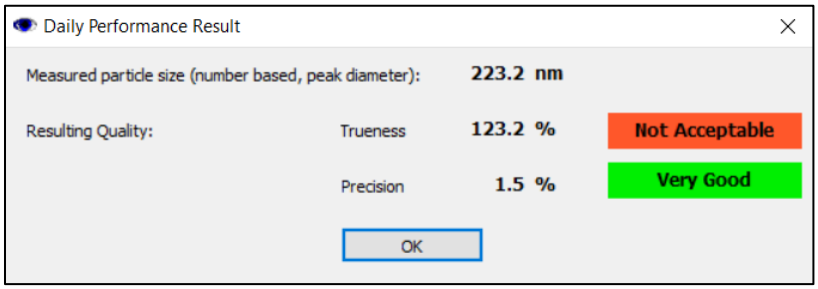


Figure 6-26: Accepted trueness deviation from 100 nm standard >18%. The instrument is not ready for measurements.

Procedure for performing a Daily Performance measurement:

To prepare the alignment suspension, two consecutive dilution steps are recommended (e.g. two consecutive 1:500 dilutions, or one 1:1,000 followed by a 1:250 dilution).

1. Preparation of **dilution 1:**

   Add 5 µl of 100 nm polystyrene beads to 5 ml of particle-free water. Scale up if necessary. This results in a **1:1,000 dilution.**

   Shelf life of solution 1 is approximately 2-3 days when stored at 4°C.

2. Preparation of **dilution 2:**

   Add 80 µl of the 1:1,000 dilution to 20 ml of particle-free water. Scale up if necessary. This yields a **1:250,000** alignment suspension.

   Shelf life of dilution 2 is around 30-60 minutes.

   If reproducible (< 5%) values for concentration and size distribution measurements are desired, it is recommended to prepare solutions fresh prior to the measurement.

3. Inject 100 nm standard solution (= dilution 2)

4. Press “Daily Performance“. The instrument automatically adjusts the instrument parameters for 100 nm particles and performs the particle size distribution measurement.

5. After measurement, the result of the daily performance measurement shows up. If the result shows “very good” or “good”, resume with “OK”. The instrument is ready for measurements

6. If the result shows “Not Acceptable” please check the following:

   a. Check for cleanliness of the measuring cell.

   b. Verify that the water is particle-free.

   c. Standard suspension might be agglomerated. Prepare a new 1:250,000 dilution by using the already existent 1:1,000 dilution or prepare both dilutions (1:1,000 and 1:250,000) completely freshly.

   d. Verify that the instrument is located on a vibration-free table or lab bench during measurement.

6.3 Pump & Temp tab

6.3.1 Pump control

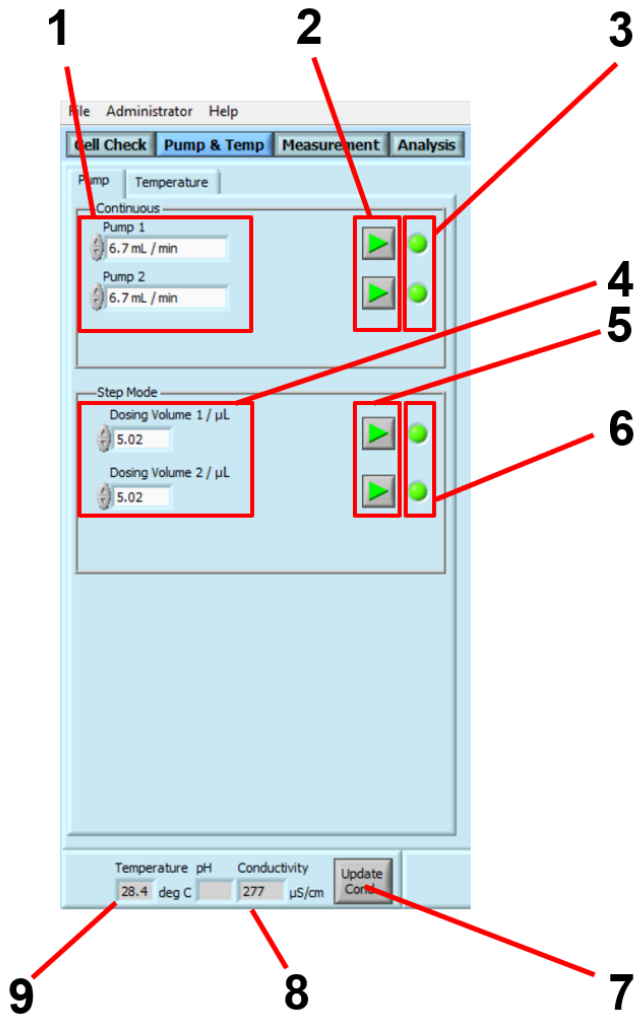


Table 6.3: Description of parameters and functions in the pump menu.

Number	Function	Remarks
1	For adjusting the flow rate of pump 1 and pump 2 in the continuous mode	
2	Button for switching the pumps on and off	When the pumps are in use the button turns to a red square.
3	Green light indicate that the pumps are ready to use.	Green light turns red if pump is in use or temporarily inactive due to heat protection.
4	For adjusting the dosing volume of pump 1 and pump 2 in the step mode	

Number	Function	Remarks
5	Button for switching the pumps on and off	When the pumps are in use the button turns to a red square.
6	Green light indicate that the pumps are ready to use.	Green light turns red if pump is in use.
7	Button for checking the conductivity of the liquid inside the measuring cell	Only available with zeta potential capability.
8	Result of the latest conductivity measurement	Only available with zeta potential capability.
9	Current temperature of the measuring cell	

The ZetaView® device has two internal peristaltic pumps named Pump1 and Pump2. In addition, an external third pump (Pump3) in combination with an NTA Cell assembly (see section 4.4) can optionally be connected to the device.

Please note that the external pump (Pump3) is not hot-pluggable. During instrument operation, the external pump is not recognized if it is plugged in. A system restart is therefore necessary.

The screenshot below shows where both internal pumps and the external third pump are selectable.

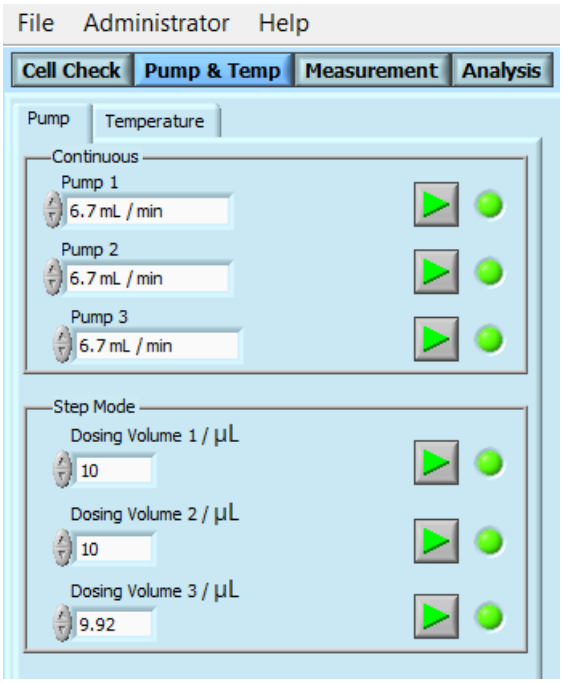


Figure 6-27: Pump menu with all three available pumps. The optional third pump is only available if connected externally to the ZetaView®.

All pumps can be operated in two modes.

Continuous mode: The pump can be continuously operated to prime, wash or rinse the instrument with an appropriate buffer or water. Especially after removing and replacing the measuring cell or the Cell assembly, this step is recommended so that all air in the fluidics of the ZetaView® device is completely removed and replaced by water or buffer (see chapter 5).
In the dropdown menu of the continuous mode, the flow rate of the pumps can be adjusted. The default value for the pump rate is 5ml / min. However, the pump rate may be adjusted by clicking the arrow up or down. The following figure shows which values can be set for the pump rate.

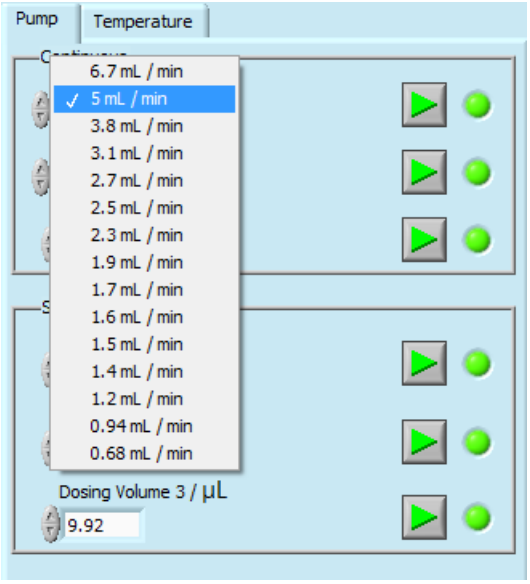


Figure 6-28: The pump rate can be adjusted by means of the drop down menu.

After the pump rate has been set, the pumps can be activated by clicking on the start button.

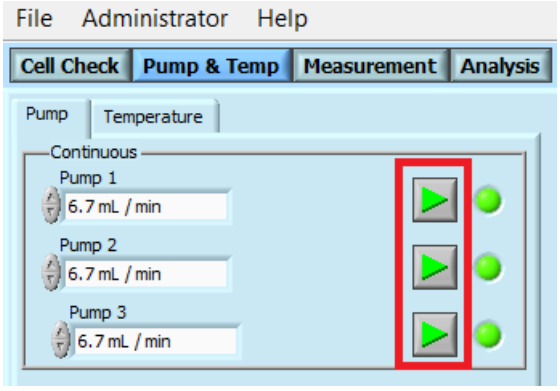


Figure 6-29: All pumps can be activated by clicking on the start button highlighted with a red frame.

Several pumps can be operated simultaneously. While the pump is running, the start button changes to a red square which is shown below.

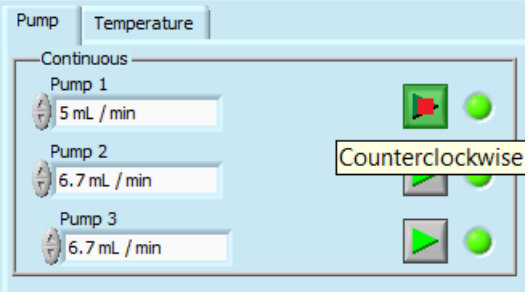


Figure 6-30: A red square indicates a running pump.

Once the pumps are started, they will run until they stop automatically. However, it is possible to stop the pumps at any time by clicking the red square again. After a pump has been manually or automatically stopped, the green dot to the right of the start button of the corresponding pump changes from green to red.

This is an overheating protection of the pump motors. In this state, a new activation of the pump is not possible and only works again when the dot switches back to green and the pump motor has cooled down. The longer a pump was activated (maximum 90 seconds), the longer the time in which the pump cannot be re-activated.

Step mode: In the step mode, the pumps can be operated step by step. After pressing the green start button, the corresponding pump is activated and pumps the volume shown in the window through the measuring cell.

The pump volume can be adjusted in the input field. Typical volumes to push the sample through the measuring cell is 10µl to 100µl. After entering the pump volume and starting the pump, the ZetaView® instrument will push the adjusted volume of water or buffer (depending on which fluid is connected to the pump) through the fluidic system. As a result, the sample in the measuring cell is pushed forward by the adjusted volume, which introduces a new sub-volume of the sample into the field of view of the detection optics. In this way, measurements of several sub-volumes of the same sample are possible and can thus make statistics more robust. The step mode can be executed manually or automatically in an SOP (see chapter 9).

It should be noted that the step mode only works reliably if the fluidic system of the ZetaView® has been completely filled with water or with buffer by using the continuous mode of the pumps. If the step mode is activated without the fluid system of the ZetaView® being filled, it may happen that the remaining air inside the system prevents or restricts a reliable displacement of the sample in the measuring cell.

6.3.2 Temperature control

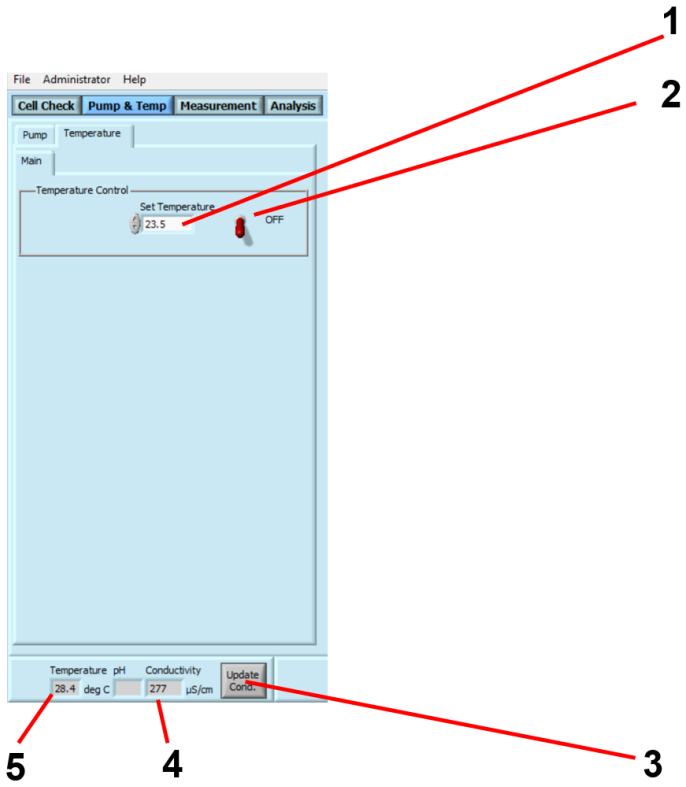


Table 6.4: Description of parameters and functions in the temperature menu.

Number	Function	Remarks
1	For entering the desired temperature	The maximum supported range is from 5°C – 45°C. However, the eligible temperature setpoint depends on the ambient temperature and humidity (dew point). .
2	Switch to turn the temperature control on and off	The switch turns green once the temperature control is on.
3	Button for checking the conductivity of the liquid inside the measuring cell	Only available with zeta potential capability.
4	Result of the latest conductivity measurement	Only available with zeta potential capability.
5	Current temperature of the measuring cell	If the temperature control is switched on, the temperature will be indicated with a green, yellow or red background depending on how close the adjusted temperature is to the current temperature.

The ZetaView® instrument has a temperature control, which makes it possible to keep the measuring cell at a fixed temperature. The temperature control is accessible in the Pump & Temp menu.

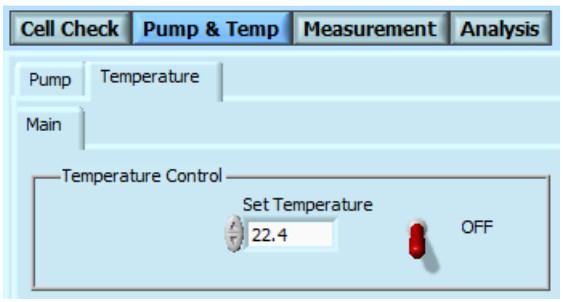


Figure 6-31: Temperature can be set by entering the desired value in the window or by clicking on the arrows located on the left side of the temperature value up and down. To turn the temperature control on, click on the red switch right next to the temperature value.

The ZetaView® systems have an ambient temperature and humidity sensor from which the dew point is derived (data are displayed on the Temperature control tab in Administrator mode only). In order to prevent condensation on the outer wall of the cell, the ZetaView software supports set temperature values 5°C above the dew point. As closest approximation, you can adjust the temperature from 5°C below the temperature in the cell assembly to 45°C. However it is not recommended to set the temperature too high as excessive vertical particle drift (see section 6.1.7) due to heat development may occur.

A tempered and slightly cooled down cell may be very important for some measurements, especially if the location of the ZetaView® is quite warm, if the samples are sensitive to temperature changes, or if excessive vertical particle drift becomes an issue.
After the temperature control has been switched on for example to 20°C (see figure below), the sample in the measuring cell will be tempered according to the adjusted value.

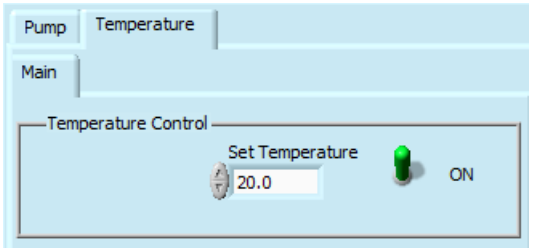


Figure 6-32: Temperature control switched to 20°C.

This process takes a few minutes. During that time, a color code at the bottom left of the ZetaView® software shows via a traffic light system (red, yellow, green), whether a measurement can be performed or not.

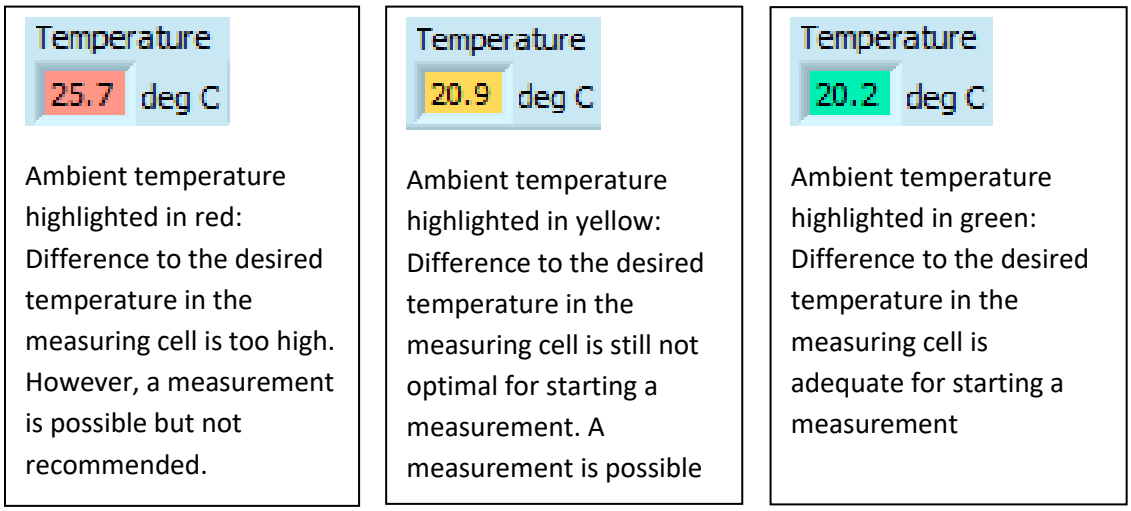


Figure 6-33: Color code of the ambient temperature indicator of the bottom left of the ZetaView® software. Please note that a measurement is always possible no matter what color code is displayed.

Even when the temperature control is switched off, the temperature inside the measuring cell is still detected but the color code is not shown.

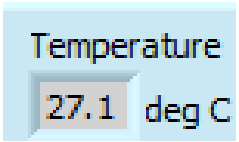


Figure 6-34: Temperature indicator without activated temperature control. Temperature reading is not highlighted.

Measurements without temperature control are still accurate because the current temperature of the sample is always detected when measuring the size, concentration and zeta potential. The detected temperature value is always considered when evaluating the results in the Stoke-Einstein equation and in the Helmholz-Smoluchowski equation.

6.4 Measurement Tab

6.4.1 SOP tab

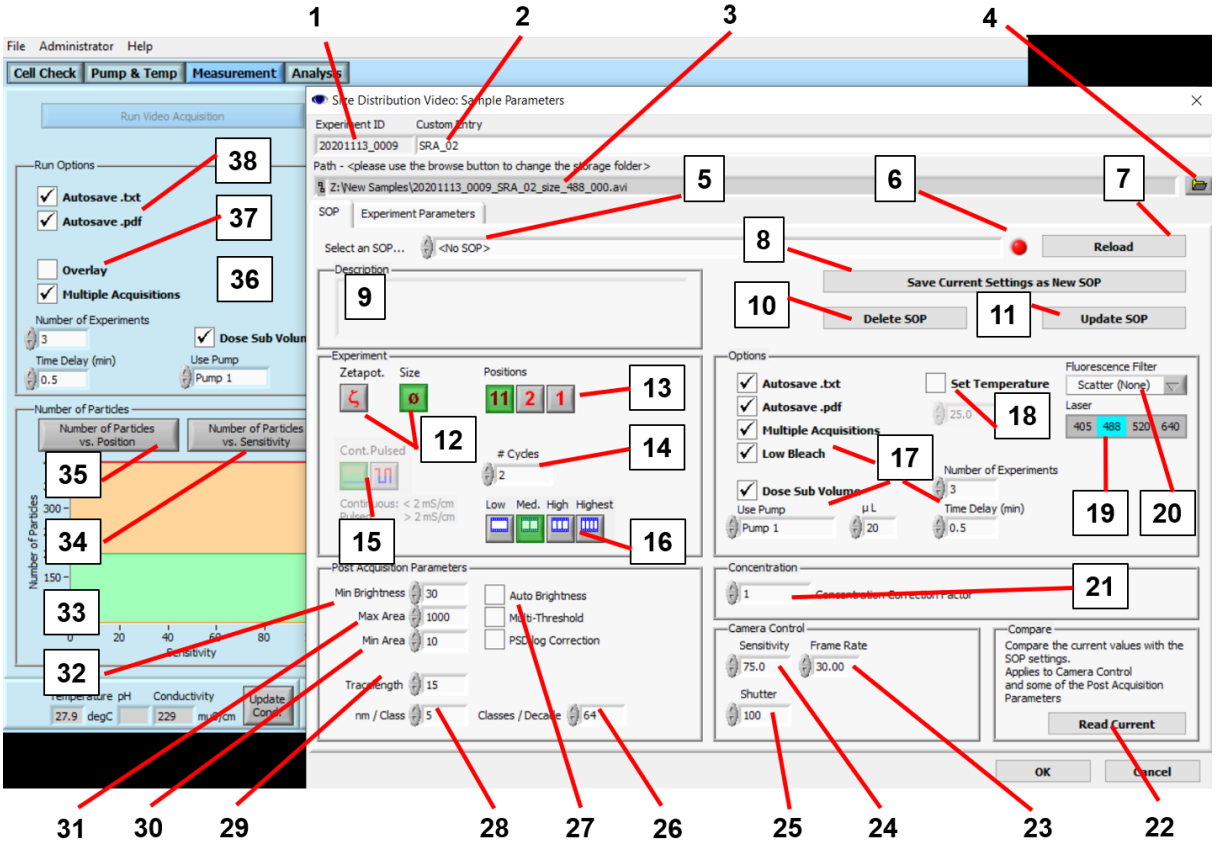


Table 6.5: Description of parameters and functions in the Measurement Menu SOP.

Number	Function (Measurement Menu SOP)	Remarks
1	Experiment ID consisting of yyyymmdd_consecutive number	Each experiment ID is unique so that measurements with the same custom entry are not overwritten.
2	Custom entry for the file name or experiment name	
3	Storage path of the measurement including information of the used laser wavelength	
4	Browse to change the storage folder	
5	Drop down menu of the SOPs	Gives access of all saved SOPs.
6	Status light of the SOPs	Indicates if an SOP is activated or inactivated.
7	Reload a selected SOP	
8	Saves the current measurement parameters as a new SOP	
9	Option for describing an SOP in more detail	

Number	Function (Measurement Menu SOP)	Remarks
10	Deletes a selected SOP	By pressing this button there will not be any further warning.
11	Updates the SOP with the current adjusted measuring parameters	
12	Switch between zeta potential measurement and size measurement	
13	Measure all 11 positions or 2 positions or 1 position (currently adjusted position) inside the measuring cell	2 positions represent the SL (stationary layer) positions.
14	Select the number of the cycles	Represents the number of measurements per position.
15	Switch between low and high conductivity zeta potential measurements	Low conductivity: sample has <2mS/cm High conductivity: sample has >2mS/cm
16	Select the number of the frames	Describes the number of pictures recorded in a video per measurement position.
17	Parameters for “Multiple Acquisitions” measurements	Important for fluorescence measurements and for strengthening the statistics.
18	Adjust the temperature control for SOPs	
19	Switch between laser wavelength for SOP	
20	Drop down menu for switching between scatter and fluorescence mode	
21	Enter the concentration correction factor if fluorescence concentration calibration becomes an issue	
22	To compare the current settings with settings of a selected SOP	
23	Adjust the frame rate of the camera	This camera parameter directly affects the measurement result.
24	Adjust the sensitivity of the camera sensor	This camera parameter directly affects the measurement result.
25	Adjust the exposure time of the camera	This camera parameter directly affects the measurement result.
26	Adjust the number of classes per decade	Applies only for the logarithmic values in the text file.

Number	Function (Measurement Menu SOP)	Remarks
27	Automatic Brightness	Should be used on particles that have low diffusion behavior. Re-calculates the minimum brightness for each frame of a video. This method prevents overexposure of the particles with regard to its brightness values.
28	Select the width of the bin class	Applies only for the linear values in the text file.
29	Adjust the Tracelength	Minimum number of frames in which a particle must be traced for being included in the analysis.
30	Adjust Min Size (=Min Area)	The minimum number of pixels per spot (particle) allowed can be adjusted.
31	Adjust Max Size (=Max Area)	The maximum number of pixels per spot (particle) allowed can be adjusted.
32	Adjust Min Brightness	Represents the threshold of when a grey value is recognized as a white pixel. When the grey value is lower than the threshold, the pixel will be black. All the information for the subsequent image processing of tracking and analysing is in the white pixels.
33	Miniature sheet of the “Number of particles vs Sensitivity”	
34	Button for executing “Number of particles vs Sensitivity”	
35	Button for executing “Number of particles vs Position”	
36	Ref. 17	
37	When active, multiple histograms can be overlayed	
38	When active, a text file and a pdf report are automatically generated after the measurement	

6.4.2 Experiment Parameters tab

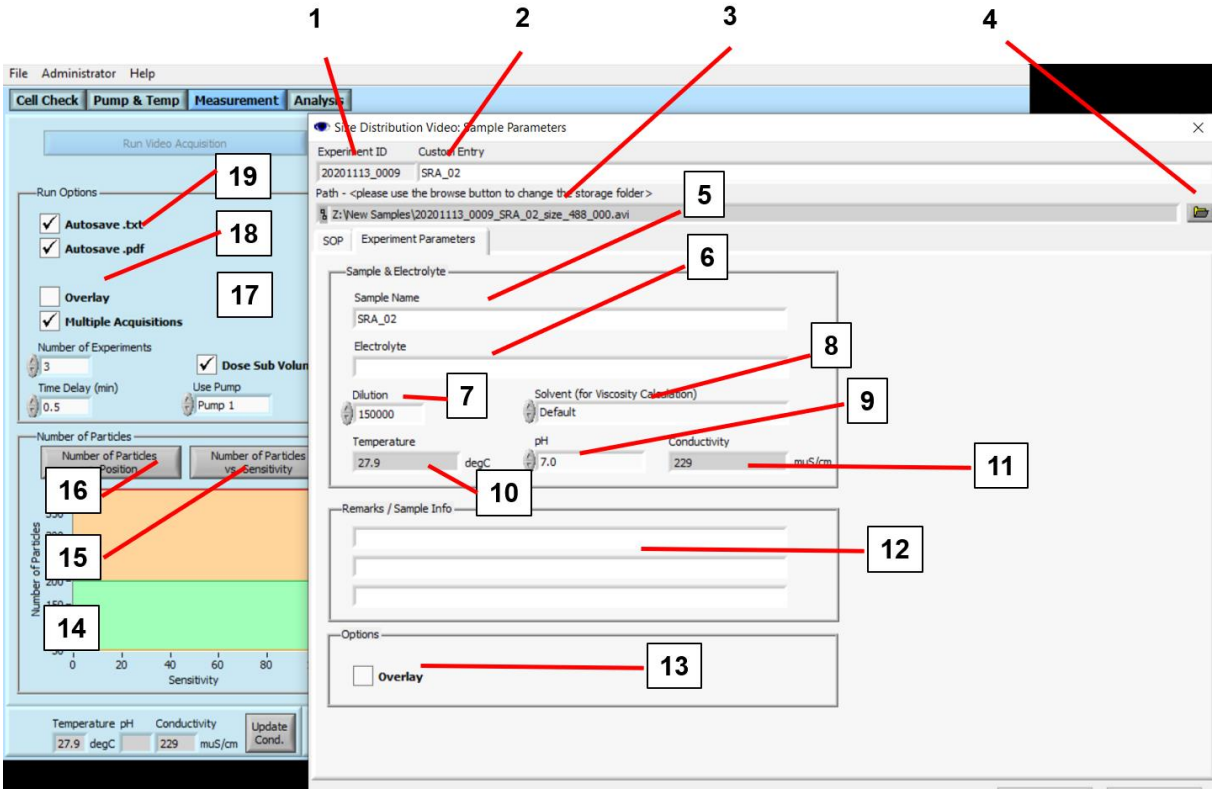


Table 6.6: Description of parameters and functions in the Measurement Menu Experiment Parameters.

Number	Function (Experiment Parameters Menu)	Remarks
1	Experiment ID consisting of yyyymmdd_consecutive number	Each experiment ID is unique so that measurements with the same custom entry are not overwritten.
2	Custom entry for the file name	
3	Storage path of the measurement including information of the used laser wavelength and the experiment type (size or prof)	
4	Browse to change the storage folder	
5	For entering an additional sample name displayed on the measurement report	The sample name is independent from the custom entry file name and must be entered for each measurement (no auto-fill function).
6	For entering an electrolyte (PBS, HEPES etc.)	This entry will be shown on the measurement report and does not affect the result of the measurement.

7	For entering the dilution factor	This entry will be shown on the measurement report and it is used to calculate the “Original Concentration”
8	Drop-down menu for selecting different viscosities	Additional viscosities must be entered by the user before selecting them via the drop-down menu (ref. to section 9.6).
Number	**Function (Experiment Parameters Menu)**	**Remarks**
9	For entering the pH of the sample	This entry will be shown in the measurement report and does not affect the result of the measurement.
10	Sensed temperature inside the measuring cell	
11	Sensed conductivity of the sample	
12	For entering additional remarks or sample information	This entry will be shown in the measurement report and does not affect the result of the measurement.
13	When active, multiple histograms can be overlayed	
14	Miniature sheet of the “Number of Particles vs Sensitivity”	
15	Button for executing “Number of particles vs Sensitivity”	
16	Button for executing “Number of particles vs Positions”	
17	Parameters for “Multiple Acquisitions” measurements	Important for fluorescence measurements and for strengthening the statistics of the measurement.
18	When active, multiple histograms can be overlayed	
19	When active, a text file and a pdf report are automatically generated after the measurement	

6.4.3 Autosave .txt & Autosave .pdf

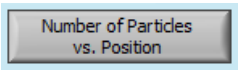
Activate “Autosave.txt” to automatically generate a text file after a measurement, including sample name, instrument settings, measurement parameters, and measurement data.
A detailed description of the text file can be found in chapter 11 and section 13.6.

Activate “Autosave.pdf” to automatically generate a pdf-report after the measurement. This contains a summary of the measurement results.
A detailed description of the pdf report can be found in chapter 7.3 and section 13.5.

6.4.4 Number of Particles vs. Position (NvP)

The function "Number of Particles vs. Position" (NvP) can be used to check whether the particles are homogeneously distributed in all 11 positions after injection of the sample into the ZetaView® instrument. A homogeneous distribution of the sample and the particles in the measuring cell is essential for robust statistics.

After activating the function by pressing the button "Number of Particles vs. Position",



with the current camera settings and post-acquisition parameters, a total of 19 different positions within the measuring cell are automatically scanned and the current number of particles measured for each position. The measuring positions 0.1 - 0.9 are included in the scanned positions.
During the measurement, a diagram is created at the same time, in which the positions on the X-axis and the number of particles on the Y-axis are plotted.

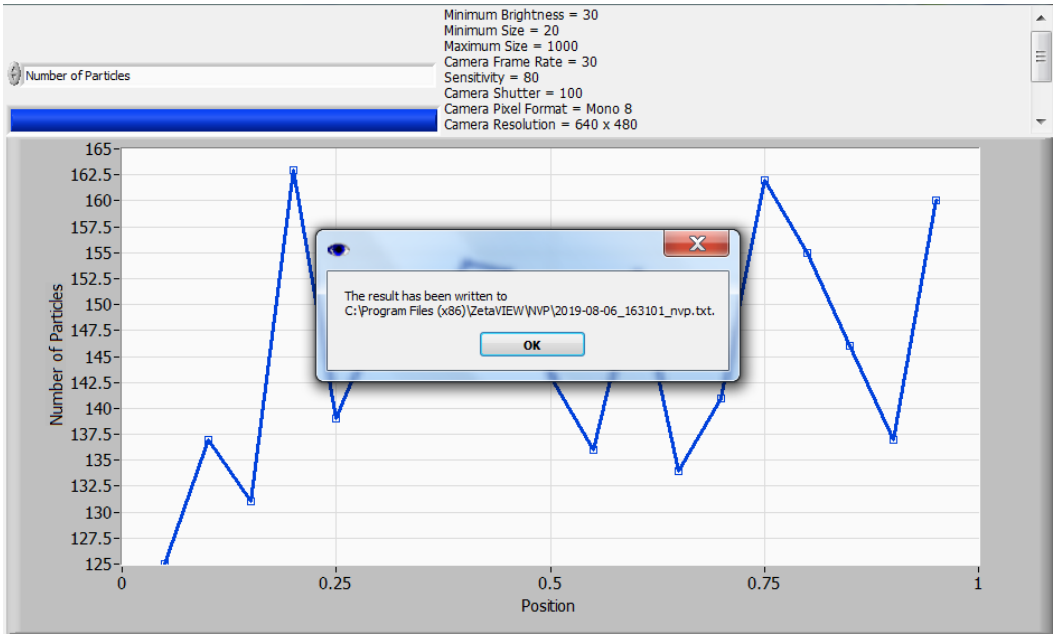


Figure 6-35: Result of an NvP measurement with message about the storage location and file name.

The result is a graph, in which the number of particles is plotted for each individual position and all measuring points are connected to each other. In addition, the storage location and name of the corresponding file is indicated. The storage location is pre-determined, and the file name of the measurement is automatically assigned. Neither can be changed by the user before saving.

In addition to the number of particles, other parameters such as "Scattering Intensity", "Mean Particle Area" and "Mean Particle Intensity" can also be selected.

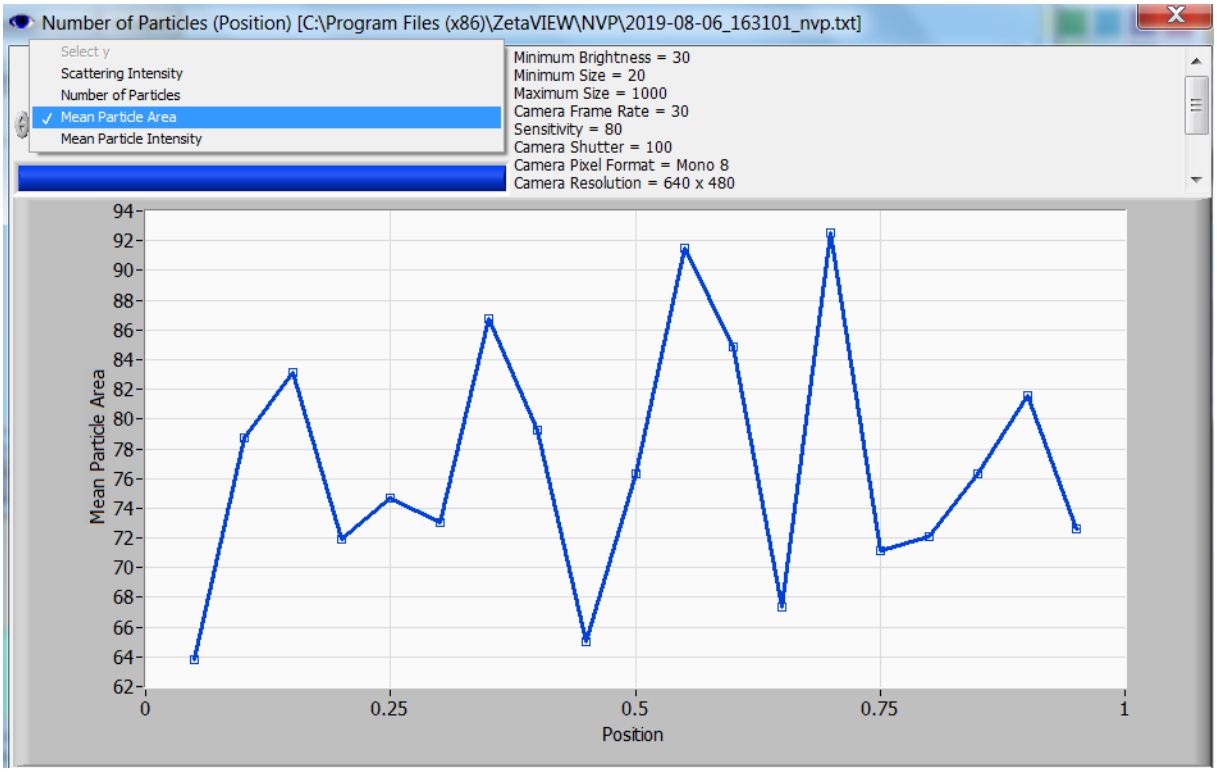


Figure 6-36: Additional parameters are selectable after a successful NvP measurement. The top right shows the camera settings and post-acquisition parameters set during the NvP measurement.

The result of the measurement is stored separately in a text file containing "Number of Particles", "Scattering Intensity", "Mean Particle Area" and "Mean Particle Intensity" for each position.

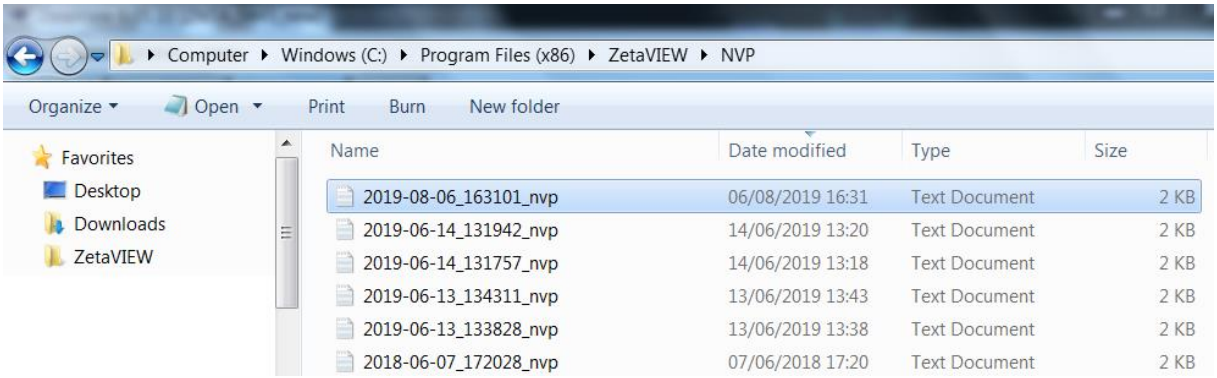


Figure 6-37: Path under which the results of NvP measurements can be found.

If you only want to make sure that all the particles in the measuring cell are largely homogeneously distributed in all 11 positions, you can also check this by manually selecting each individual measuring position and observing the bar "No. of Detected Particles ".
All measuring positions should show approximately the same number of particles with plus or minus 15 % of particles as a guideline.

It should be noted that an NvP measurement is only a snapshot because the number of particles varies continuously due to their movement. A new NvP measurement will therefore lead to a slightly different result.

6.5 Analysis tab

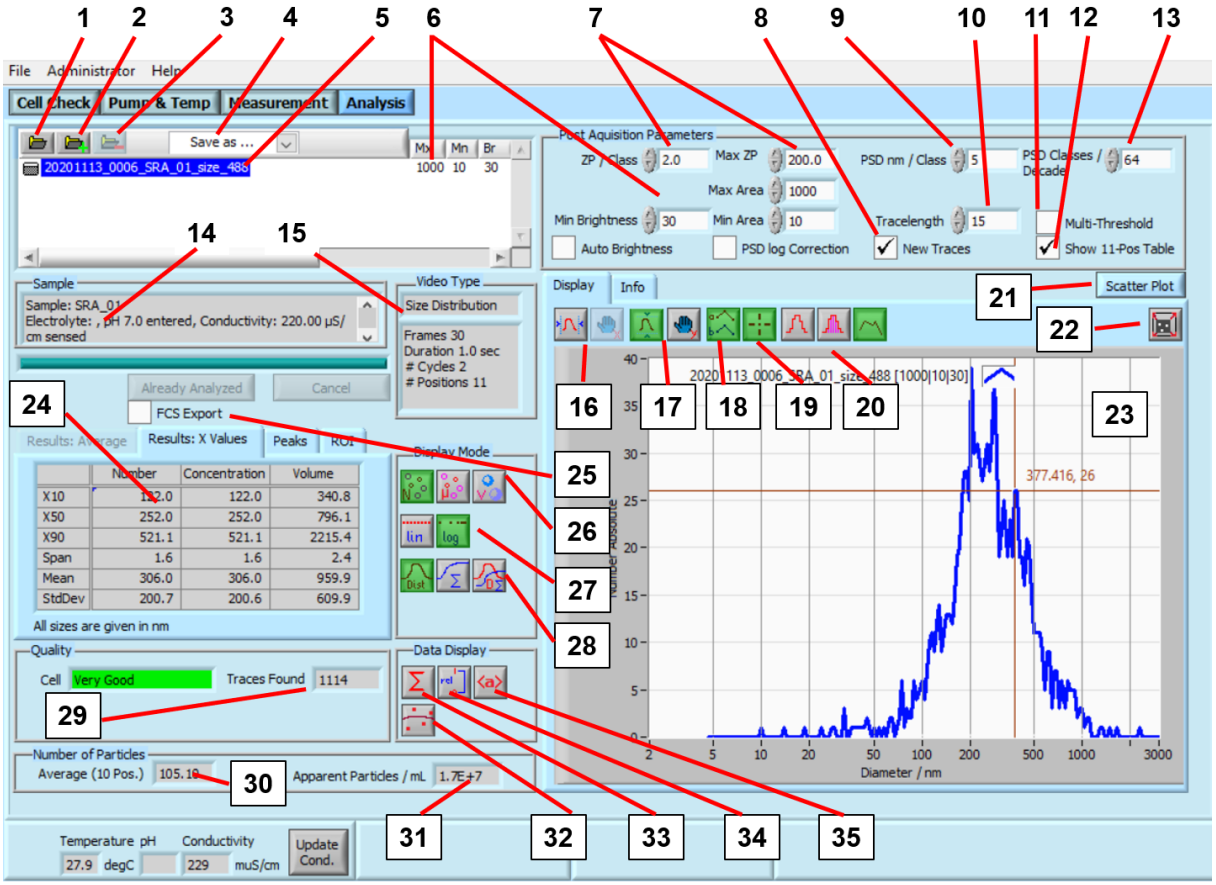


Table 6.7: Description of parameters and functions in the Analysis Menu

Number	Function (Analysis Menu)	Remarks
1	Load files / load analyses	
2	Add files / add analyses	
3	Remove selected analysis	
4	Save as text, report, pdf, save screen	
5	Shows file name incl. experiment ID of the recorded video	
6	Post-acquisition parameters	
7	Adjust the bin class of the zeta potential and max. zeta potential to be measured	
8	Adjust if new traces should be taken into account during capturing a video	Refers to individual frames recorded at one position.
9	Select the width of the bin class when doing a size measurement	Applies only for the linear values in the text file.
10	Adjust the Tracelength	Minimum number of frames in which a particle must be traced for being included in the analysis.

Number	Function (Analysis Menu)	Remarks
11	When enabled, the software calculates optimal brightness for each frame of the video. Can be combined with “Auto Brightness”	Recommended for multimodal mixtures and for particles larger than 200 nm in size and with different scattering behaviour.
12	Shows the 11-position table after each 11-position acquisition	
13	Adjusts the number of classes per decade	Applies only for the logarithmic values in the text file.
14	Provides information about sample name, electrolyte, pH and conductivity	
15	Provides information about the video type (size or zeta potential measurement) and parameters of the video	
16	Scale X-axis	Determine the range of the X-axis or let the software do this automatically.
17	Scale Y-axis	Determine the range of the Y-axis or let the software do this automatically.
18	Legend	Enables and disables the legend.
19	Cursor	Enables and disables the cursor.
20	Shows line plot, bar plot or step plot	Changes the appearance of the graph.
21	Shows the scatter plot	This function is only available if the FCS Export (see 25) is activated.
22	Enlarge graph	Displays a larger view of the result in a separate window.
23	Graph	Shows current graph of the measurement.
24	Statistic table	Average and peak statistics are available as well as the region of interest (ROI) for showing concentration in a specified size range.
25	Generates an FCS-file of the following measurement. Must be activated before the corresponding measurement starts	An existing FCS file is required for the presentation of a scatter plot.
26	Switch between number-weighted, concentration-weighted, volume-weighted distribution	According to the experiment 3 modes of distribution results can be visualized.

Number	Function (Analysis Menu)	Remarks
27	Switch between linear and logarithmic plot	
28	Switch between distribution and cumulative	Distribution only, cumulative only and distribution & cumulative is available.
29	Absolute number of traced particles	Number of particles considered in the analysis.
30	Calculated average number of particles per measuring position	
31	Measured particle concentration averaged over all measuring positions	
32	Smooth graph	Algorithm for smoothing the graph.
33	Sum up multiple graphs	Calculates the sum of multiple measurements.
34	Normalize multiple measurements	Normalizes multiple distributions to 100%.
35	Average of multiple measurements	Calculate the average of multiple measurements.

6.6 Scatter Plot tab

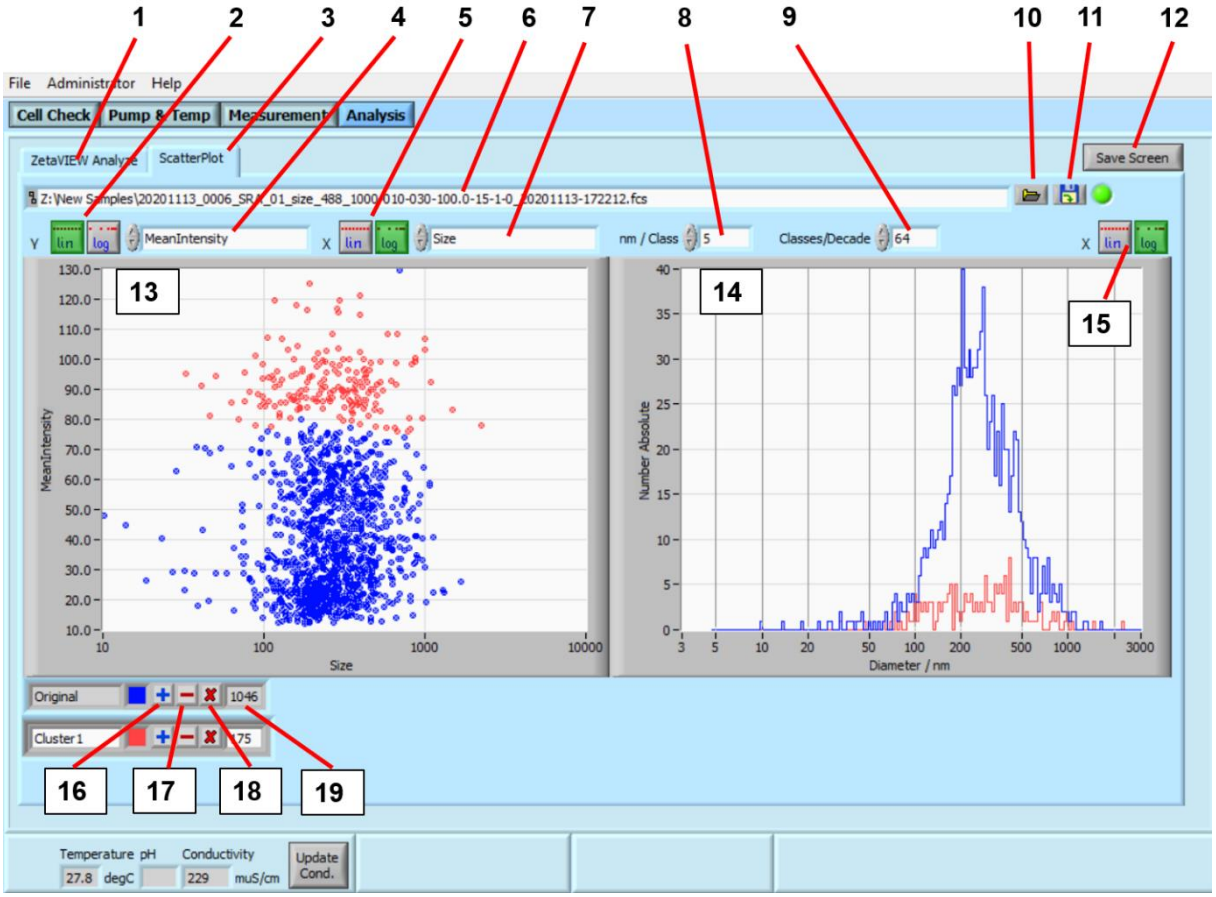


Table 6.8: Description of parameters and functions in the Scatter Plot of the Analysis Menu

Number	Function (Analysis Menu)	Remarks
1	Switch to the Analyze menu	
2	Switch between linear and logarithmic scale of the Y-axis of the scatter plot	
3	Switch to scatter plot	
4	Drop down menu for selecting parameters for plotting the Y-axis	
5	Switch between linear and logarithmic scale of the X-axis of the scatter plot	
6	Name of the FCS-file	
7	Drop down menu for selecting parameters for plotting the X-axis	
8	Select the width of the bin class	Applies only for the linear values in the text file.
9	Adjust the number of Classes per Decade	Applies only for the logarithmic values in the text file.
10	Browse button to load a new FCS file	

Number	Function (Analysis Menu)	Remarks
11	Saves an FCS-file	The FCS file will be saved in the same storage folder without any pop-up message.
12	Saves a screenshot	The screenshot is saved in png-format by default.
13	Scatter plot of the current measurement	
14	Histogram of the corresponding scatter plot	
15	Switch between linear and logarithmic scale of the X-axis of the histogram	
16	Add particles to a present cluster	
17	Remove particles from a present cluster	
18	Delete a cluster	
19	Number of traces of a cluster	

7 How to proceed for a size measurement

The particle size in a sample is determined by tracking each individual particle while recording a video while they move according to the Brownian motion. Since the ZetaView® instrument detects, localizes and tracks each particle in the field of view, the software can quantify the average mean square displacement per time interval and determines a diffusion coefficient for each individual particle. The diffusion coefficient of each individual particle is taken into the Stokes-Einstein equation. The temperature and the viscosity of the buffer in which the particles are suspended plays a decisive role as well and are considered during size determination.

$$Dt = \frac{< \overline{x,y}^2 >}{4} \qquad D = \frac{k_B T}{6\pi\eta r}$$

D = Diffusion coefficient; k_B = Boltzmann constant; T = Temperature;
η = Viscosity of medium; $< \overline{x,y}^2 >$ = Mean square displacement; r = radius

Figure 7-1: Calculation of the mean square displacement for each particle (left) and Stokes-Einstein equation (right).

The size of the particles in a sample can be measured in all 11 measuring positions, in two positions which represent the SL positions (ref. section 13.2) or only one position. The latter one represents the current position adjusted. When it comes to good statistical reproducibility, measurement in all 11 positions are highly recommended.

In general, before samples are analysed in all 11 positions, “AutoAlignment” using polystyrene 100 nm beads (see section 6.2.2) should be performed. If the instrument is equipped with an NTA Cell assembly, this ensures proper focus adjustment on the particles. If the device is supplied with a Z-NTA Cell assembly, there is also a symmetry correction.

To carry out a size measurement, the following conditions must first be met:

- The measuring cell must be clean inside and outside
- The start-up routine (auto alignment and possibly focus optimization) must have been successfully completed (see chapter 5)
- The ZetaView® device must be primed with the appropriate buffer / liquid in which the particles of the sample are dissolved
- There must be no air bubbles in the fluid system or in the measuring cell
- The particles of the sample must be in focus
- There should be approximately the same number of particles in all 11 measuring positions
- The particles should be distributed homogeneously in all 11 measuring positions
- The horizontal and vertical drift must not be too high

After the sample is injected, the particles appear in the field of view. Depending on the sample,

1. Adjust scatter or fluorescence (see chapter 12) mode and the laser wavelength
2. Adjust the camera parameters (pre-acquisition parameters) according to your needs
3. Enter the dilution factor
4. Adjust the post-acquisition parameters according to your needs

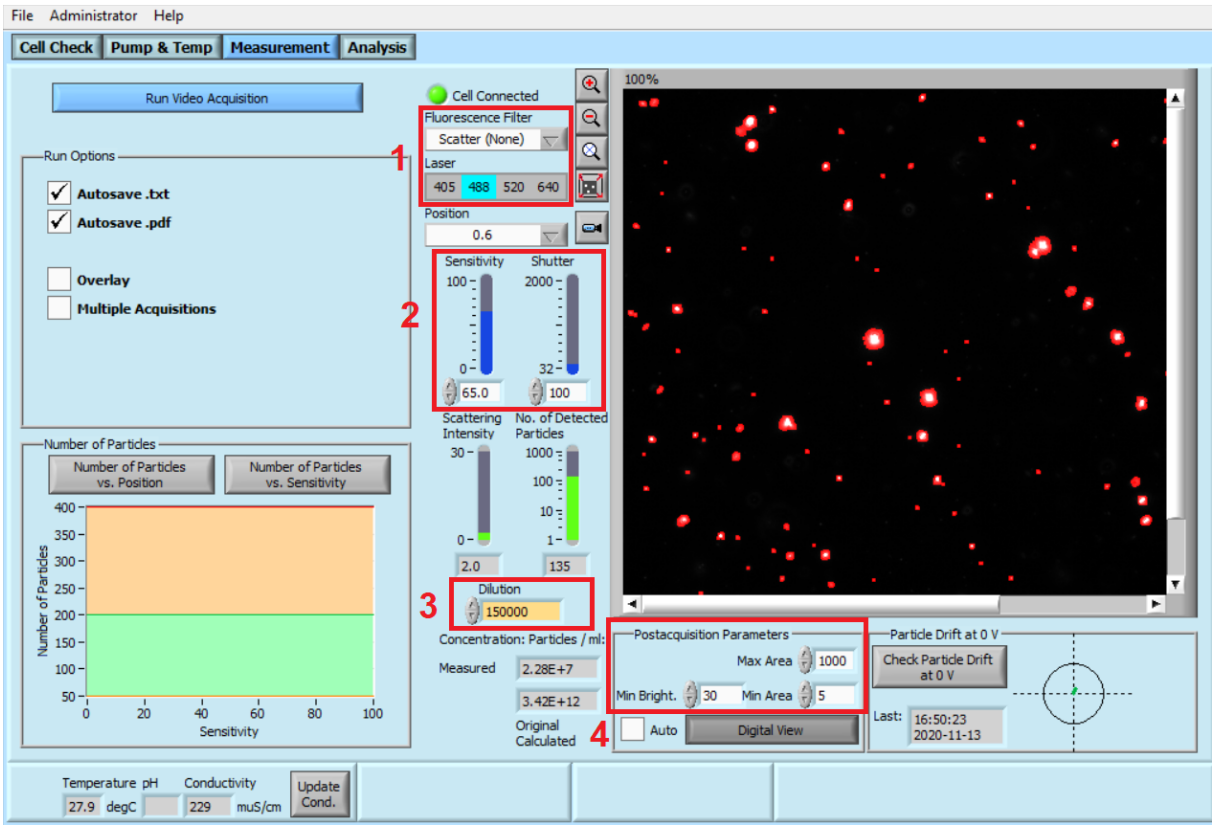


Figure 7-2: Settings to be made before doing size measurement.

The following table summarizes the ranges of camera parameters recommended for particle detection and measurement of particle size. The parameters are based on polystyrene particles and provide a hint for adjusting parameters for particles in a real sample.

The image acquisition, for example how the video is recorded, is defined by the pre-acquisition parameters. Besides sensitivity, shutter and frame rate (see section 8.2), the number of cycles, multiple acquisitions, resolution (see section 8.1) and measuring positions define the statistical part, for example how often a video is acquired.

Post-acquisition parameters define the data mining of the recorded video. Post-acquisition parameters can be varied after acquisition. This means that the same video can be processed with different sets of post-acquisition parameters.

For optimum results, the number of detected particles should be between 100 and 300.

Table 7.1: Measurement parameters for different particle sizes based on polystyrene (PS)

Parameter		PS 40 nm	PS 100 nm	PS 200 nm	PS 300 nm	PS400 nm	PS 500 nm	PS600 nm	PS >600 nm
Pre-acquisition	Sensitivity	80-90	65-70	60	60	50-60	50-60	50-60	50-60
	Shutter	50	100	100	150-200	200-300	300	500	500
	Frame rate (fps)	60-30	30	30-15	15	15-7.5	7.5	7.5-3.75	3.75
	Resolution (Number of frames)	Medium	Medium	Medium-High	High	High-Highest	Highest	Highest	Highest
Post-acquisition	Minimum Brightness	20	25	Auto	Auto	Auto	Auto	Auto	Auto
	Min Area	5	5	40	50	50	100	100	100
	Max Area	100	1000	2000	10000	10000	10000	10000	10000
	Tracelength	<15	≥15	≥30	≥30	≥30	≥45	≥45	≥45

Further settings are made in the Measurement menu.

1. Click "Run Video Acquisition"
2. Enter the file name
3. Select the storage folder
4. Select size measurement
5. Select number of positions to be measured (“11” positions, “2” SL positions, “1” <current> position adjusted)
6. Select number of cycles to measure the positions
7. Select the resolution of the video (number of frames)
8. Click OK, then the measurement starts

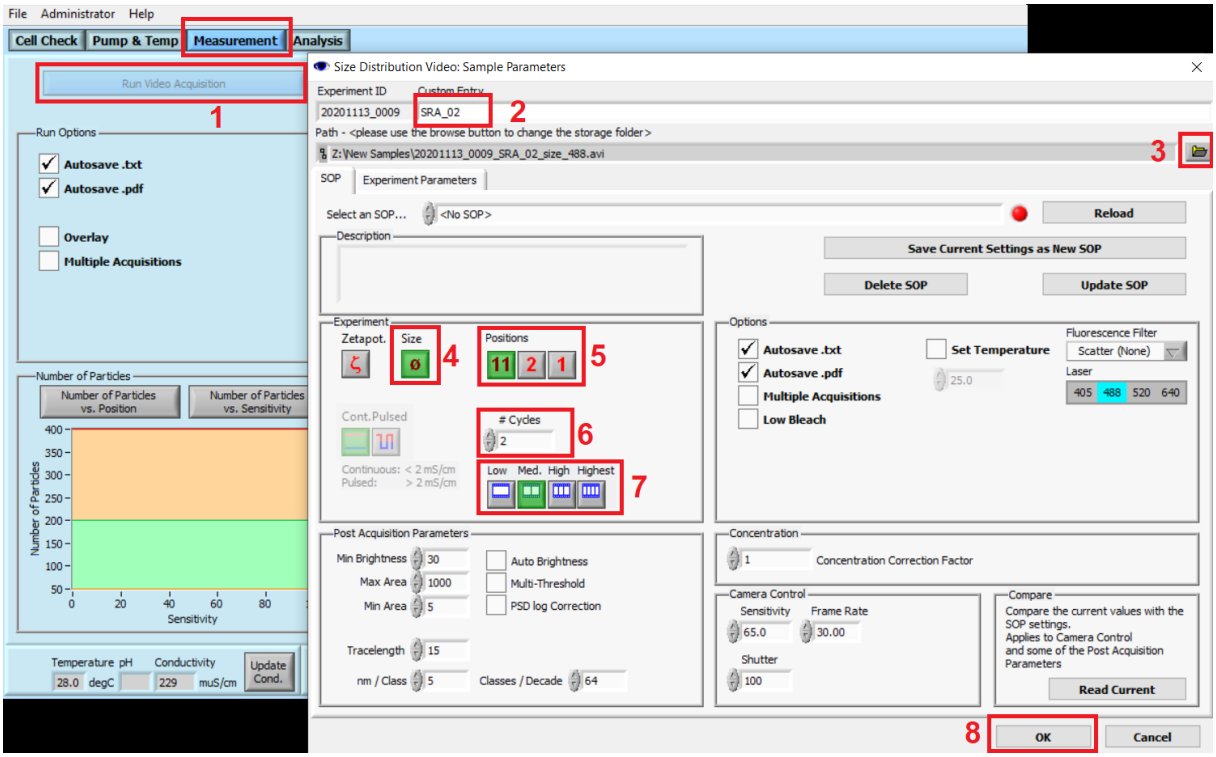


Figure 7-3: Settings to adjust in the measurement menu.

During an 11-position measurement, the scanning process of the ZetaView® optics can be observed from position to position in the "Position" field. The progress bar at the bottom right shows the progress during the measurement. The measurement can be stopped here at any time as well.

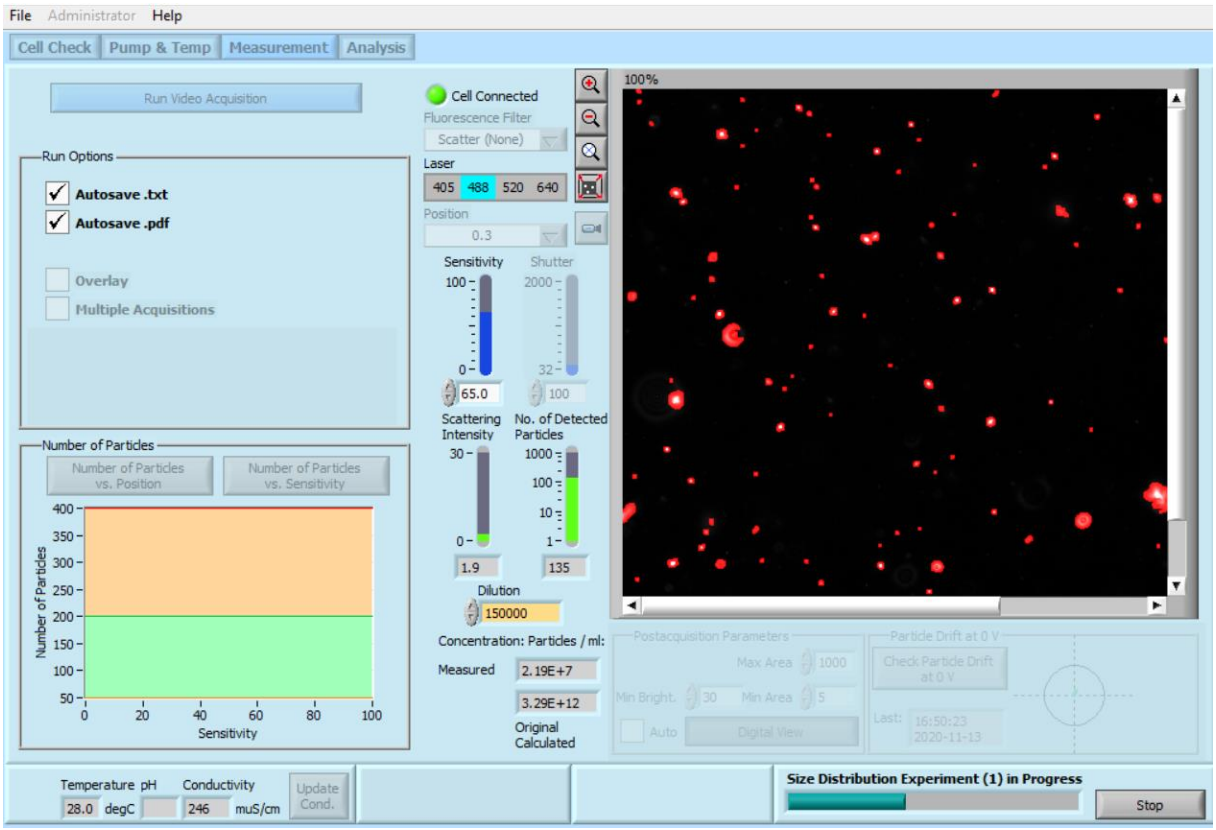


Figure 7-4: Progress during a size measurement.

After the measurement, the software automatically switches to the Analysis tab. The progress of the analysis can be observed in the progress bar in the left part of the software interface and can be stopped if desired.

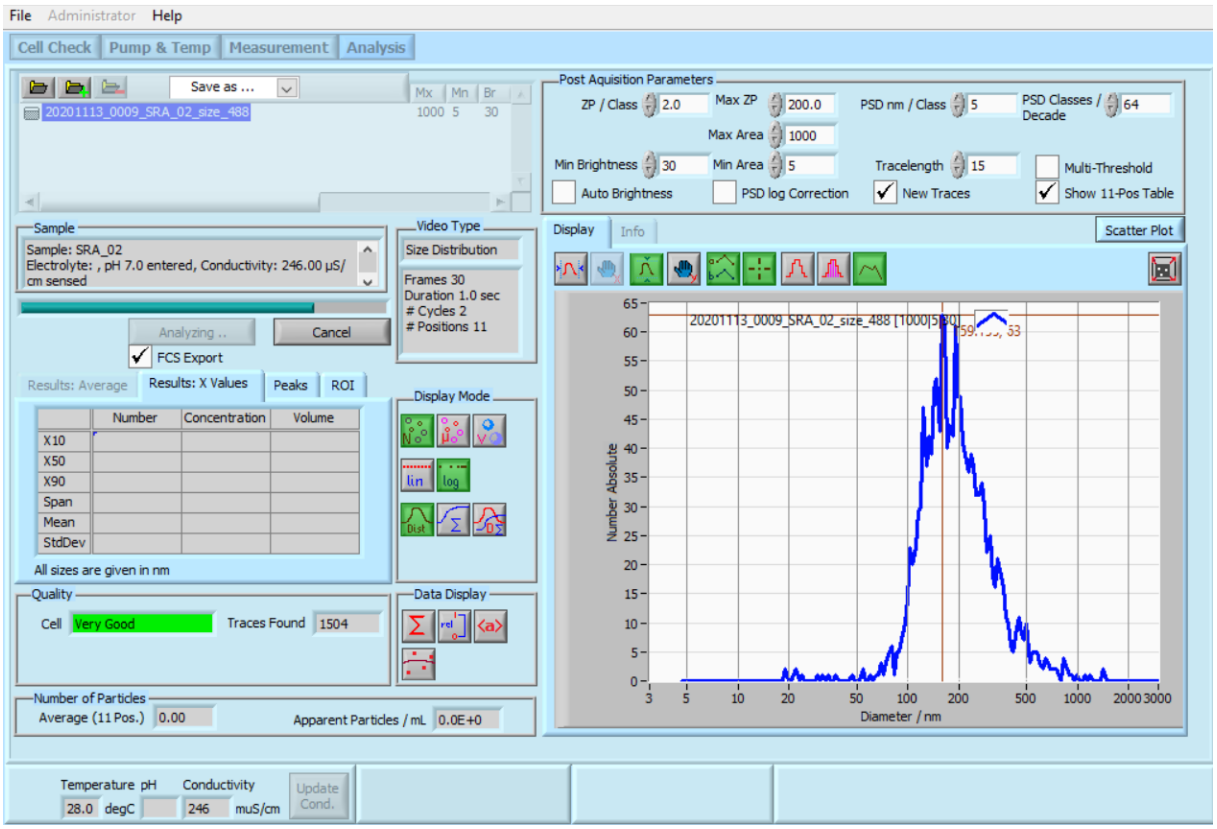


Figure 7-5: Progress of an analysis in the Analysis tab.

After analysis, the final histogram and the corresponding “X Values”, “Peaks”, “Traces Found” and the particle concentration (“Apparent Particles / ml”) are displayed. If the measurement was an 11-position measurement, the 11-position table is also displayed, provided that this was activated in the Analysis tab before starting the measurement. No table is displayed for a 1- or 2-position measurement.

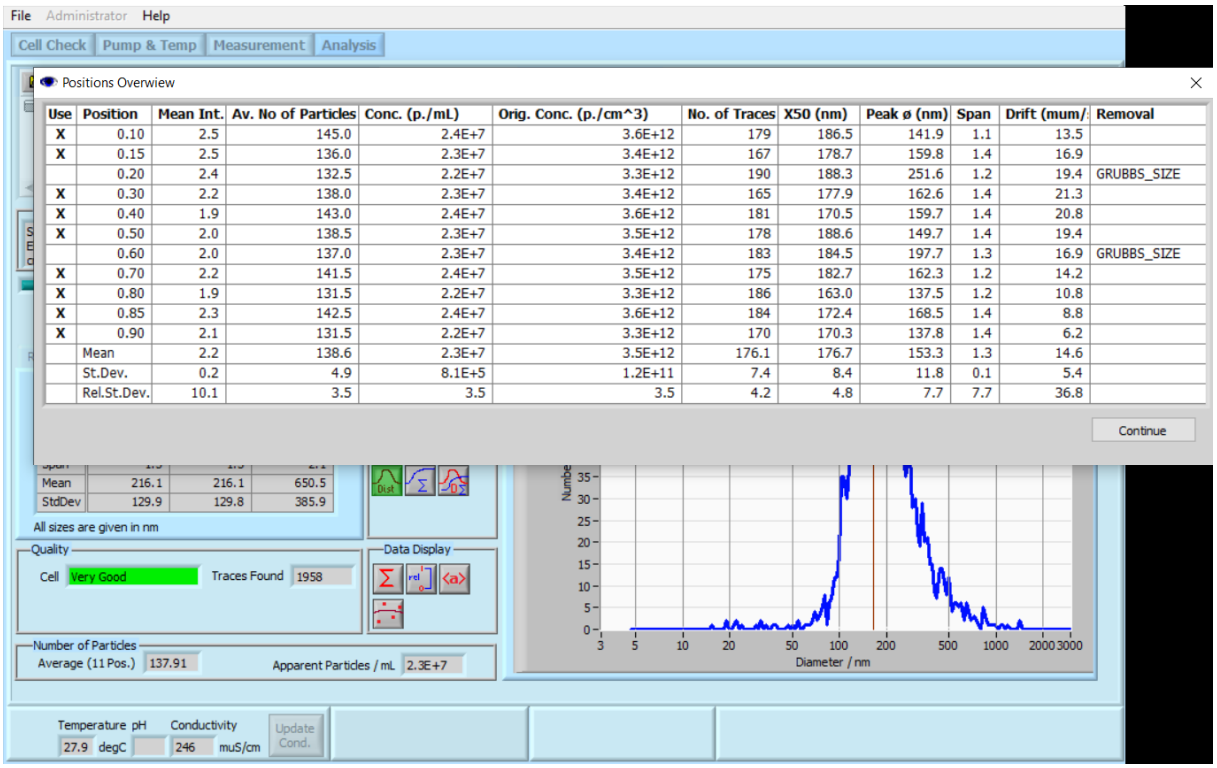
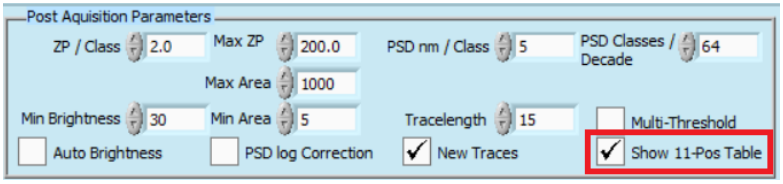


Figure 7-6: Complete analysis with an 11 position table after a size measurement.

7.1 11-Position table

If “Show 11-Pos Table” is enabled in the Analysis tab, the 11-position table is automatically displayed after every 11-position measurement, but before generation of the pdf-report (for pdf report see section 7.3).



The 11- position table shows statistical values and quality criteria for each individual measuring position (0.1 - 0.9) that can be used for a quality assessment of the sample or the entire measurement.

The figure below shows an example of an 11-position table.

Use	Position	Mean Int.	Av. No of Particles	Conc. (p./mL)	Orig. Conc. (p./cm^3)	No. of Traces	X50 (nm)	Peak ø (nm)	Span	Drift (µm/s)	Removal
X	0.10	7.3	197.0	8.5E+7	8.5E+8	39	109.1	122.8	1.0	8.6	
X	0.15	6.7	180.0	7.8E+7	7.8E+8	34	109.1	146.1	0.8	9.5	
X	0.20	7.0	184.0	7.9E+7	7.9E+8	42	111.1	116.8	0.6	9.1	
X	0.30	7.1	162.0	7.0E+7	7.0E+8	43	104.3	93.2	0.7	9.6	
X	0.40	7.4	172.0	7.4E+7	7.4E+8	28	111.1	106.6	1.3	8.9	
X	0.50	7.0	173.0	7.5E+7	7.5E+8	42	111.1	139.2	0.6	8.4	
X	0.60	7.4	186.0	8.0E+7	8.0E+8	43	85.8	82.8	0.7	7.4	
X	0.70	7.1	163.0	7.0E+7	7.0E+8	30	105.9	104.1	1.0	6.8	
X	0.80	7.1	167.0	7.2E+7	7.2E+8	38	115.1	107.4	1.0	5.9	
X	0.85	6.9	146.0	6.3E+7	6.3E+8	35	113.1	106.6	0.8	4.8	
X	0.90	6.7	134.0	5.8E+7	5.8E+8	33	118.3	104.6	0.9	4.0	
	Mean	7.1	169.5	7.3E+7	7.3E+8	37.0	108.5	111.8	0.9	7.5	
	St.Dev.	0.2	18.1	7.8E+6	7.8E+7	5.3	8.5	18.5	0.2	1.9	
	Rel.St.Dev.	3.3	10.7	10.7	10.7	14.5	7.8	16.6	22.5	25.8	

Figure 7-7: 11-position table after a measurement of all 11-positions without showing any outliers.

For each measuring position 0.1 to 0.9 (column 2; “Position”) statistical average values are displayed which derive from the detected particles in the field of view for the corresponding measuring position.

Table 7.2 explains the columns of the 11-position table:

Column	Headline of the column	Meaning
1	Use	A cross in this measuring position indicates inclusion in the evaluation
2	Position	Shows the measuring position
3	Mean Intensity	Mean scattered light intensity of all detected particles in the field of view averaged over all video frames for the corresponding measurement position
4	Av. No of Particles	Number of detected particles in the field of view, averaged over all video frames for the corresponding measurement position
5	Conc. (Particles/ml)	Measured particle concentration
6	Orig. Conc. (Particles/ml)	Calculated particle concentration, taking into account the dilution factor, if entered
7	Number of Traces	Number of found traces of all particles in the field of view
8	X50 (nm)	Median size of all detected particles considered in the field of view
9	Peak Ø (nm)	Mode size of the detected particles in the field of view
10	Span	Measure of the width of the distribution. The span is calculated from (X90-X10) / X50
11	Drift	Average non-Brownian related movement of the particles in the field of view within the measuring cell
12	Removal	Shows the reason a statistical outlier has been rejected in the corresponding measurement position

The last 3 lines of the 11-position table show “Mean”, “Standard Deviation” and “Relative Standard Deviation” across all measurement positions (0.1-0.9) for the parameters in the columns 3-11 (Mean Intensity - Drift).

Outlier control:

The 11-position measurement features an automatic outlier control. When “Show 11-Pos Table” function is enabled in the Analysis tab, the 11-position table will be displayed after the measurement.
In general, we recommend having at least 8 valid positions to accept the data, but sometimes with difficult samples (for example only low concentrations of particles available, high scattering background, very heterogeneous particles in the sample) you will need to compromise and accept less valid positions, or even run the system at just the center position.

If the ZetaView® software detects potential outliers in one or several measuring positions, these positions are not marked with a cross in the first column (Use) and the positions will not be included in the analysis by default. In addition, the last column of the 11-position table (Removal) indicates the reason why the software does not consider this measurement position for evaluation.
The following figure shows an example of an 11-position table with detected outliers.

Use	Position	Mean Int.	Av. No of Particles	Conc. (p./mL)	Orig. Conc. (p./cm^3)	No. of Traces	X50 (nm)	Peak ø (nm)	Span	Drift (µm/s)	Removal
	0.10	10.4	381.0	1.6E+8	1.6E+11	1	91.1	93.9	0.0	9.5	MIN_TRACES
	0.15	11.1	507.0	2.2E+8	2.2E+11	4	58.1	83.3	0.5	12.0	MIN_TRACES
	0.20	10.7	413.0	1.8E+8	1.8E+11	1	63.6	65.5	0.0	17.1	MIN_TRACES
	0.30	10.1	313.0	1.3E+8	1.3E+11	2	50.3	50.8	0.6	17.9	MIN_TRACES
	0.40	9.8	273.0	1.2E+8	1.2E+11	5	55.1	54.3	1.0	17.7	GRUBBS_NUMBER
	0.50	9.6	222.0	9.6E+7	9.6E+10	1	216.1	222.7	0.0	15.5	MIN_TRACES
	0.60	9.9	280.0	1.2E+8	1.2E+11	1	55.1	56.8	0.0	10.2	MIN_TRACES
	0.70	10.0	297.0	1.3E+8	1.3E+11	2	119.3	120.5	1.0	7.0	MIN_TRACES
	0.80	10.4	359.0	1.5E+8	1.5E+11	2	62.5	62.9	0.5	5.4	MIN_TRACES
	0.85	10.8	425.0	1.8E+8	1.8E+11	1	37.1	38.2	0.0	2.9	MIN_TRACES
	0.90	10.7	406.0	1.7E+8	1.7E+11	7	76.1	80.1	0.9	2.5	GRUBBS_MI
	Mean	0.0	0.0	0.0E+0	0.0E+0	0.0	0.0	0.0	0.0	0.0	
	St.Dev.	0.0	0.0	0.0E+0	0.0E+0	0.0	0.0	0.0	0.0	0.0	
	Rel.St.Dev.	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	

Continue

Figure 7-8: Example of an 11-position table with outliers. None of the positions is considered for the final analysis.

Column 1 (Use) of the table above does not consider a measurement position for the evaluation, because the ZetaView® software's outlier control has identified all positions as outliers. The last column (Removal) shows the reasons why the measurement positions are declared as outliers; therefore in this example, “Mean”, “St.Dev and “Rel. St.Dev.” have no values.

The outlier control of the ZetaView® software checks a total of 7 parameters, which are determined during an 11-position measurement. By comparing with established default values and by comparing the measurement positions among each other by using the GRUBBS significance test, the ZetaView® software recognizes potential outliers within the measurement positions.

The following 7 parameters are queried and checked in the outlier control in the order shown below. Furthermore, for each parameter there are possible reasons listed as to why the software suggests removing the measuring position.

Table 7.3: Summary of parameters checked by the outlier control and reasons for removal

Priority of the query	Outlier message	Refers to column	Meaning	Possible reason for removal
1	Min_Traces	No. of Traces	A particle must be followed for at least 5 or more traces. This message typically comes up when only few particles are in the sample	Too few particles; concentration too low
2	Max_Drift	No column; Motion of the Particles; See also “Check Particle Drift at 0mV” (section 6.1.7)	Flagged when the speed of the particles exceeds 30µm/sec	Temperature issues; Buffers of different viscosity or salt content were mixed; Leakage or bubbles in the fluidic system
3	Range_Size	Peak Diameter	Flagged when particles are detected that are lower than 10 nm and larger than 3000 nm.	Detected particles are too large or too small for reliable size determination
4	Range_Number	Av. No. of Particles	Flagged when the average number of particles is lower than 10 and larger than 800. This message may come up particularly, when only a few particles are in the sample	Too few or too many particles in the sample
5	GRUBBS_Number	Av. No. of Particles	Deviation of the average number of particles is too large compared to other positions	Heterogeneous distribution of particles throughout the cell
6	GRUBBS_MI	Mean Int.	Deviation of the mean intensity of the particles is too large compared to other positions	Air bubble; large particles; High scattering contaminations in the sample; Background from free fluorescence dye or protein monomers; Optics too close to one cell wall (in case of misalignment)
7	GRUBBS_SIZE	Peak Diameter	Deviation of the size of the particles is too large compared to other positions	Heterogeneous distribution of large particles throughout the cell

The GRUBBS Significance Test refers to the parameters “Average Number of Particles”, “Mean Intensity”, and “Peak Diameter”.

The automatic result of the outlier control within the 11-position table can be over-ridden manually by clicking on the desired measurement position in column 1 (Use). As a result, the corresponding message in column 12 (Removal) disappears.
This is shown in the following two example tables.

Use	Position	Mean Int.	Av. No of Particles	Conc. (p./mL)	Orig. Conc. (p./cm^3)	No. of Traces	X50 (nm)	Peak ø (nm)	Span	Drift (µm/s)	Removal
X	0,10	2,6	67,0	1,4E+7	1,4E+7	19	135,4	115,9	1,2	14,0	
	0,15	2,5	53,0	1,1E+7	1,1E+7	11	180,5	227,9	0,8	15,4	GRUBBS_SIZE
X	0,20	2,6	41,0	8,5E+6	8,5E+6	20	142,9	143,5	0,8	18,2	
X	0,30	2,5	48,0	9,9E+6	9,9E+6	15	145,5	150,1	1,6	20,8	
X	0,40	2,6	76,0	1,6E+7	1,6E+7	19	142,0	151,1	0,8	19,8	
X	0,50	2,5	88,0	1,8E+7	1,8E+7	20	119,3	146,4	0,8	16,5	
X	0,60	2,6	105,0	2,2E+7	2,2E+7	26	128,3	168,5	0,8	14,6	
	0,70	3,2	118,0	2,4E+7	2,4E+7	20	122,3	122,7	0,7	11,7	GRUBBS_MI
X	0,80	2,5	129,0	2,7E+7	2,7E+7	17	135,4	145,0	0,7	7,4	
X	0,85	2,6	157,0	3,2E+7	3,2E+7	16	128,3	171,6	1,0	5,4	
	0,90	2,8	157,0	3,2E+7	3,2E+7	21	118,6	124,3	0,5	4,2	GRUBBS_MI
	Mean	2,6	88,9	1,8E+7	1,8E+7	19,0	134,6	149,0	1,0	14,6	
	St.Dev.	0,1	39,9	8,2E+6	8,2E+6	3,4	8,9	17,1	0,3	5,6	
	Rel.St.Dev.	2,8	44,8	44,8	44,8	17,8	6,6	11,5	32,9	38,4	

Continue

Figure 7-9: 11-position table after measurement. The ZetaView® software has detected 3 outliers.

Use	Position	Mean Int.	Av. No of Particles	Conc. (p./mL)	Orig. Conc. (p./cm^3)	No. of Traces	X50 (nm)	Peak ø (nm)	Span	Drift (µm/s)	Removal
X	0,10	2,6	67,0	1,4E+7	1,4E+7	19	135,4	115,9	1,2	14,0	
	0,15	2,5	53,0	1,1E+7	1,1E+7	11	180,5	227,9	0,8	15,4	GRUBBS_SIZE
X	0,20	2,6	41,0	8,5E+6	8,5E+6	20	142,9	143,5	0,8	18,2	
X	0,30	2,5	48,0	9,9E+6	9,9E+6	15	145,5	150,1	1,6	20,8	
X	0,40	2,6	76,0	1,6E+7	1,6E+7	19	142,0	151,1	0,8	19,8	
X	0,50	2,5	88,0	1,8E+7	1,8E+7	20	119,3	146,4	0,8	16,5	
X	0,60	2,6	105,0	2,2E+7	2,2E+7	26	128,3	168,5	0,8	14,6	
X	0,70	3,2	118,0	2,4E+7	2,4E+7	20	122,3	122,7	0,7	11,7	
X	0,80	2,5	129,0	2,7E+7	2,7E+7	17	135,4	145,0	0,7	7,4	
X	0,85	2,6	157,0	3,2E+7	3,2E+7	16	128,3	171,6	1,0	5,4	
	0,90	2,8	157,0	3,2E+7	3,2E+7	21	118,6	124,3	0,5	4,2	GRUBBS_MI
	Mean	2,6	92,1	1,9E+7	1,9E+7	19,1	133,3	146,1	0,9	14,3	
	St.Dev.	0,2	38,5	8,0E+6	8,0E+6	3,2	9,3	18,2	0,3	5,3	
	Rel.St.Dev.	8,2	41,8	41,8	41,8	16,6	7,0	12,5	32,6	37,4	

Continue

Figure 7-10: Position 0.7 has been activated by a cross manually. As a result, the message "GRUBBS_MI" has disappeared. This position is now added into the analysis when clicked "Continue".

Use	Position	Mean Int.	Av. No of Particles	Conc. (p./mL)	Orig. Conc. (p./cm^3)	No. of Traces	X50 (nm)	Peak ø (nm)	Span	Drift (µm/s)	Removal
X	0,10	2,6	67,0	1,4E+7	1,4E+7	19	135,4	115,9	1,2	14,0	
	0,15	2,5	53,0	1,1E+7	1,1E+7	11	180,5	227,9	0,8	15,4	GRUBBS_SIZE
X	0,20	2,6	41,0	8,5E+6	8,5E+6	20	142,9	143,5	0,8	18,2	
X	0,30	2,5	48,0	9,9E+6	9,9E+6	15	145,5	150,1	1,6	20,8	
	0,40	2,6	76,0	1,6E+7	1,6E+7	19	142,0	151,1	0,8	19,8	MANUAL
X	0,50	2,5	88,0	1,8E+7	1,8E+7	20	119,3	146,4	0,8	16,5	
X	0,60	2,6	105,0	2,2E+7	2,2E+7	26	128,3	168,5	0,8	14,6	
	0,70	3,2	118,0	2,4E+7	2,4E+7	20	122,3	122,7	0,7	11,7	MANUAL
X	0,80	2,5	129,0	2,7E+7	2,7E+7	17	135,4	145,0	0,7	7,4	
X	0,85	2,6	157,0	3,2E+7	3,2E+7	16	128,3	171,6	1,0	5,4	
	0,90	2,8	157,0	3,2E+7	3,2E+7	21	118,6	124,3	0,5	4,2	GRUBBS_MI
	Mean	2,5	90,7	1,9E+7	1,9E+7	19,0	133,6	148,7	1,0	13,8	
	St.Dev.	0,1	42,7	8,8E+6	8,8E+6	3,7	9,1	18,4	0,3	5,6	
	Rel.St.Dev.	2,6	47,0	47,0	47,0	19,2	6,8	12,4	33,4	40,5	

Continue

Figure 7-11: The positions 0.4 and 0.7 were manually removed, as indicated by the message "Manual" in the last column.

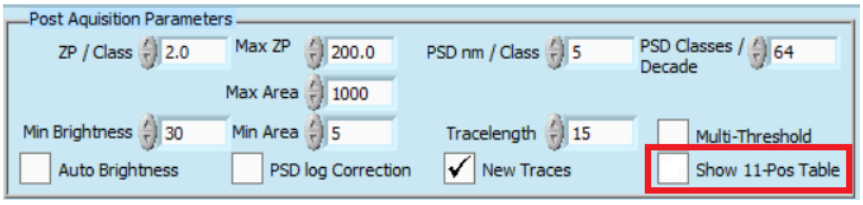
Please note that the message “MANUAL” only appears when one or more positions are removed manually but not when they are added manually.
In this example above, the positions 0.15, 0.4, 0.7 and 0.9 are not included in the evaluation.

In addition, note that the software leaves it to the operator to make the final determination regarding the outlier test. The outliers shown in the result table are therefore not to be considered "carved in stone", but rather as a statistical recommendation.

After clicking on "Continue" in the 11-position table, only those measuring positions activated with a cross in column 1 (Use) will then be analyzed. This is independent of whether the measurement positions were manually activated or deactivated by a cross, or whether the operator follows the automatic outlier control completely.

Only after "Continue" has been clicked on will the evaluation and generation of the pdf-report (see section 7.3) take place.

If “Show 11-Pos Table” is not enabled in the Analysis tab, the 11-position table is not automatically displayed, however, the outlier control is still done in the background. This means that the ZetaView® software automatically removes those measurement positions that contain potential outliers. In this case, the user has no influence on the table for enabling or disabling distinct measuring positions.



7.2 Multiple Acquisitions

The feature “Multiple Acquisitions” can be used to automatically perform a pre-determined number of measurements. The time interval between measurements and the liquid volume dispensed through the pump's step mode can be controlled as well. This feature can be used for size measurements, zeta potential measurements and fluorescence measurements. In fluorescence measurements, “Multiple Acquisitions” can be used simultaneously with the "Low Bleach" function to reduce rapid bleaching of dyes (ref. section 12.6). With the “Multiple Acquisitions” function, the statistics of the measurements can be significantly increased.

In addition, with this feature it is possible to observe a sample in the measuring cell over a defined period. At certain time intervals, which can be adjusted by the operator, the same measurement of the sample can always be repeated automatically in order to examine changes in the sample, such as agglomeration of the particles, or a shift in the size distribution. In this way, a kinetics experiment can be run very easily.

All measuring parameters can be freely adjusted by the operator of the ZetaView® instrument, but it should be noted that sensible parameters should be set for the respective experiment.

The following example shows how to adjust the ZetaView® to automatically perform three size measurements with a time delay of 1 minute:

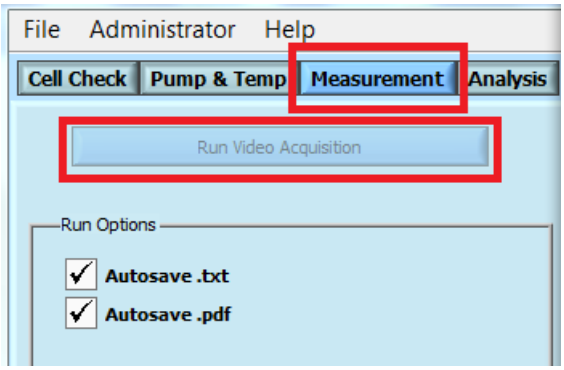


Figure 7-12: Click „Run Video Acquisition” in the Measurement tab.

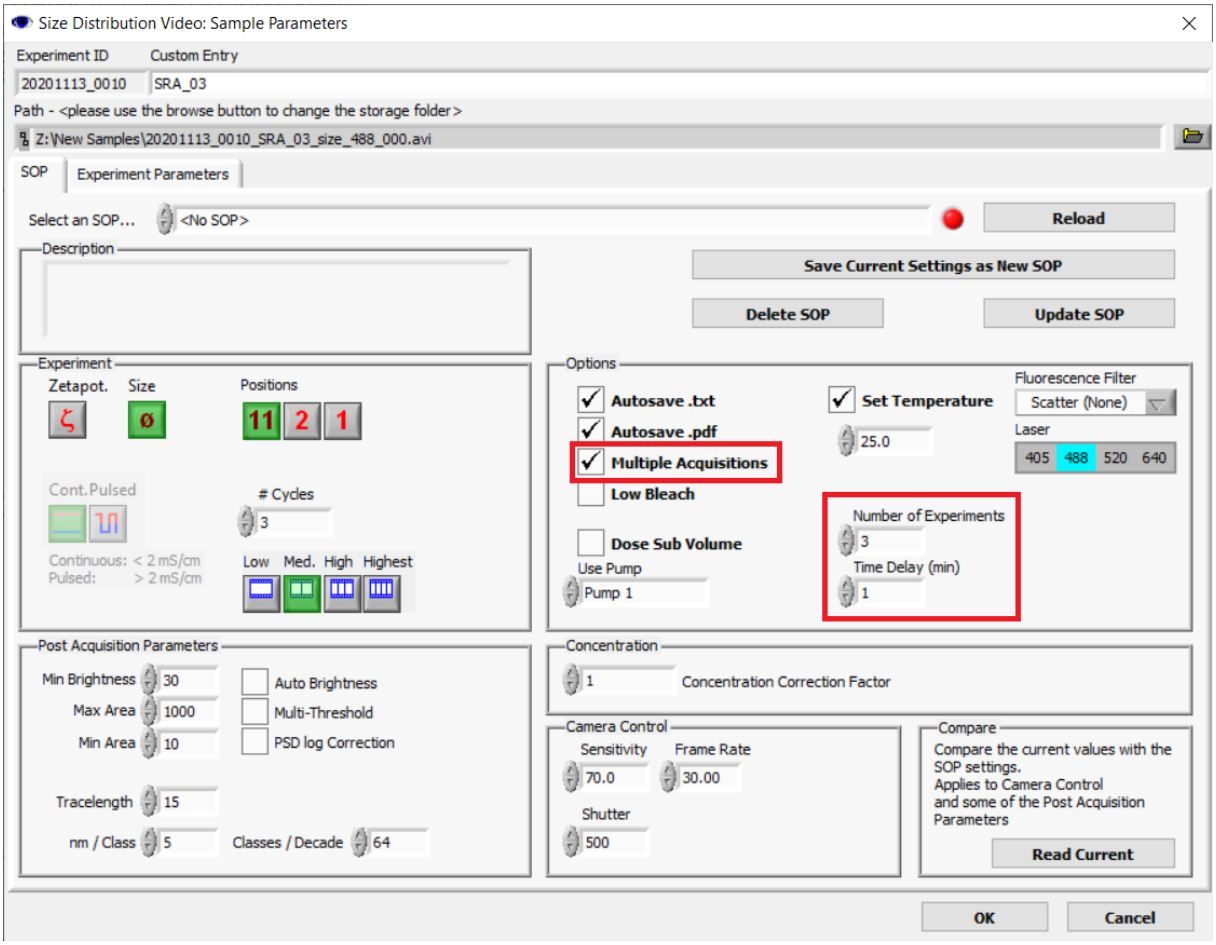


Figure 7-13: The settings for the Multiple Acquisition function are highlighted in red.

1. Activate “Multiple Acquisitions“
2. Enter „Number of Experiments“: 3
3. Enter „Time Delay (min)“: 1
4. „Dose Sub Volume“ is not activated

A series of 3 measurements of the same samples will start. Between each measurement, a 1-minute pause is taken before the next measurement starts. Depending on the adjusted number of measurements, a temporal kinetics experiment can be carried out in this way. The following figure shows schematically what the ZetaView® is doing if adjusted with the settings above.

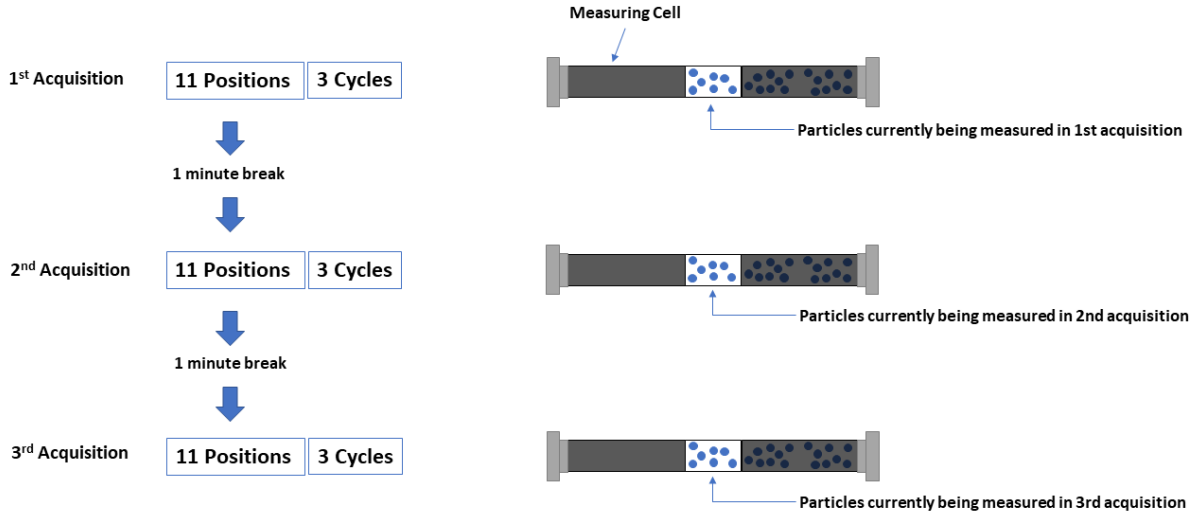


Figure 7-14: Schematic representation of a "Multiple Acquisitions" measurement with 3 acquisitions. In each acquisition, all 11 positions are measured 3 times. After each acquisition, a 1-minute pause is taken before proceeding to the subsequent acquisition. In all acquisitions, the same sub volume is measured.

It is important to note that the "Show 11-position table" function needs to be unchecked for the acquisitions to proceed without user intervention (see figure below). Otherwise, the experiment hangs up after one acquisition while waiting for the user to hit "continue".

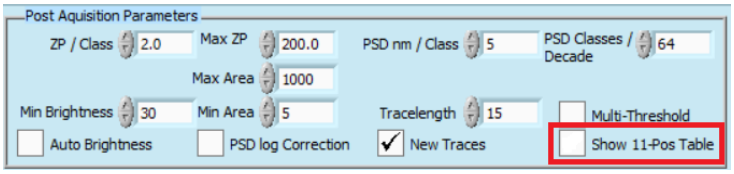


Figure 7-15:"Show 11-Pos Table" must be unchecked if "Multiple Acquisitions" includes 11-positions measurements. This does not apply for "Multiple Acquisitions" comprising 2 positions or 1 position.

The "Dose Sub Volume" feature extends "Multiple Acquisitions" by moving the sample through the cell after the pause (time delay), so that a new sub volume of the same sample enters the ZetaViews measurement optics. In this way, a completely different sub volume of the same sample can be measured. This may be important for samples that tend to agglomerate quickly due to heat and is recommended for fluorescent samples to prevent rapid bleaching. The following illustrations show an example of adjusting 4 acquisitions with a 1-minute pause (time delay 1min) and activated "Dose Sub Volume" function.

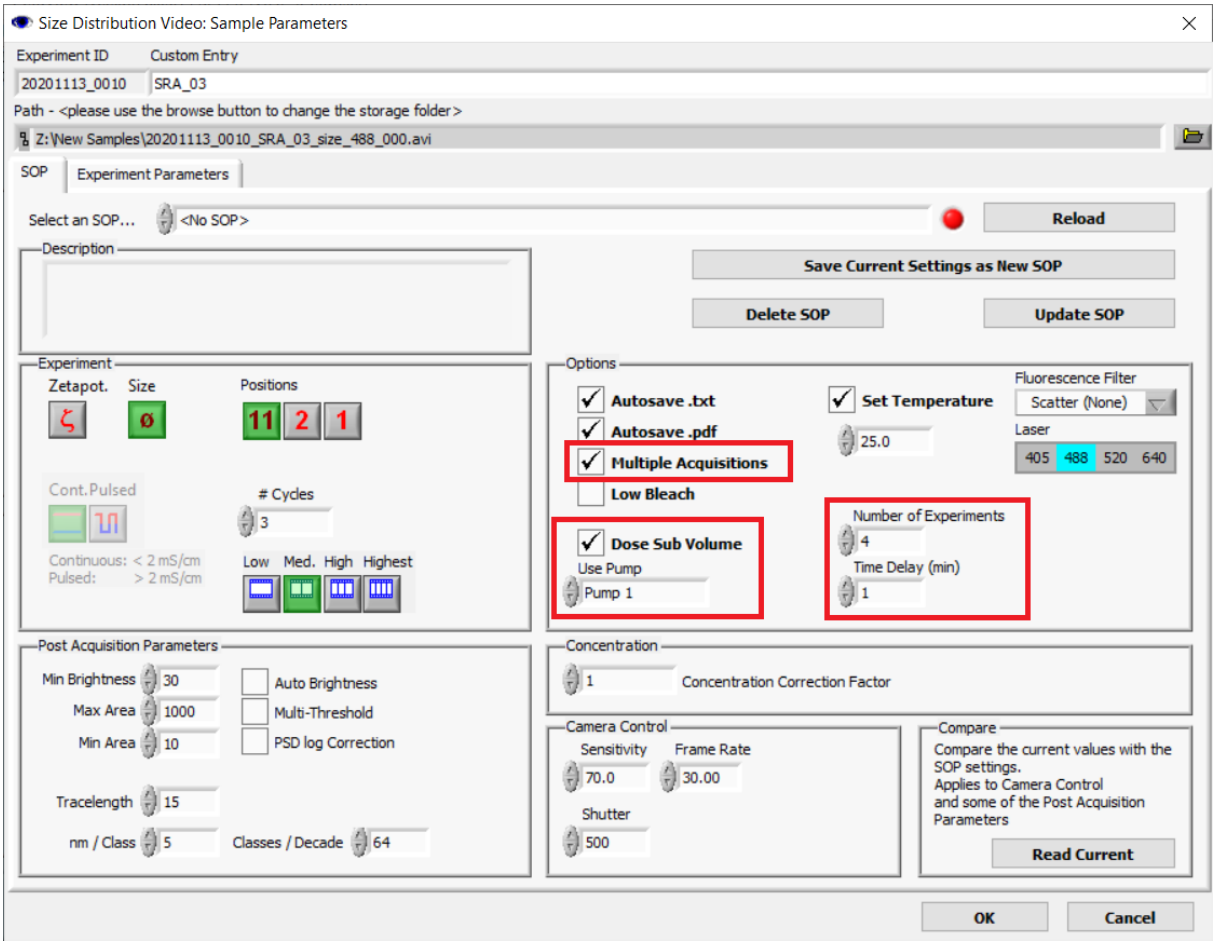


Figure 7-16: The settings for the number of frames (resolution of the video) are highlighted in red.

1. Activate “Multiple Acquisitions“
2. Enter „Number of Experiments“:4
3. Enter „Time Delay (min)“:1
4. Activate „Dose Sub Volume“
5. Select the pump used to move the sub volume through the cell

A series of 4 measurements of the same sample will start. Between each measurement, a time interval of one minute is taken. After this pause, a new sub volume of 20µl is pushed into the field of view of the cell. This sub volume is fixed and cannot be changed. This ensures that all particles that were previously in the field of view of the measuring cell are completely replaced by new particles of the same sample. A minimum of 20 sub volumes can be measured in this way. This is shown schematically in the following figure.

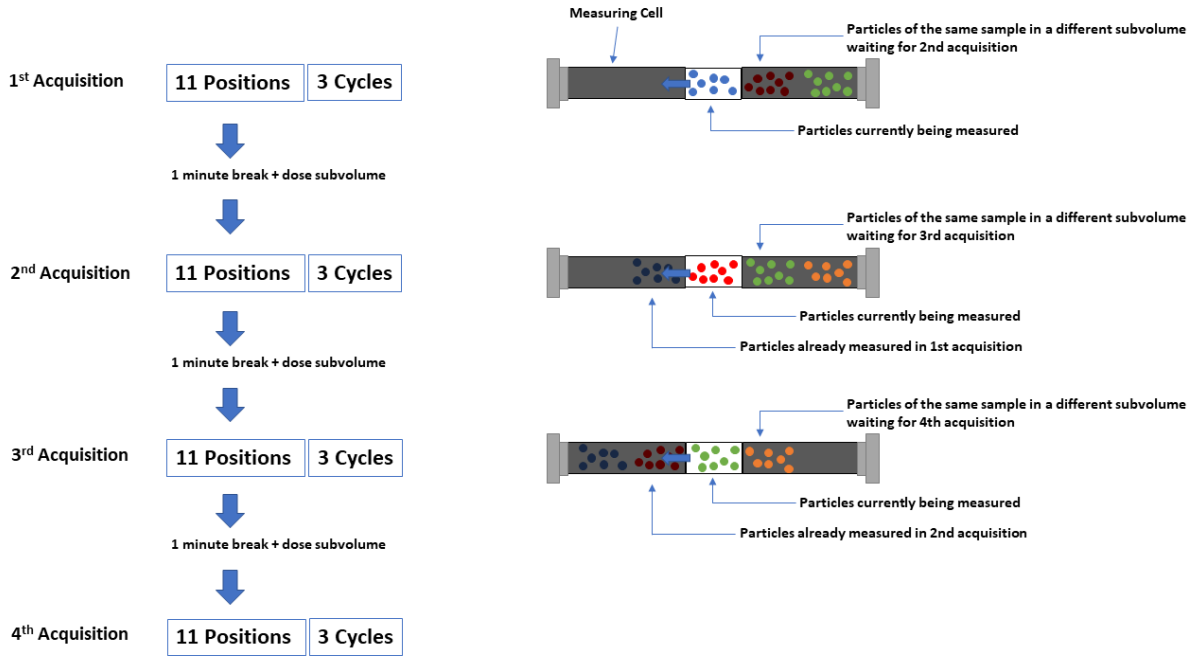


Figure 7-17: Schematic representation of a multiple acquisition measurement with 4 acquisitions and with "Dose Sub Volume" function switched on. In each acquisition, all 11 positions are measured 3 times.

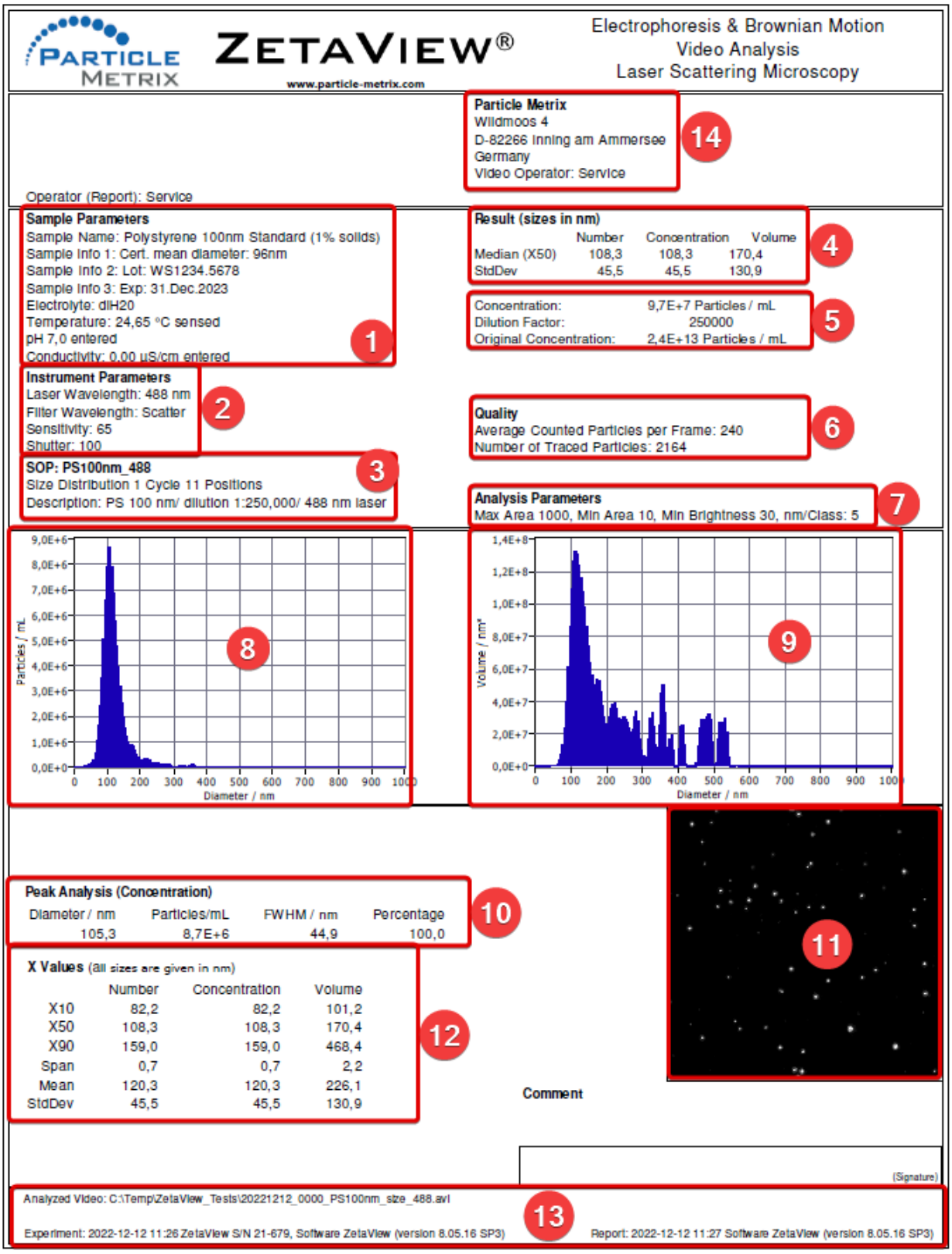
After each acquisition, a 1-minute pause is taken. Subsequently, new particles in a new sub volume are moved into the field of view of the measuring optics by one of the two pumps (red particles will be measured in the 2nd acquisition, green particles in the 3rd acquisition, etc.) before the subsequent acquisition is begun. As a result, in each acquisition, other particles of the same sample are measured.

Since the function "Multiple Acquisitions" has been introduced for strengthening the statistical power of the measurements, each acquisition comes with its own batch of result data files such as pdf report, txt-files, and video files. This is important because you can compare several acquisitions of a single sample.

In contrast to that, if the "Multiple Acquisitions" feature is switched off, an 11-position, 2-position, or 1-position measurement will always show only one batch of result data files, no matter how many cycles have been measured. Hence, the number of result files is generated from the number of acquisitions and not from the number of cycles.

In contrast to single acquisitions, the pdf reports from multiple acquisitions will not show up automatically. They must be called up manually in the corresponding directory.

7.3 Description of the PDF report (size measurement)



1. Sample Parameters

Here, the sample information that was entered on the measurement menu are documented. All those information (comment, sample info 1, sample info 2, sample info 3, Electrolyte, Temperature, and pH) can be found and entered in the „Experiment Parameters“ tab (see below).

Note: The *Sample Name* has to be entered each time the measurement menu is opened, there is no auto-fill of that field or any linkage to the *Custom Entry File Name*.

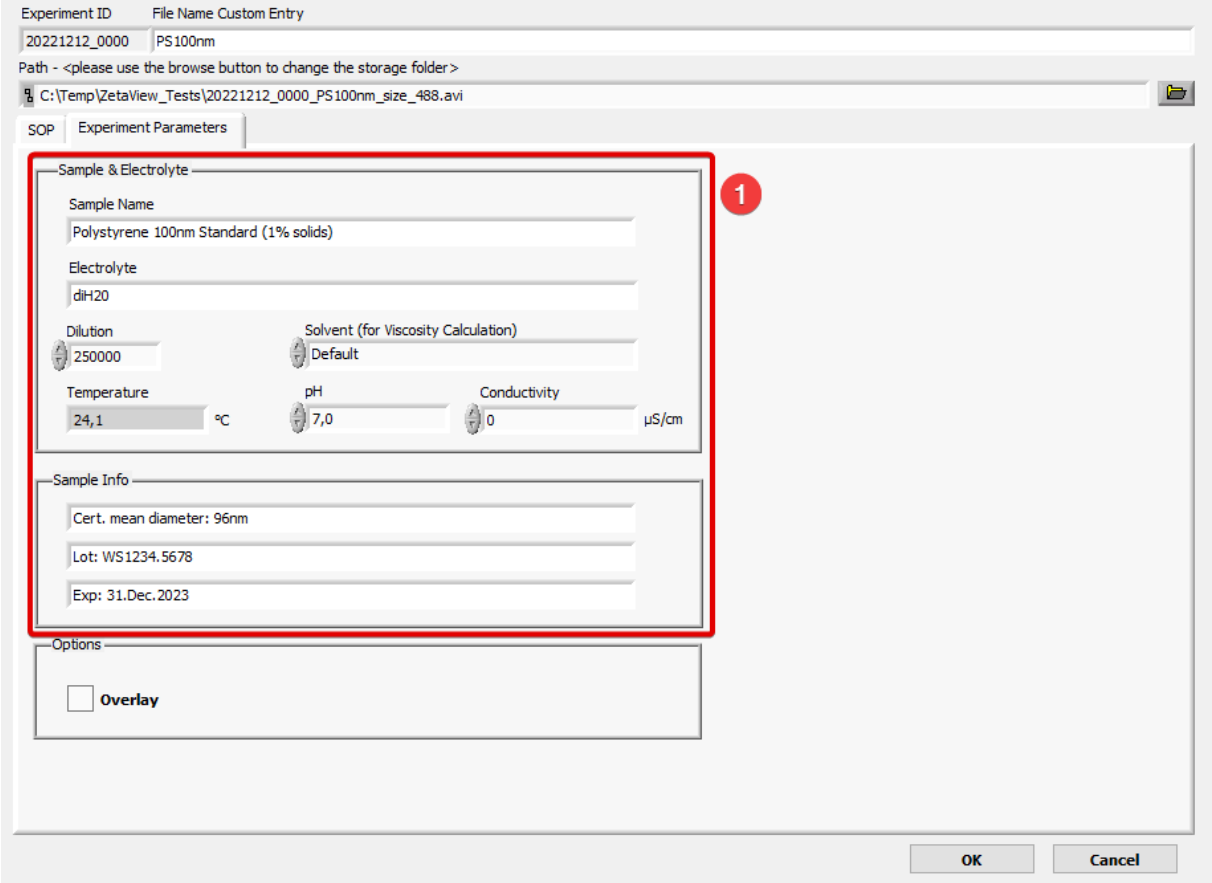


Figure 7-18: The settings for the Experiment Parameters are highlighted in red.

2. Instrument Parameters

This section lists which laser and camera settings have been used during the measurement (e.g. laser wavelength, filter wavelength, shutter and sensitivity settings). If no fluorescence filter was used, “Scatter” is stated on the report.

3. SOP

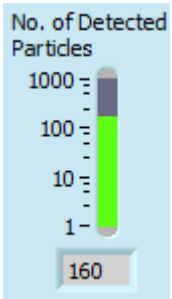
This section lists the name and description of the used SOP and what kind of measurement has been performed (size distribution or zeta potential measurement). In addition, it lists the number of the cycles. In case an SOP was not saved before starting it, (e.g. status of the SOP shows red), the reports states < No SOP >.

4. **Result** (sizes in nm)
   The median (X50 value) is documented for
   - Number-weighted distribution,
   - Concentration-weighted distribution
   - Volume-weighted distribution.

   Since the graph of the number-weighted distribution and concentration-weighted distribution is the same, the values for the number-weighted distribution and concentration-weighted distribution are equal.

5. **Measured concentration** of the particles, dilution factor and original calculated concentration is documented. The original concentration is calculated by the dilution factor multiplied by the measured concentration.

6. **Quality**
   Average counted particles per frame is a calculated value and describes the number of particles that are detected on average within one video frame. This number is very similar to the „No. of Detected Particles“ that you can see in the lower right bar in the software.



   Number of traced particles is the total number of particles that are analysed in the particle size statistics.

7. **Analysis Parameters** (see post-acquisition parameters in section 6.1.6) that were used for the measurement
   - Max Area (maximum number of pixels that a particle contains to be analysed in the statistics)
   - Min Area (minimum number of pixels that a particle contains to be analysed in the statistics)
   - Min Brightness (grey level of the particles) describes the minimum grey level that a particle has for analysis in the statistics

8. **Histogram** of the number-weighted distribution or concentration-weighted distribution, depending on which plot was shown in the Analysis tab when the pdf was created

9. **Histogram** of the volume-weighted distribution

10. **Peak Analysis**
    For every detected peak in the histogram the peak diameter (in nm) is shown. In addition, the absolute number of particles (Number Absolute) related to the corresponding peaks, the FWHM (Full Width Half Maximum) and the percentage is documented.

11. **Picture** of the first image taken during the measurement

12. **X-Values**
    - **X10** = 10% of all analysed particles are smaller than the stated value
    - **X50** = 50% of all analysed particles are smaller and 50% are larger than the stated value
    - **X90** = 90% of all analysed particles are smaller than the stated value

      Example: For the number-weighted distribution and for concentration-weighted distribution 10% of all analysed particles are smaller than 77.1nm.
      For the volume-weighted distribution 10% of all analysed particles are smaller than 114.5nm.

    - **Span** describes the width of the histogram. The span is calculated in the following way:

      $$\mathbf{Span} = \frac{\mathbf{X90} - \mathbf{X10}}{\mathbf{X50}}$$

    - **Mean** is also called average. It is the sum of the values (in nm) divided by the number of values (number of all particles).

    - **StdDev** quantifies the amount of variation of the measured size in nm.

13. **Experiment and Report Details**
    This section lists the details about the analyzed video file (e.g. Windows path and file name) and when the experiment was conducted (e.g. time stamp, ZetaView serial number and software version used). It lists also details when the report was created (e.g. time stamp and ZetaView software version).

14. **Company Information**
    Starting the ZetaView software in Administrator mode (see section 15.1), the Measurement tab offers a “PDF Report Settings” button to customize the report header.

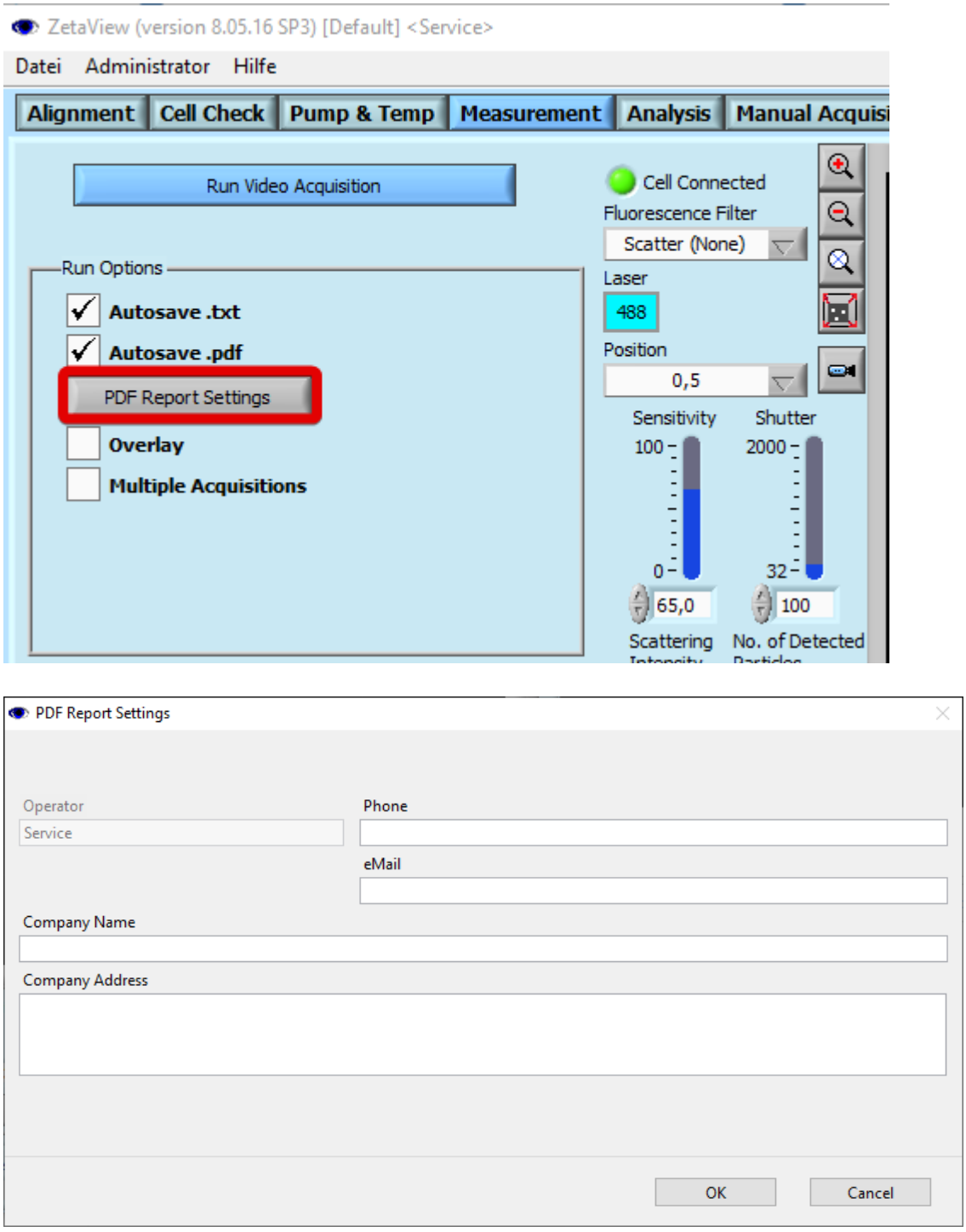


Figure 7-19: PDF Report Settings - dialogue window for entering customized header information.

8 Additional video parameters and settings

8.1 Number of Frames

The ZetaView® instrument determines the size and concentration of the particles based on a video recorded and stored during a measurement. If, for example, an 11-position measurement is performed, a separate video is recorded for each individual measuring position. All 11 videos from such an 11-position measurement are then merged into a single video during the analysis.

Basically, a video of the ZetaView® is a sequence of frames in which the particles are detected and tracked. The number of frames of a video per measurement position can be adjusted in the Measurement tab under "Run Video Acquisition". The number of frames equals the resolution of the video recorded by the ZetaView® instrument.

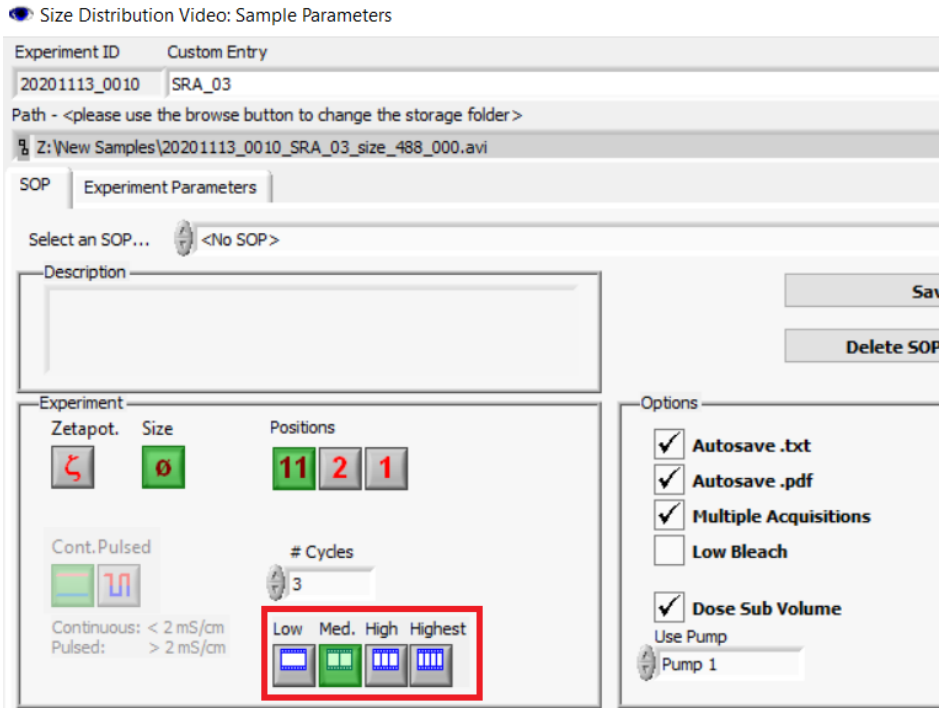


Figure 8-1: The settings for the number of frames (resolution of the video) are highlighted in red.

Table 8.1: „Low“, „Med.“, „High“ and „Highest“ indicates the number of consecutive frames in a video per measuring position.

Setting	Low	Med.	High	Highest
Number of frames	15 frames	30 frames	60 frames	90 frames

In addition, the number of frames contributes to the duration of the video per position as well as to the total length of the final video after a measurement. For example, the

setting “Low” results in a video that contains 15 frames (pictures) “Med.” yields to a video that contains 30 frames. All detected particles are followed frame by frame until the end of the video.

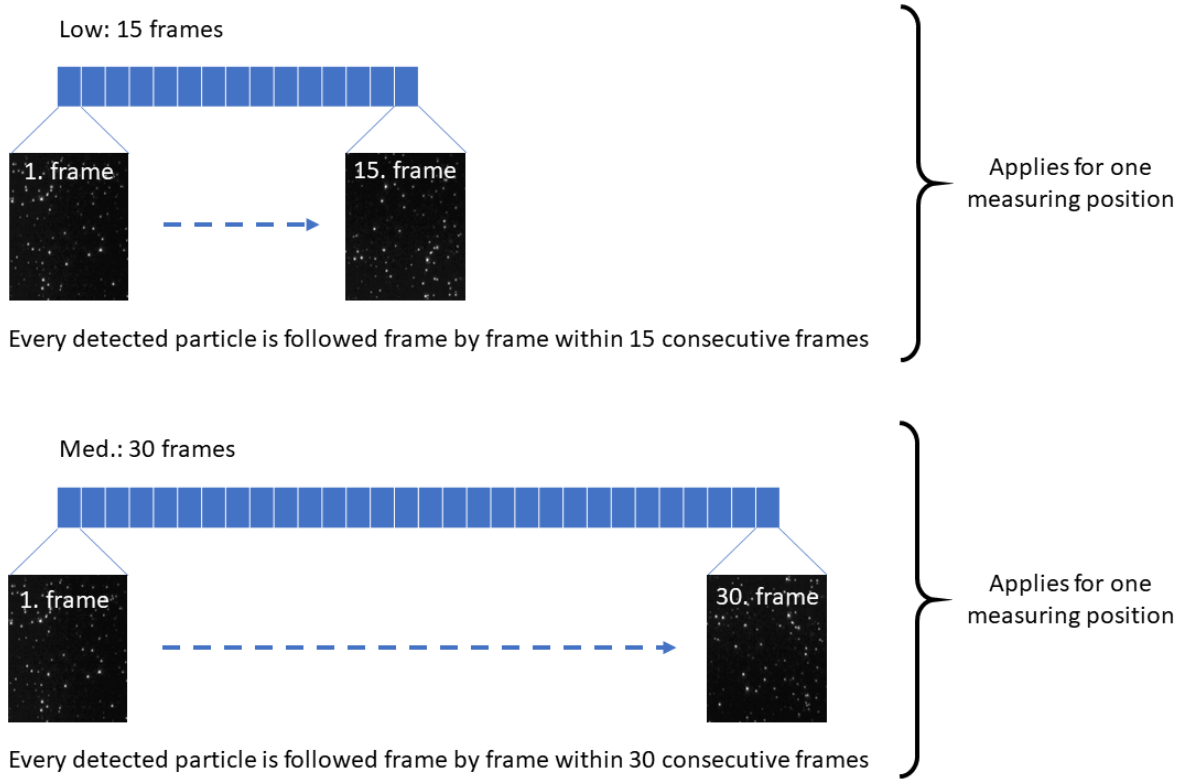


Figure 8-2: The settings “Low” and “Med.” are shown as an example for the adjusted number of frames. The number applies for one position inside the measuring cell.

If, for example, “Med.” and 11 positions is selected for measurement, a video will be recorded that contains 30 pictures per measurement position resulting in 330 pictures for the final video of all 11 positions.

Table 8.2: The number of frames is user-selectable and allows measurement flexibility. The table below shows typical measurement conditions for using the corresponding number of frames:

Low	15 frames	• For a short, fast measurement, since the trace length is of course limited by the number of frames • To keep the exposure of the laser or duration of the electric field as short as possible (for example, profile measurement of zeta potential) • when running a fluorescence measurement with a fast photo-bleaching fluorophore • Please note that the received distributions (size, zeta potential) are somewhat wider in proportion as the estimate is based on fewer data points (per trace as well as number of traces) • if the sample is not changing in the cell on a short time frame, the number of frames selection will have no impact on the concentration calculation
Med.	30 frames	• Default setting • Optimal compromise between acquisition time and accuracy of the data • The received distributions are narrower in proportion as the results are based on much more data points (per trace as well as number of traces)
High	60 frames	• If a better resolution than with “Med” (default) is required • Acquiring data with this resolution can allow for the measurement of a more accurate diffusion coefficient and thus a more accurate size can be obtained • Acquisition time is longer
Highest	90 frames	• If the best resolution is required • advantageous for large particles, from about 300 nm and larger • a long tracelength (60) is possible

At low concentration, for example below 50 particles in the image (corresponds to the orange color in the “Number of Detected Particles” indicator; see section 6.1.3), an increase in the number of cycles (e.g., 3x measurement of 11 position) is more advantageous than an extension of the video.

It should be noted that the number of the frames should not be mixed up with the frame rate (see section 8.2) which indicates how many frames per second are recorded.

8.2 Frame Rate

The frame rate indicates the number of frames recorded or played back per time period and is given in the unit fps (frames per second). Because the ZetaView® captures a video for a size or zeta potential measurement, it takes a certain number of pictures to be analyzed for the determination of the diffusion coefficient for each individual particle.

The frame rate sets the camera speed and thus the time interval between two subsequent images. There are two ways for changing the frame rate. It can be adjusted either by clicking on the camera control button, or in the Measurement tab by clicking “Run Video Acquisition”.

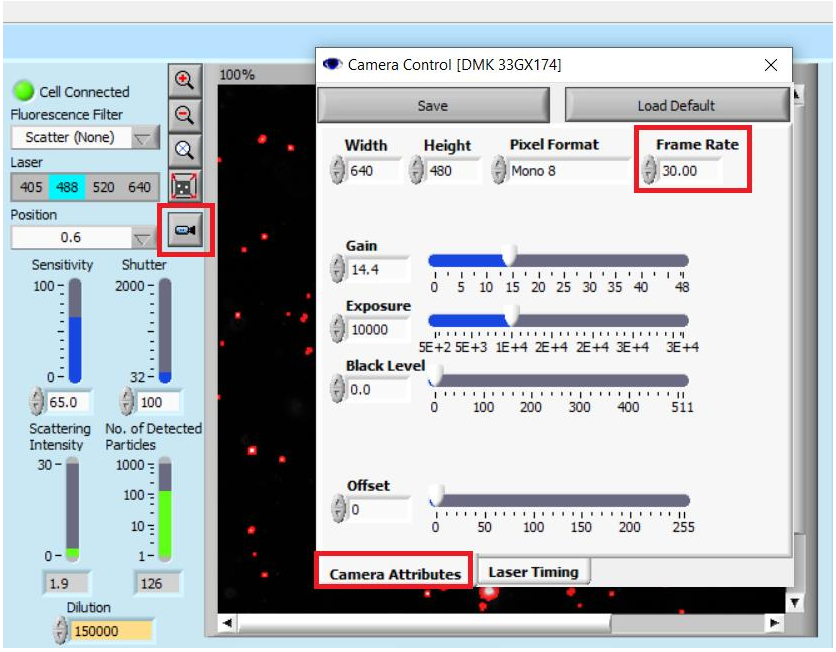


Figure 8-3: The frame rate can be adjusted in the camera control.

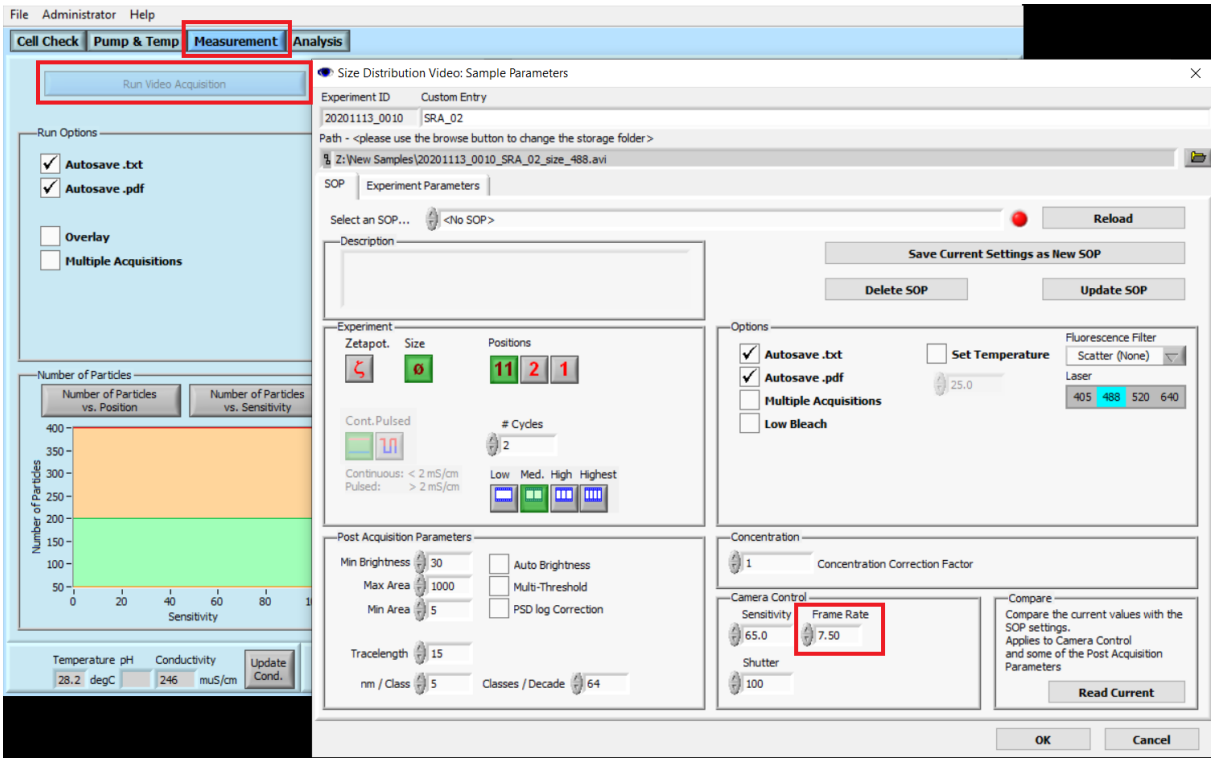


Figure 8-4: The frame rate can be adjusted in the Measurement tab as well.

Small particles require a higher frame rate in order not to underestimate the mean square displacement. The diffusion properties and thus the mean square displacement will be smaller for larger the particles. The resolution of the displacement can be improved by extending the time interval between two subsequent frames, i.e. reducing the frame rate. Recommendations for camera settings are summarized in the next table.

Table 8.3: Summary of pre- and post-acquisition parameters. Frame Rate is highlighted in blue.

	Parameter	PS 40 nm	PS 100 nm	PS 200 nm	PS 300 nm	PS 400 nm	PS 500 nm	PS 600 nm	PS >600 nm
Pre-acquisition	Sensitivity	80-90	65-70	60	60	50-60	50-60	50-60	50-60
	Shutter	50	100	100	150-200	200-300	300	500	500
	Frame Rate (fps)	**60-30**	**30**	**30-15**	**15**	**15-7.5**	**7.5**	**7.5-3.75**	**3.75**
	Resolution (Number of frames)	Medium	Medium	Medium-High	High	High-Highest	Highest	Highest	Highest
Post-acquisition	Minimum Brightness	20	25	Auto	Auto	Auto	Auto	Auto	Auto
	Min Area	5	5	40	50	50	100	100	100
	Max Area	100	1000	2000	10000	10000	10000	10000	10000
	Tracelength	<15	≥15	≥30	≥30	≥30	≥45	≥45	≥45

Table 8.4: The length of the video per position is dependent on the framerate and on the selected number of frames (Low, Med, High, Highest) that are adjusted for a measurement.

		Frame Rate (fps)				
		3.75	7.5	15	30	60
Video	Low (15 frames)	4 sec.	2 sec.	1 sec.	0.5 sec.	0.25 sec.
	Med. (30 frames)	8 sec.	4 sec.	2 sec.	1 sec.	0.5 sec.
	High (60 frames)	16 sec.	8 sec.	4 sec.	2 sec.	1 sec.
	Highest (90 frames)	24 sec.	12 sec.	6 sec.	3 sec.	1.5 sec.

The video lengths given in the table above are approximate and do not include the computational analysis of the measurement after completion of the videos.

8.3 Tracelength

Tracelength can be selected in the Measurement Menu under "Run Video Acquisition" as well as in the Analysis tab and represents a post-acquisition parameter.

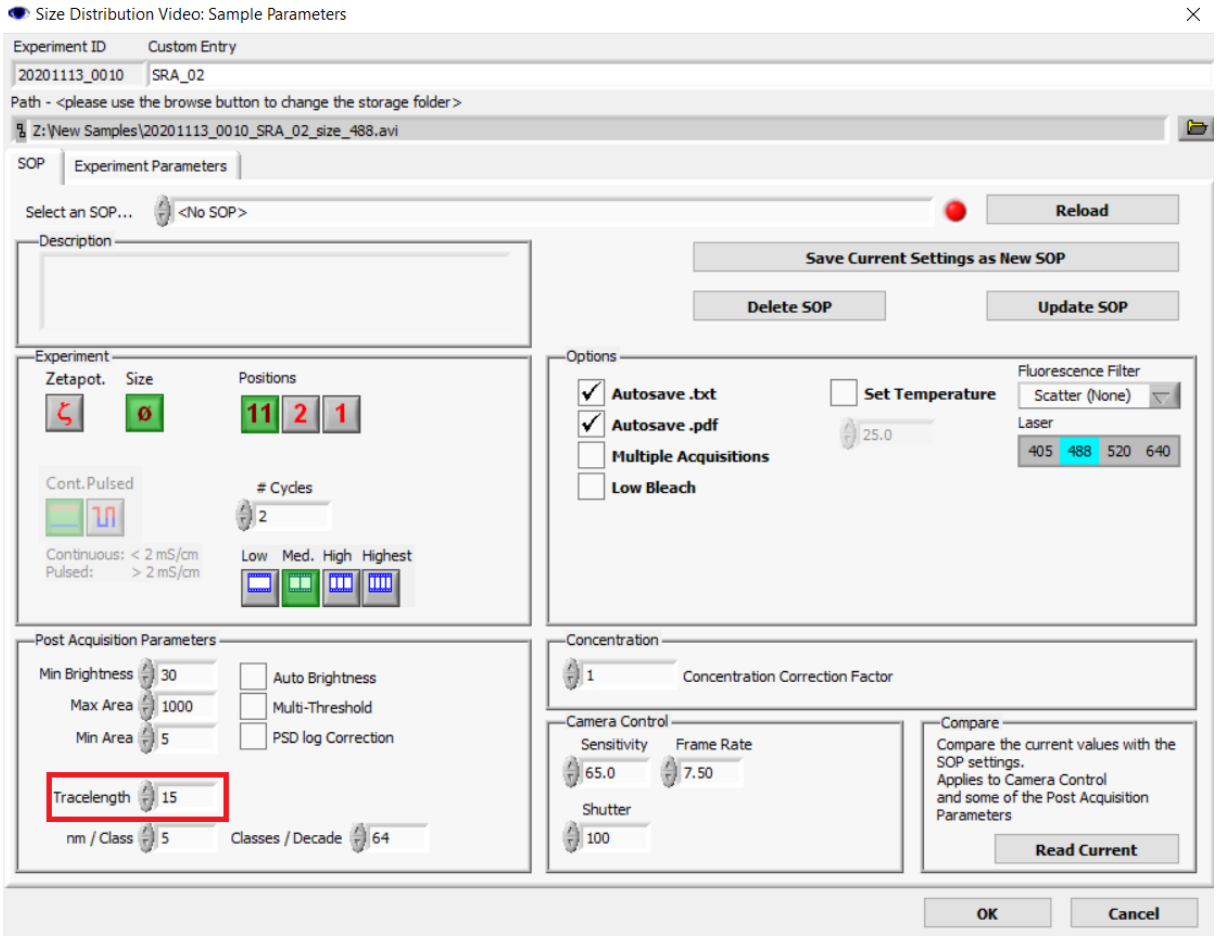


Figure 8-5: Tracelength is selectable in the "Run Video Acquisitions" menu.

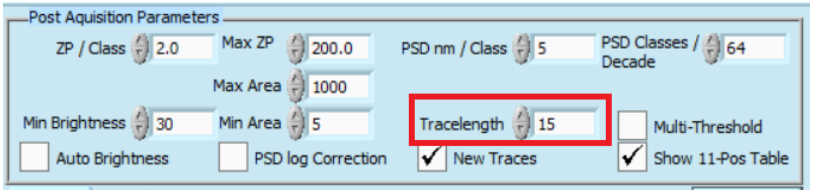


Figure 8-6: Tracelength is selectable in the Analysis tab as well.

Tracelength specifies the minimum number of consecutive video frames in which a particle must be tracked before being included in the final analysis and statistics. Generally, the resolution of the particle size distribution is improved as the tracelength is increased. For a 100 nm particle it is adequate to set the tracelength to 15.

This means that the particle must be present in at least 15 consecutive frames (per video per measuring position) without leaving the focal plane or the field of view. It is not recommended to set the tracelength to values below 10.

In the following figure, this is illustrated by way of example for an adjusted tracelength of 15.

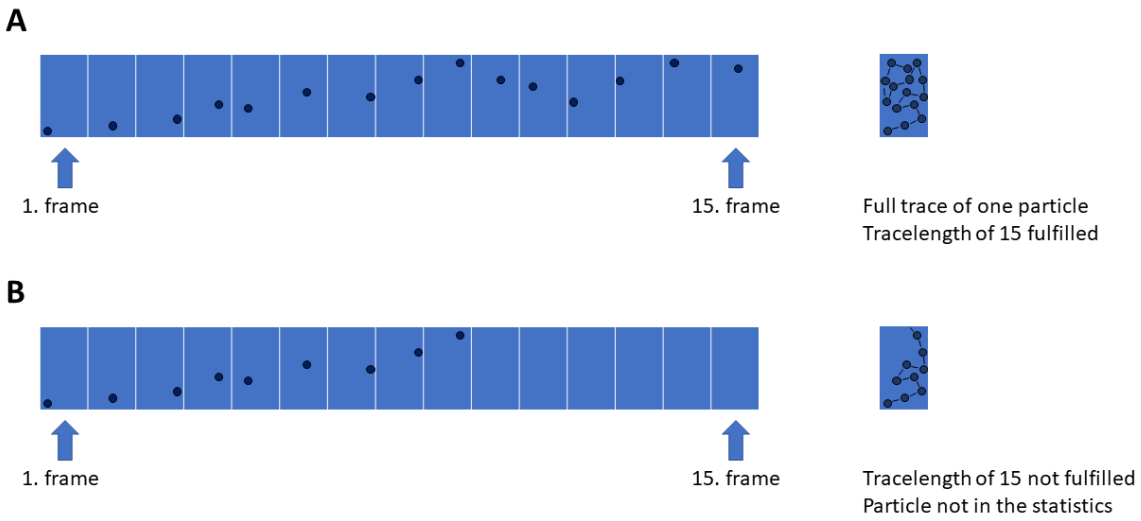


Figure 8-7: A: A particle is tracked frame by frame over 15 consecutive frames in its movement. With a default tracelength of 15, this particle is included in the analysis. B: A particle can only be tracked for 9 consecutive frames before it leaves the focus or the field of view. This particle is not included in the statistics.

Please note: If the tracelength is to be set to larger values, the resolution (Number of Frames) may need to be increased. Otherwise, the advantage of being able to trace particles with a higher number of frames is nullified because in this case the tracelength exceeds the number of frames. The following table shows the relationship between the resolution setting, number of frames and tracelength settings.

Table 8.5: Resolution setting and corresponding tracelength settings.

Resolution	Number of Frames	Tracelength settings	Recommended Tracelength settings
Low	15	10…15	7.5
Medium	30	10…30	15
High	60	10…60	30
Highest	90	10…90	45

It is recommended, to adjust “Tracelength” to half of the value of the Number of Frames (Resolution) as shown in the last column of table 8.5.

8.4 Auto Brightness

“Auto Brightness” can be selected in the Measurement tab, under "Run Video Acquisitions" as well as in the Analysis tab and represents a post-acquisition parameter (ref. section 6.1.6).

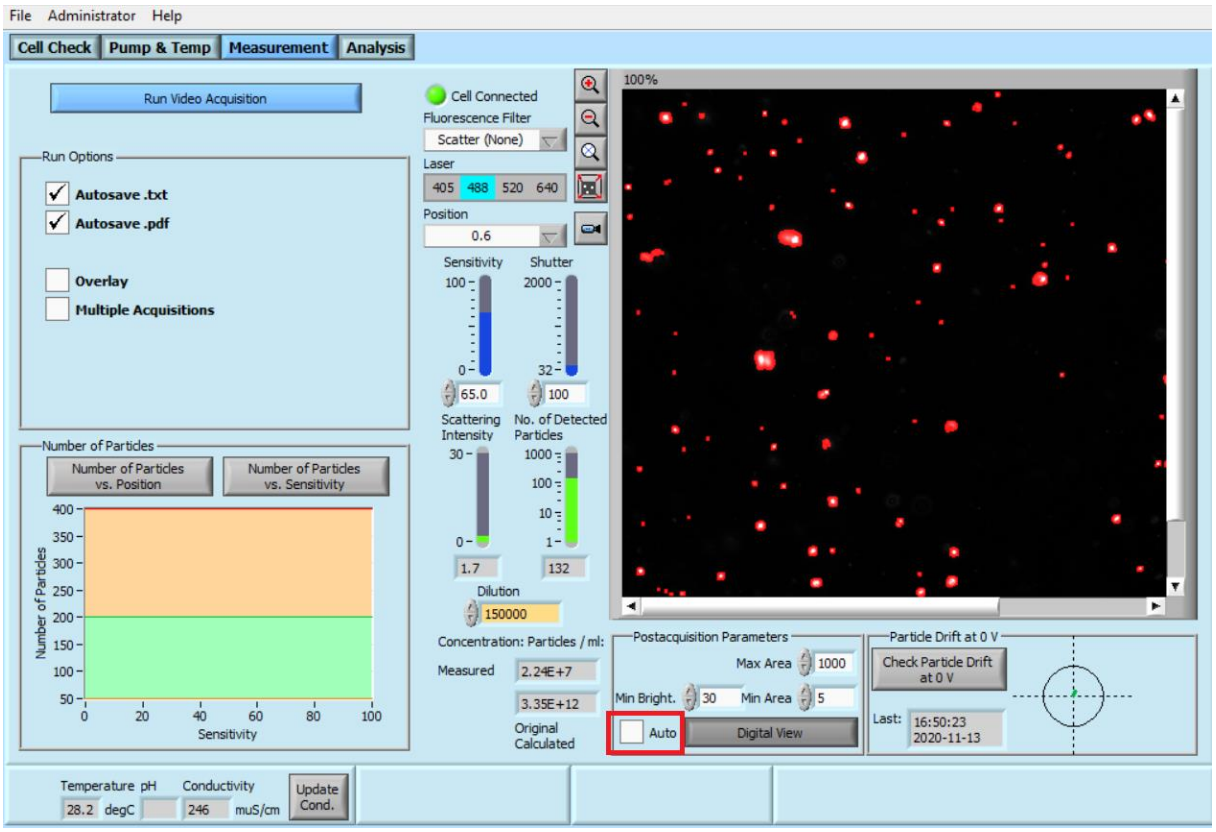


Figure 8-8: “Auto Brightness” is selectable in the Measurement tab.

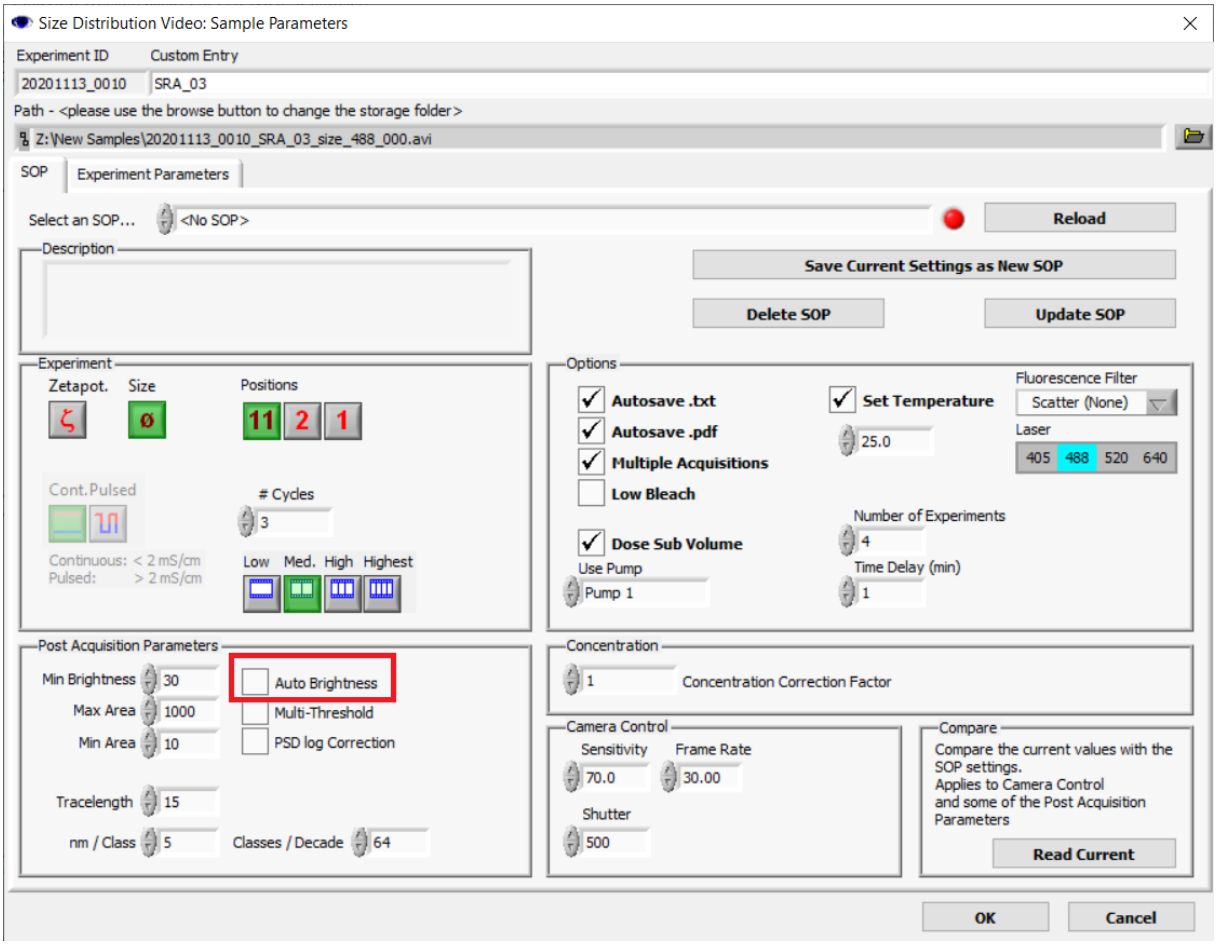


Figure 8-9: "Auto Brightness" is selectable in the "Run Video Acquisitions" tab.

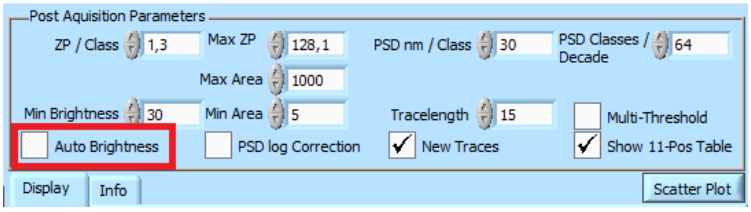


Figure 8-10: "Auto Brightness" is selectable in the Analysis tab.

"Auto Brightness" should be used on particles larger than 200 nm that have low diffusion behavior. When "Auto Brightness" is enabled, the ZetaView® device does not use the selected minimum brightness but re-calculates the minimum brightness for each frame of a video. This method prevents overexposure of the particles with regard to the brightness values and ideally makes the particles appear round.

For monomodal distributions this works well, for multimodal distributions the main mode is increasingly identified. For better results, the “Auto Brightness” function can be combined with the “Multi-Threshold” function. Please note: if the number of detected particles is rather low (<50), and the scattered light of the particles is weak, the “Auto Brightness” function cannot calculate optimum minimum brightness.

8.5 Multi-Threshold

“Multi-Threshold” refers to the minimum brightness setting. It is especially recommended for multimodal mixtures and for particles larger than 200nm in size and with different scattering behavior. When “Multi-Threshold” is enabled, “Auto Brightness” calculates optimal brightness for each frame of the video.

With this method, the center-of-gravity coordinate of each individual particle can be more accurately determined. In addition, this method also stabilizes the center-of-gravity coordinate to increase tracking efficiency. For better results, the “Multi-Threshold” function can be combined with the “Auto Brightness” feature.

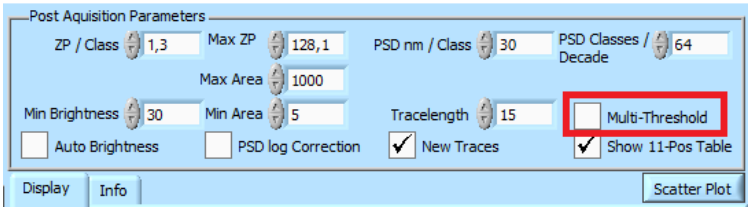


Figure 8-11: “Multi-Threshold” is selectable in the Analysis tab.

8.6 PSD log correction

PSD log correction is a function that was originally introduced to correct the logarithmic PSD histograms class by class with a correction value.

Although this feature can still be activated, it is strongly recommended not to use it. Instead, the post-acquisition parameters (ref. section 6.1.6) comprise a feature to specify the classes / decade (ref. section 11.2).

9 Standard Operating Procedures (SOPs)

If certain samples or sample sets must always be measured with precisely defined settings, it makes sense to save all these settings and measurement parameters in an SOP (Standard Operating Procedure). This avoids having to enter these settings again and again manually. Furthermore, the convenience of operating the ZetaView® is thereby significantly increased. In the ZetaView® software, all pre-acquisition parameters and post-acquisition parameters can be easily saved in an SOP, they can be updated, compared with the current settings, and deleted as well. In the figure below, the parameters which can be stored in an SOP are highlighted in red.

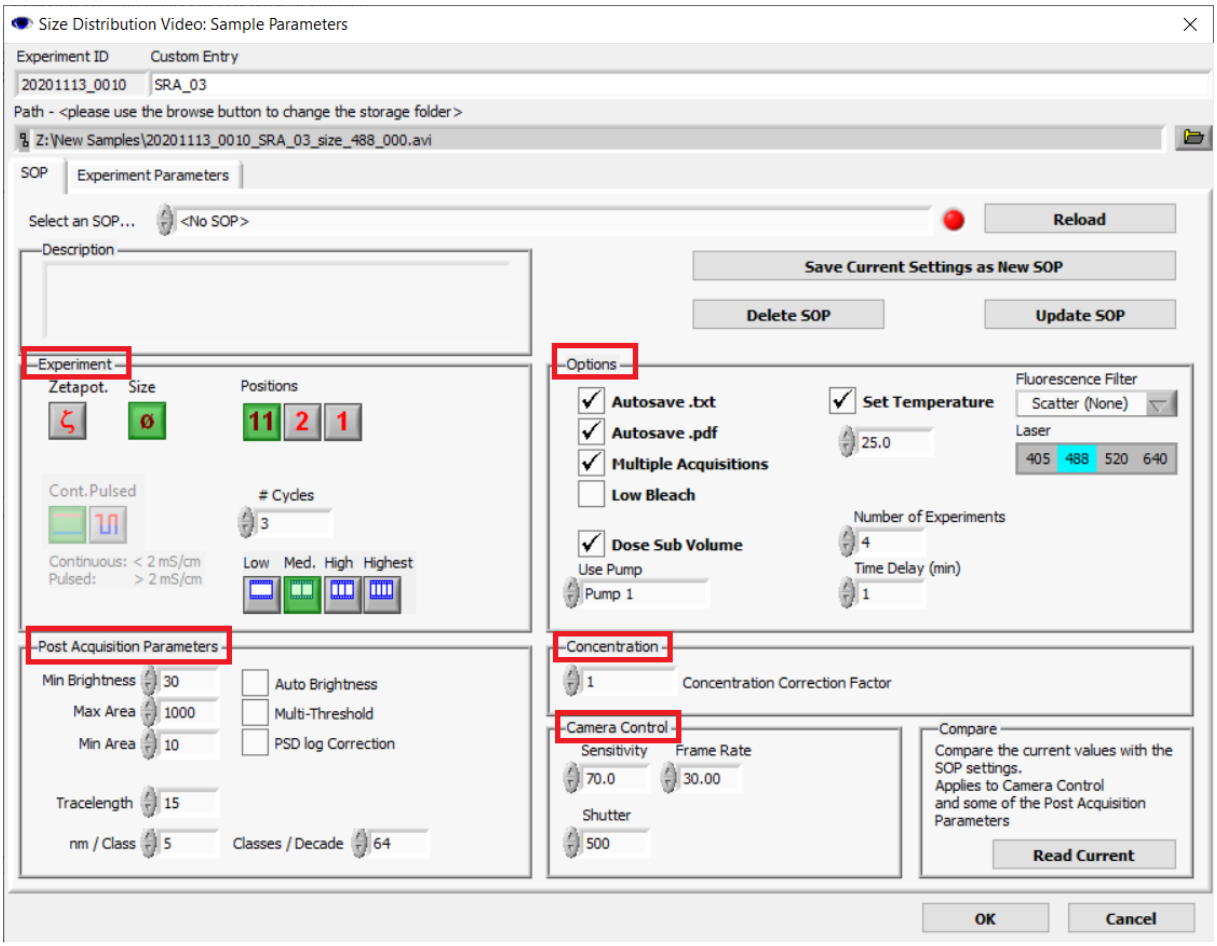


Figure 9-1: All parameters highlighted in the red boxes can be saved in an SOP.

All saved SOPs can be easily selected via the drop down menu.

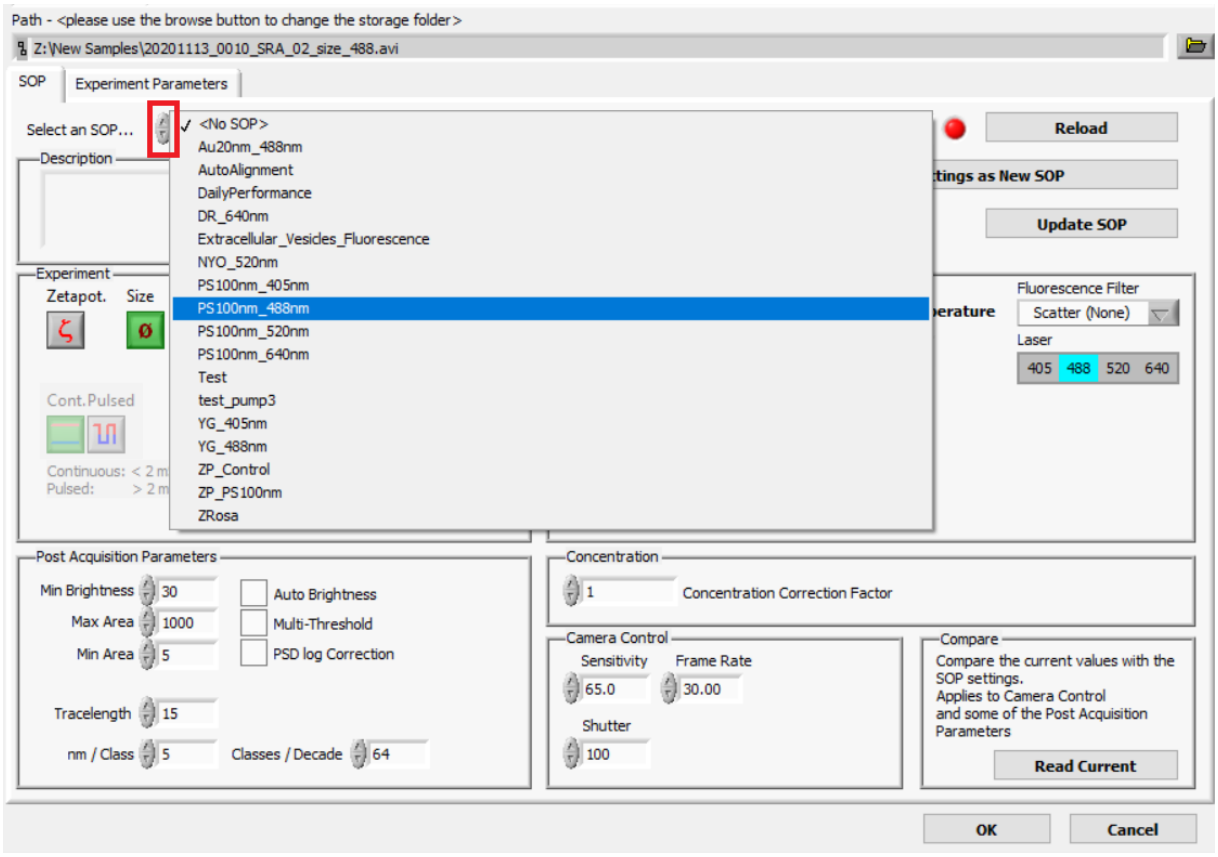


Figure 9-2: Dropdown menu, where all saved SOPs can be accessed.

The parameters of the “AutoAlignment” and “Daily Performance” (see sections 6.2.2 and 6.2.5) are also saved as an SOP. Basically, both functions are optimized for the 100 nm polystyrene standard particles and must be started via the Cell Check tab. However, if these two functions are to be used with other particles (e.g. SiO2 or other technical particles), the user can adjust the parameters “AutoAlignment” and “Daily Performance” in the SOP tab according to his requirements.

9.1 How to create an SOP

In order to create an SOP, the procedure is the same as when you want to start a measurement. First you need to adjust all parameters for the measurement in the SOP tab under "Measurement" and "Run Video Acquisition".

Once all settings have been made, a new SOP can be saved by clicking on "Save current settings as new SOP" and specifying an SOP name. In the example below, an SOP is stored under the name Mesenchym (extracellular vesicles derived from mesenchymal cells).

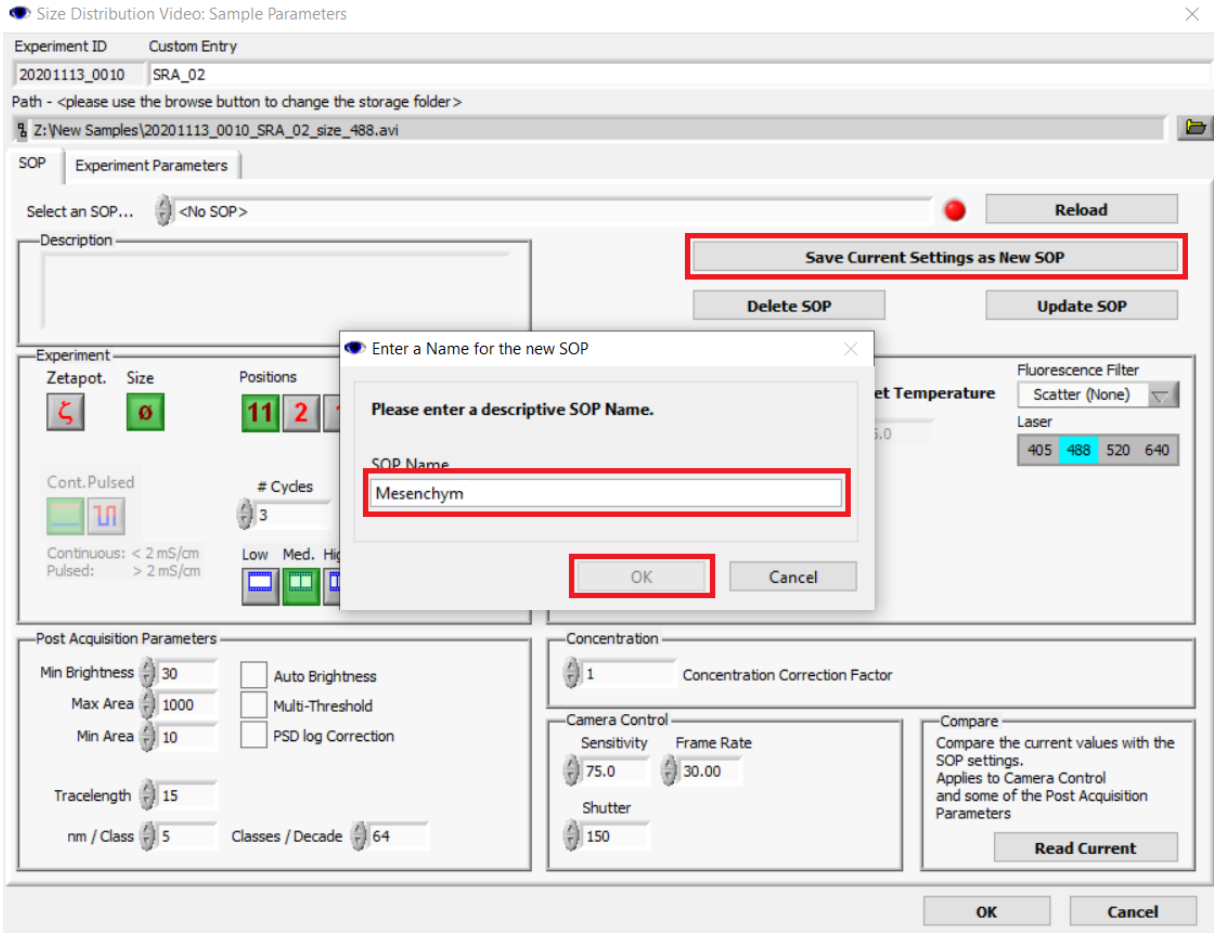


Figure 9-3: In this example, a size measurement with 11 positions and the corresponding pre- (camera control) and post-acquisition parameters are saved as an SOP. After specifying the SOP name, the "OK" button becomes active after clicking "Enter" on the keyboard.

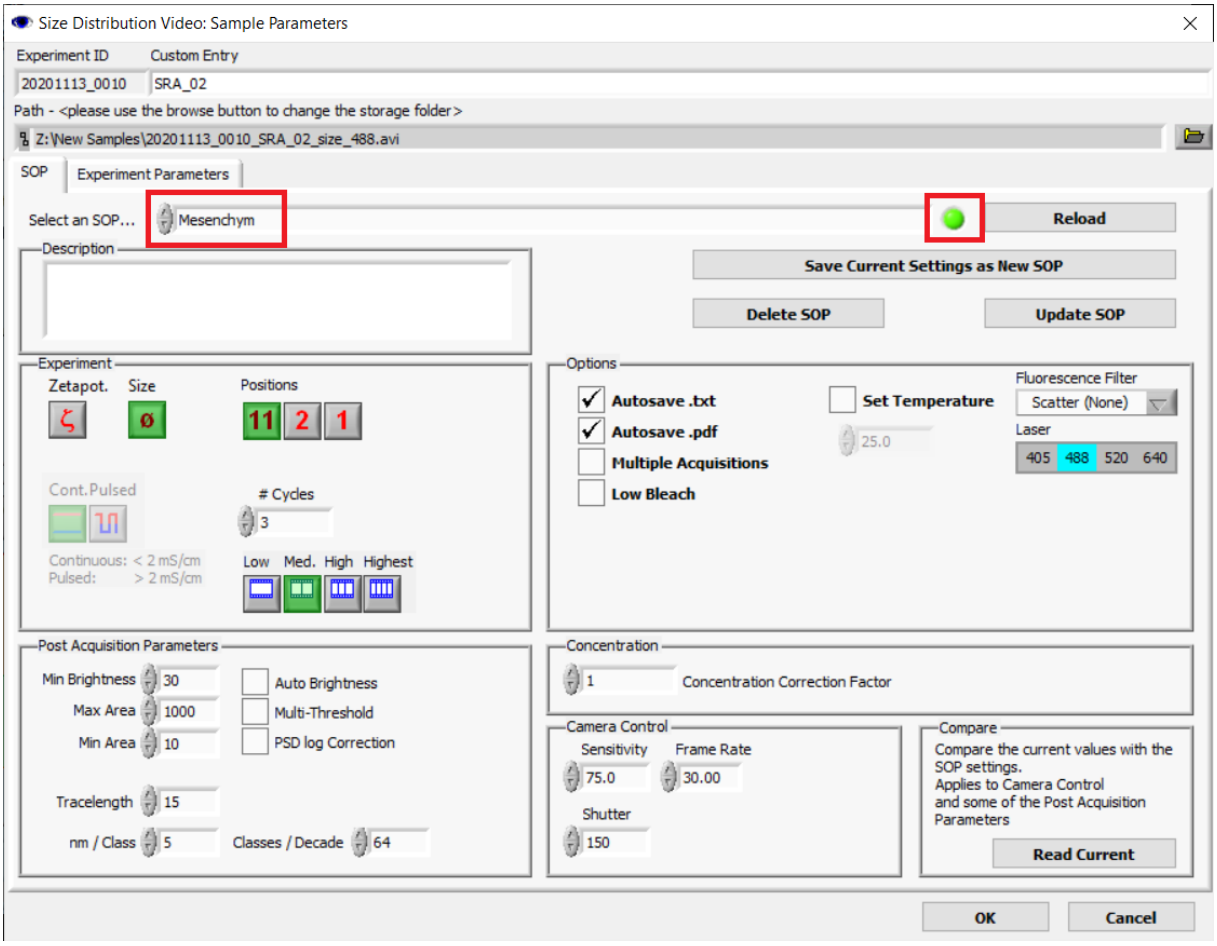


Figure 9-4: Once a new SOP has been saved, it is directly selected and activated. This can be recognized by the fact that the corresponding SOP name can be seen in the drop-down menu "Select an SOP" and that a green light is visible. With a click on "OK", a measurement is started with the parameters in this SOP.

It should be noted that a selected and activated SOP will be disabled immediately, as soon as at least one of the saved settings is changed. This is confirmed by the fact that the green light jumps to red. The corresponding SOP is then still selected but inactive. A subsequent measurement is then performed with the measurement parameters set last. This is shown in the next two screenshots.

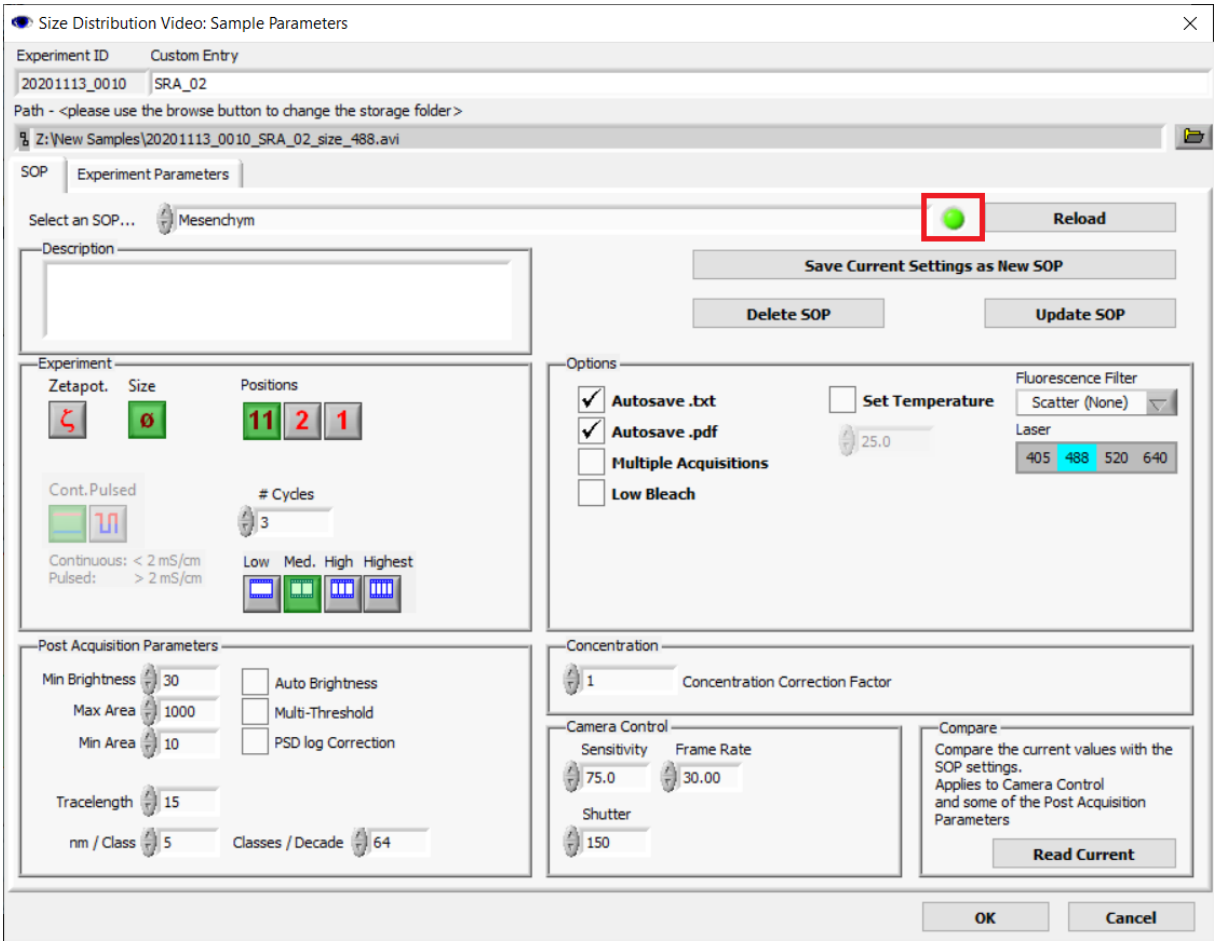


Figure 9-5: The SOP "Mesenchym" is selected and activated, which is indicated by the green light.

If you change at least one parameter of an activated SOP, this SOP becomes inactive, but the name of the SOP is still visible.

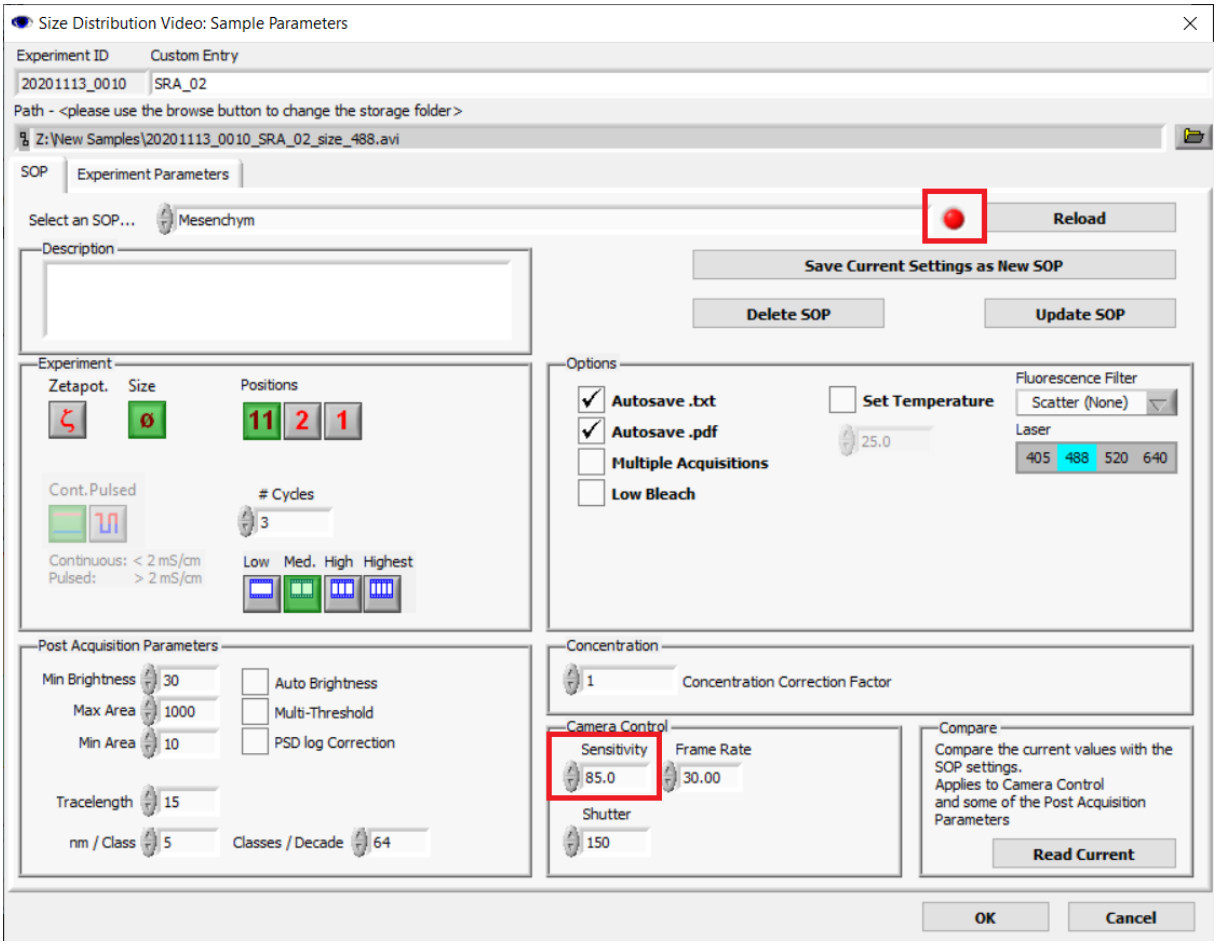


Figure 9-6: In this example, the sensitivity has been changed from 75 to 85 compared to the previous screenshot. The SOP is now inactive, indicated by the red light.

9.2 Update of an SOP

If an existing SOP is to be updated with updated measurement parameters, it is saved under the same name by clicking on "Update SOP". The measurement parameters of the original SOP will be overwritten.

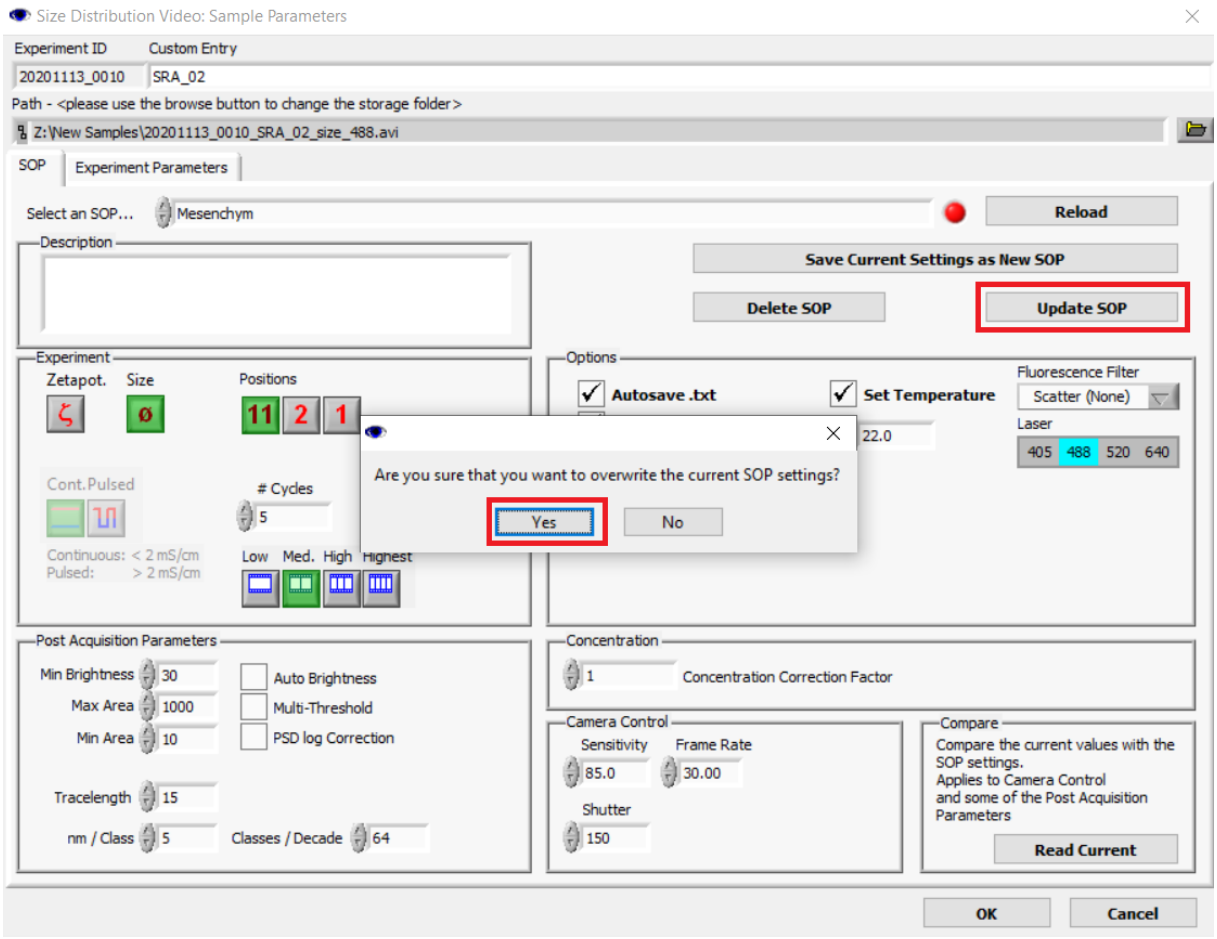


Figure 9-7: Measurement parameters can be updated by clicking the button "Update SOP". The settings of the original SOP are overwritten. The SOP name is retained.

Once the SOP has been updated, it is selected and activated, as indicated by the green light.

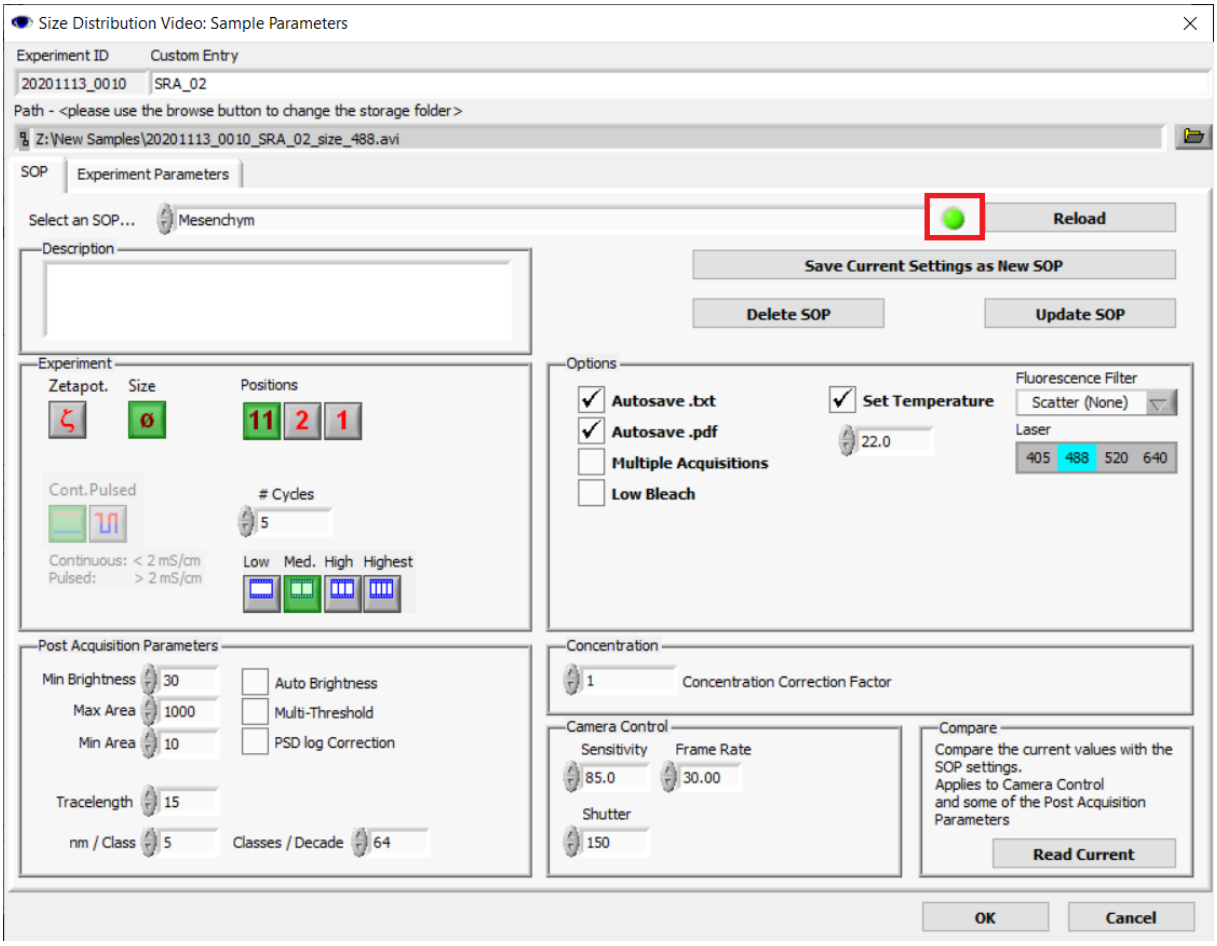


Figure 9-8: After an SOP has been updated, it is activated, recognizable by the green light.

9.3 Reload of an SOP

As mentioned above, an SOP is activated immediately, once it is selected from the drop-down menu. This means that a subsequent measurement is performed with the corresponding parameters that are saved in the SOP. A selected and active SOP becomes inactive if at least one parameter is changed either in the Measurement tab or in the SOP menu. For example, if, after a measurement with an active SOP, the shutter in the Measurement tab is changed, the selected SOP is inactive. The same applies if at least one setting in the SOP menu is changed.

With the button "Reload" the already selected, but inactive SOP can be quickly loaded and activated again without having to go the more complicated way via the drop-down menu.

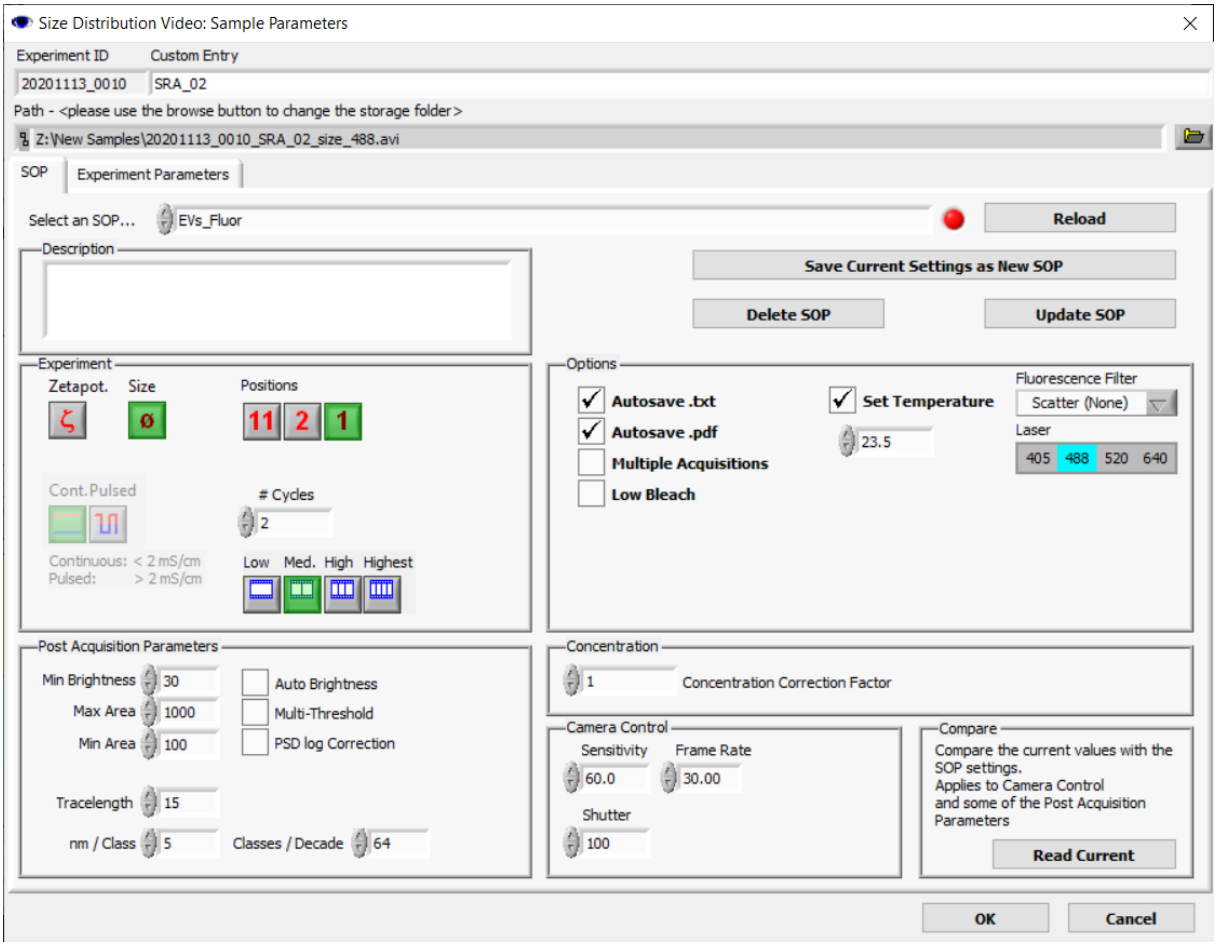


Figure 9-9: In this example, the SOP "EVs_Fluor" is selected but not activated.

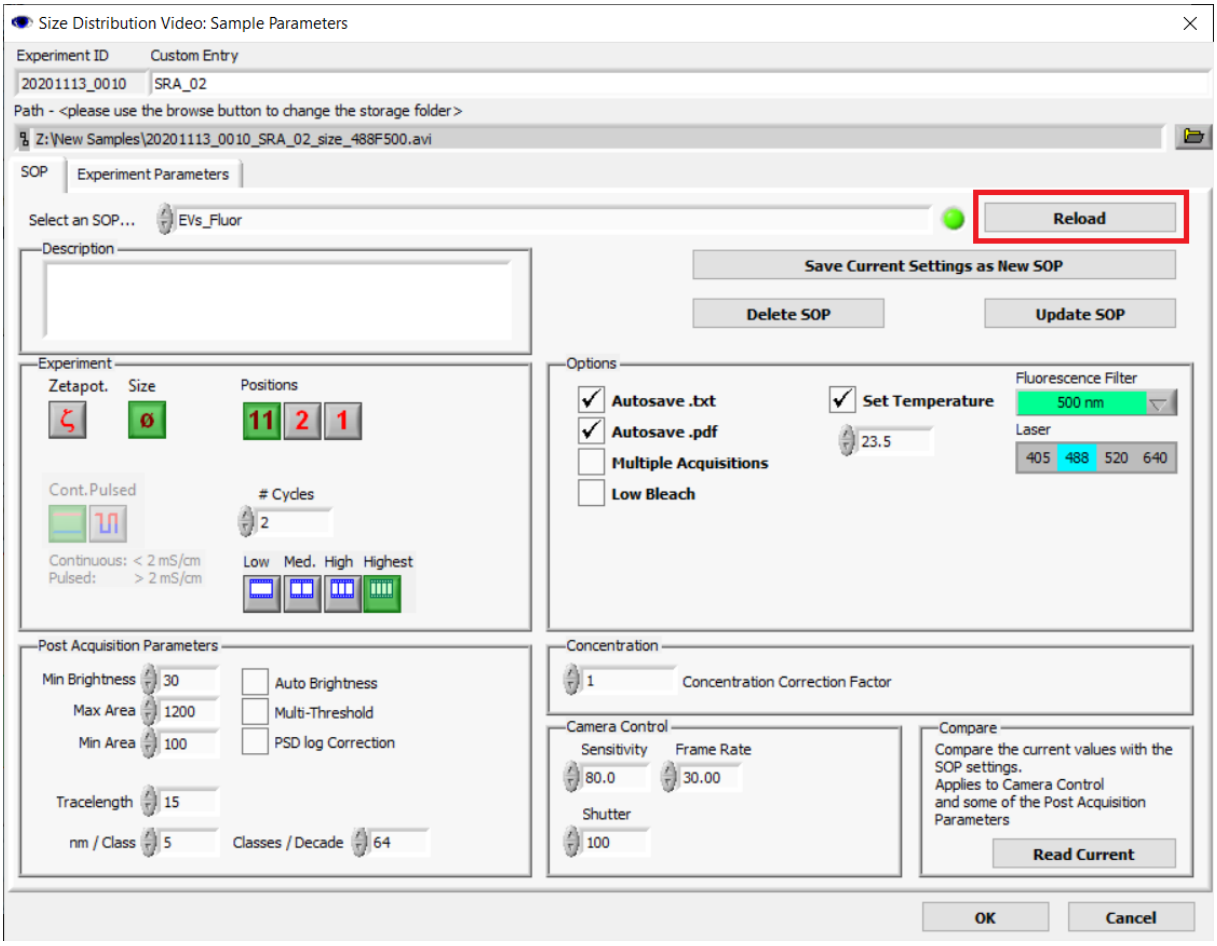


Figure 9-10: After clicking "Reload", the selected SOP "EVs_Fluor" is reloaded and becomes activated.

9.4 Delete an SOP

The deletion of an SOP is done with the button "Delete SOP". The SOP to be deleted must be selected. The SOP can be deleted either in the active (green light) or in the inactive (red light) state.

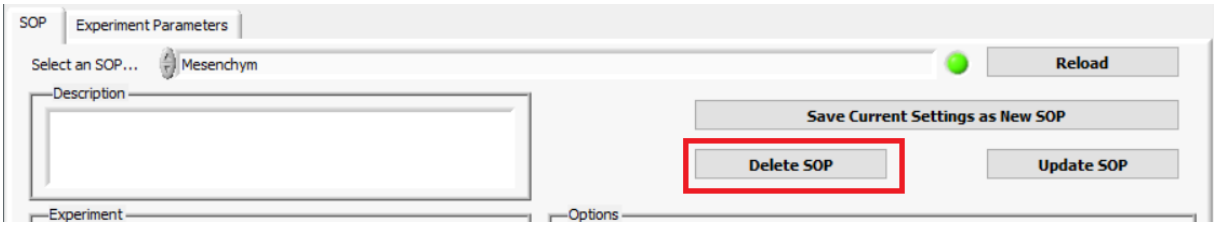


Figure 9-11: Deleting the SOP "Mesenchym".

9.5 Compare the current SOP settings with the current measurement parameters

Sometimes it is useful to compare the settings made manually in the "Measurement" main menu (see screenshot below) with those parameters of a currently selected SOP. Such a comparison can be done quickly and easily with the button "Read Current". As an example of such a comparison, first the measurement parameters are shown, which were set manually in the Measurement tab.

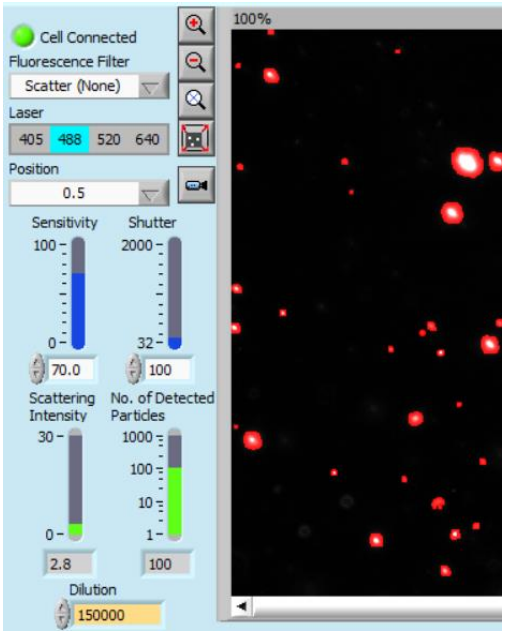


Figure 9-12: Manually adjusted current measurement parameters (pre- and post-acquisition).

If you now select and activate an SOP in the SOP menu, the previous manually adjusted measurement parameters are obscured by the SOP menu (see below).

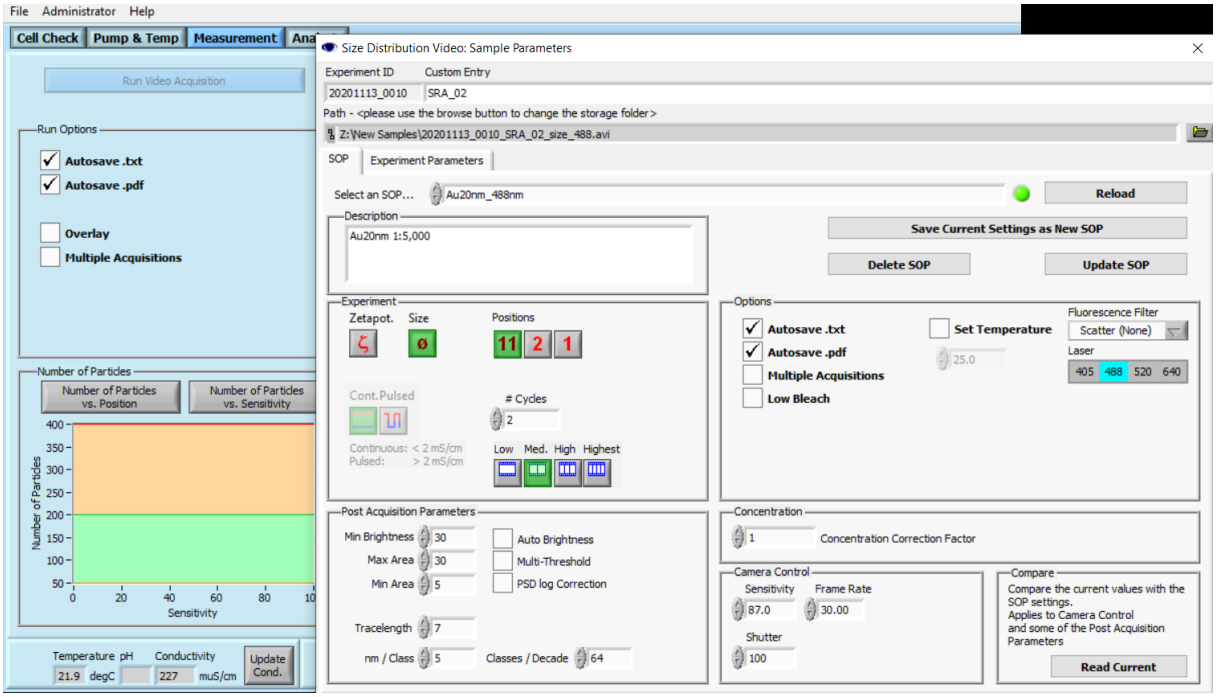


Figure 9-13: SOP menu with a selected and activated SOP. The currently adjusted measurement parameters, as shown in the previous figure, are obscured by the SOP menu here.

By clicking on "Read Current", the current, manually adjusted measuring parameters can now be called up without leaving the SOP menu (see below).

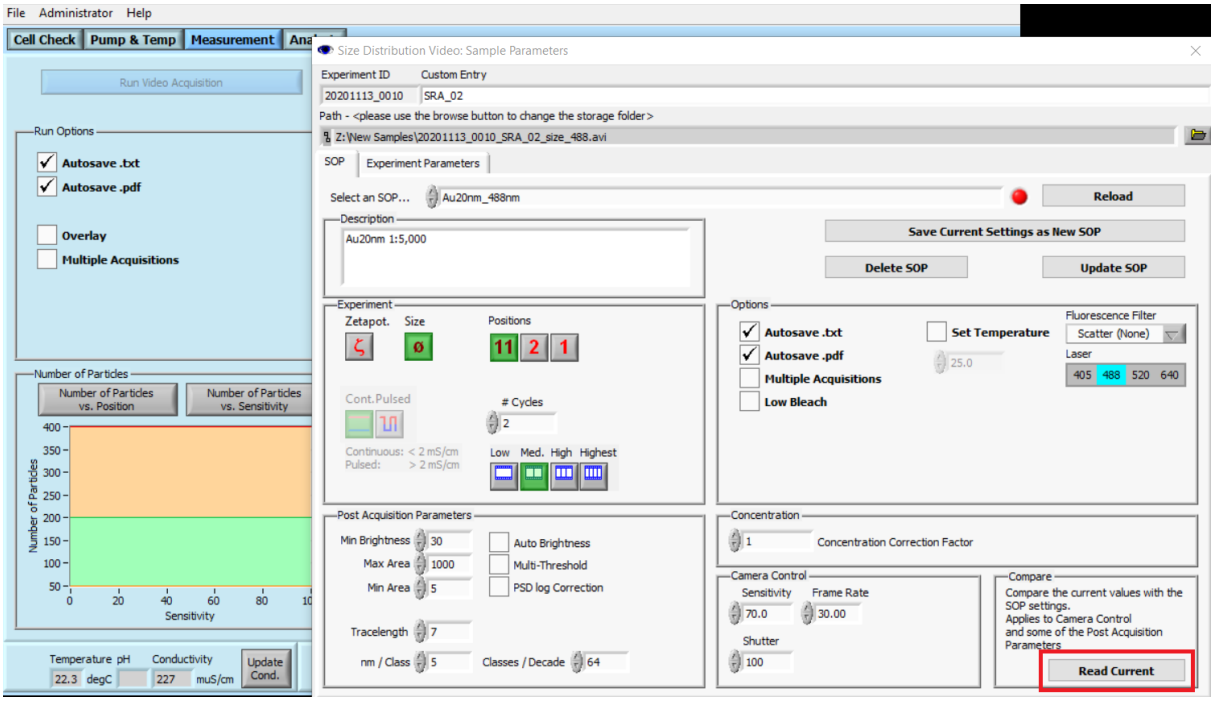


Figure 9-14: The button "Read Current" shows the current measurement parameters, which were set manually in the foreground of the measurement tab.

By alternately clicking "Read Current" and "Reload", the current measurement parameters ("Read Current") and the measurement parameters of the corresponding selected SOP ("Reload") can be displayed in the SOP menu, so that they can be compared very easily.

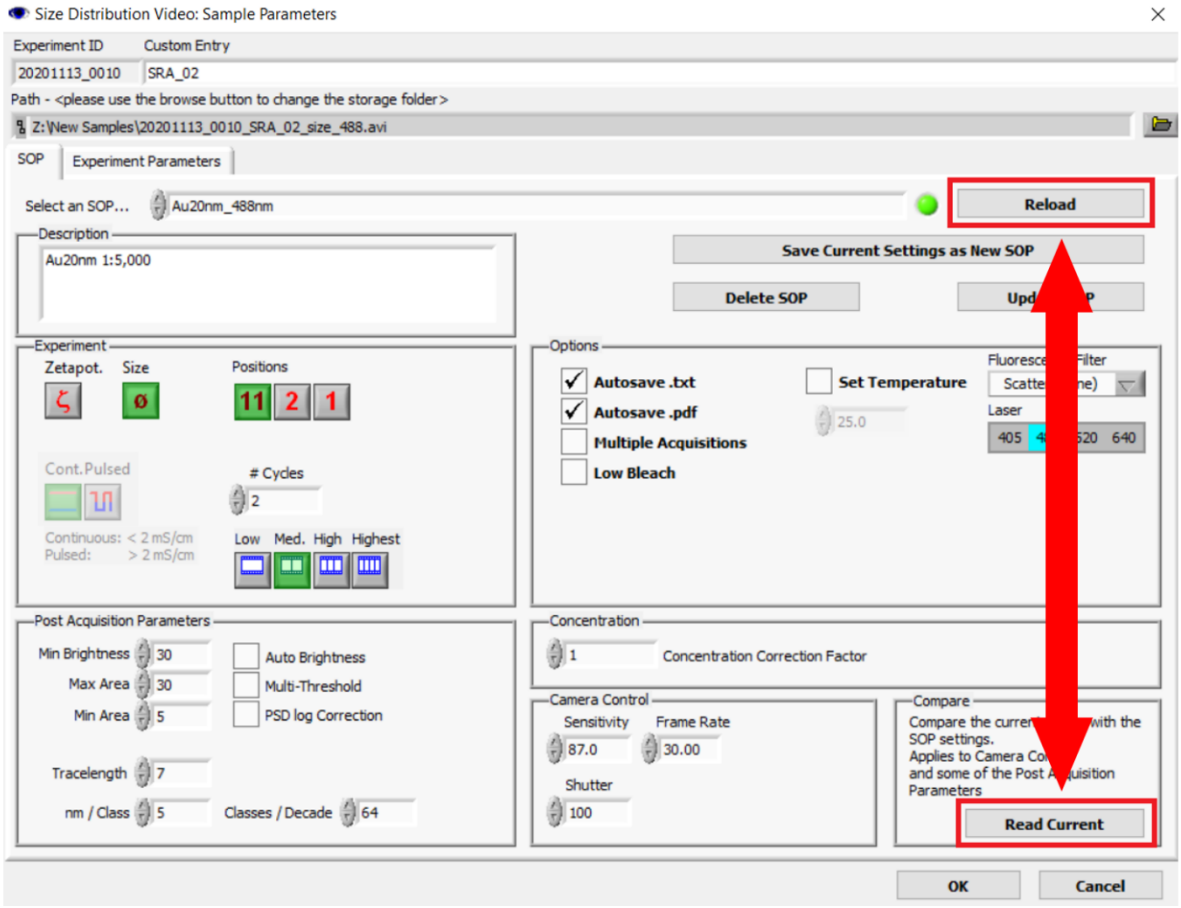


Figure 9-15: Alternate clicking on "Read Current" and "Reload" allows a comparison between the settings of the selected SOP and the currently set measurement parameters.

9.6 Change and add viscosity settings

As already mentioned in chapter 7, the ZetaView® instrument uses the Stokes-Einstein relationship to determine the diffusion coefficient and thus the hydrodynamic diameter of a particle. An essential role is played by the viscosity of the liquid in which the particles are suspended. By default, the ZetaView® device is calibrated to the viscosity of water. Alternate solvents have different viscosities, depending on their composition. Since the number and composition of candidate solvents is large, a complete collection of all solvents and their corresponding viscosities cannot be offered by Particle Metrix, rather, the software allows the user to customize the software to match requirements.

It is up to the user to add viscosities from other liquids to the ZetaView® software at a later date. The viscosity of water is stored in a text file named "default" in the viscosity folder.

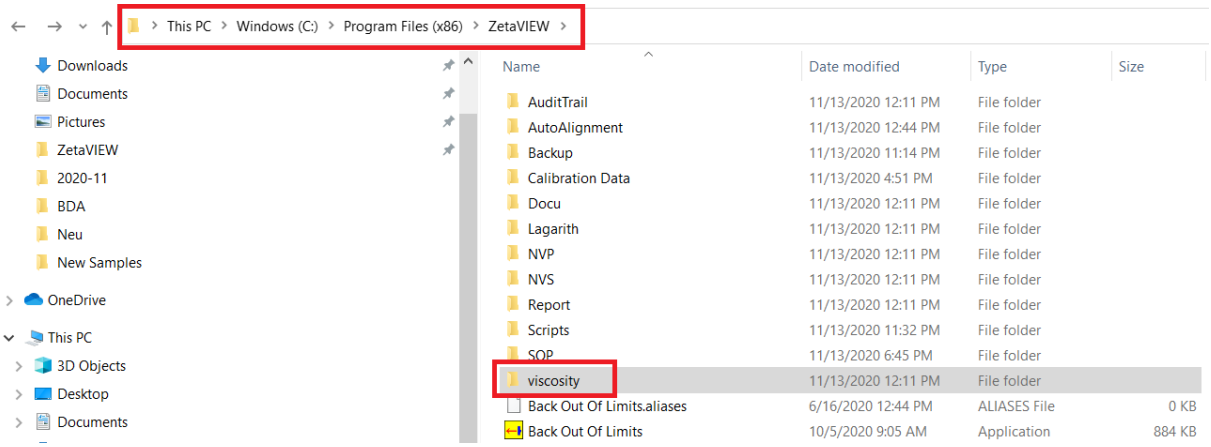


Figure 9-16: Location of the "default" text file for the viscosity of water.

If you want to measure particles dissolved in a liquid that has a viscosity other than water, you will need to create a new text file with new viscosity values. For this the "default" file can be used as a template. In the viscosity folder, open the "default" text file. The text file contains a table with different temperatures and associated viscosities in mPas (millipascals).

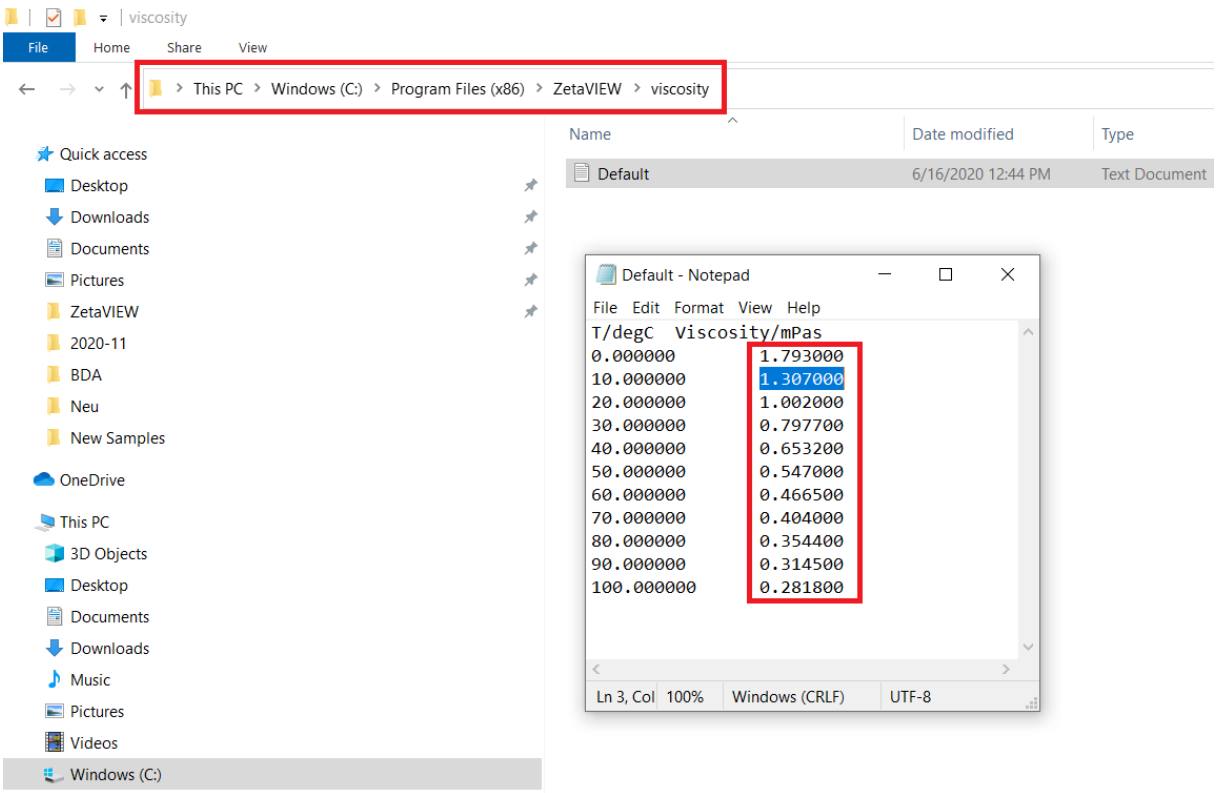


Figure 9-17: "Default" text file with the viscosity values (marked in red) for water.

In order to enter the viscosity for the desired solvent, it is necessary to change the viscosity values for the corresponding temperatures and then save the text file with a new name in the viscosity folder. Please note that it is not necessary to enter the viscosity for all temperatures. Most measurements are done at room temperature, so values above 50°C and below 10 °C can be omitted. It is necessary to include at least two viscosity values in the temperature range of interest.

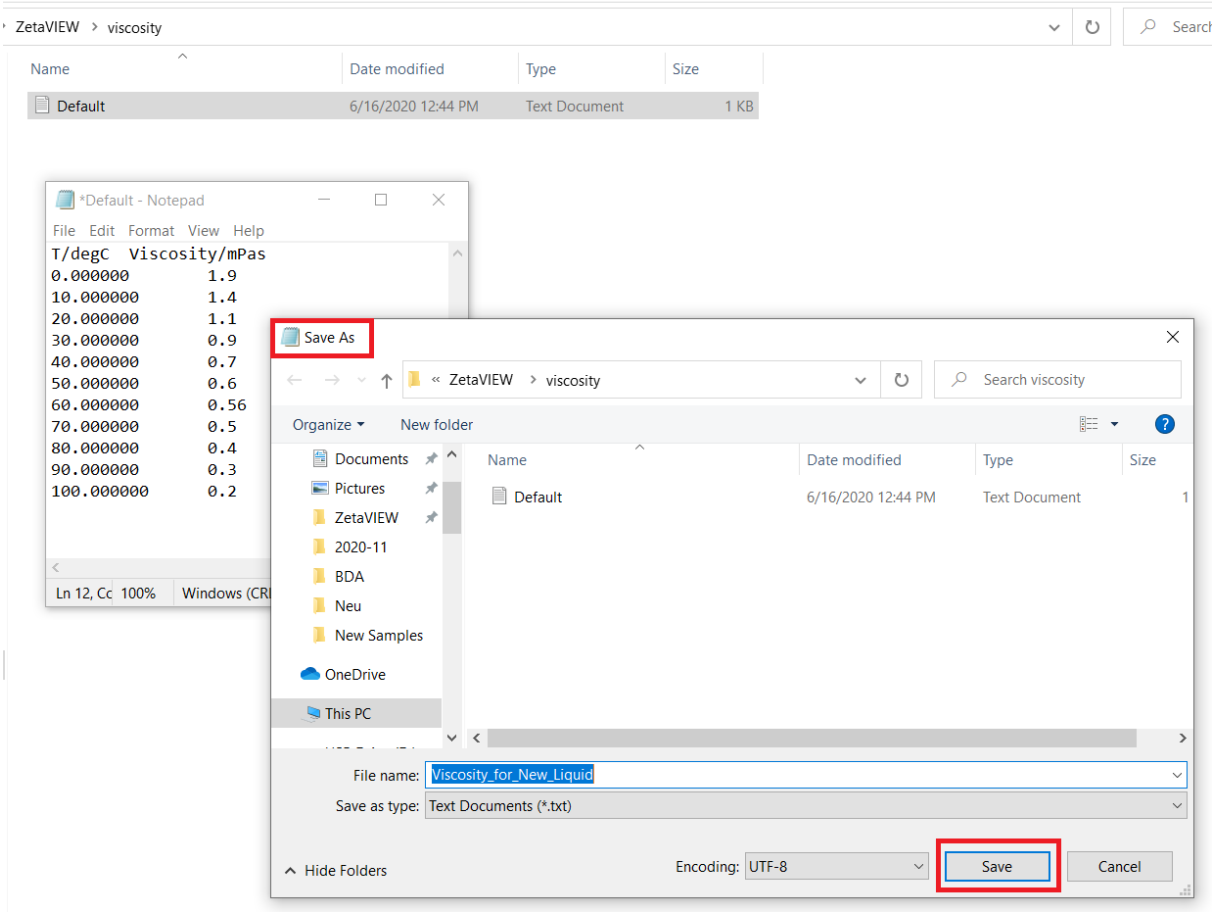


Figure 9-18: Example of changed viscosity values. Changes should be saved with a new name in the viscosity folder.

For each solvent used, the viscosity values for different temperatures must either be determined experimentally by the user or looked up in the relevant literature. Once the new viscosity table has been created and saved in the viscosity folder, it can be selected in the ZetaView® software from the drop-down menu under the appropriate file name.

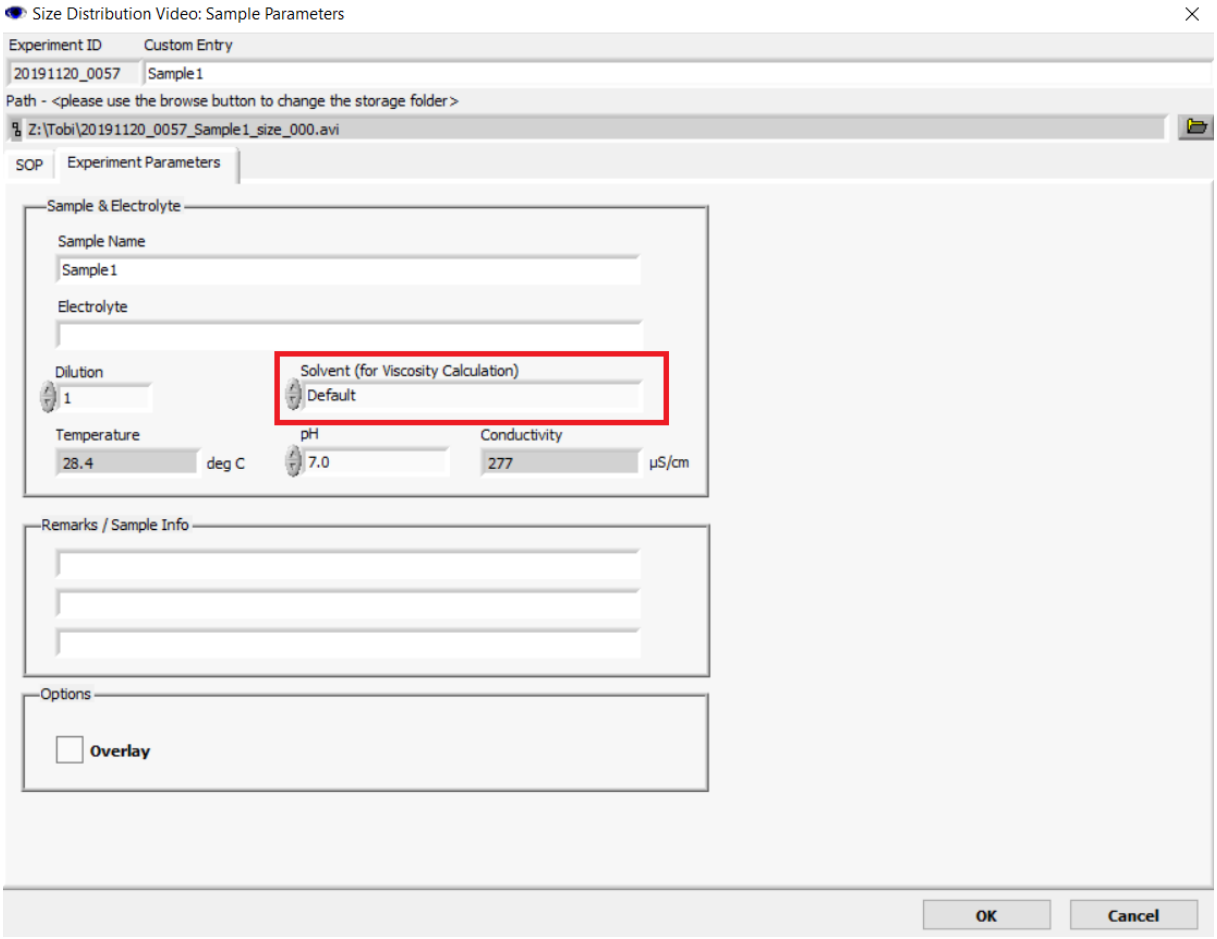


Figure 9-19: The new viscosity can be selected in the "Experiment Parameters" tab from the drop-down menu.

10 Analysis menu (size measurement)

The Analysis Menu is called automatically after a size measurement is completed. In the left part of the Analysis Menu, the empirical percentiles (X values), peaks, concentration of the particles in a desired size range (region of interest; ROI), the traces found, and the total measured particle concentration are shown. The right part of the Analysis Menu shows the histogram of the particle size distribution (PSD).

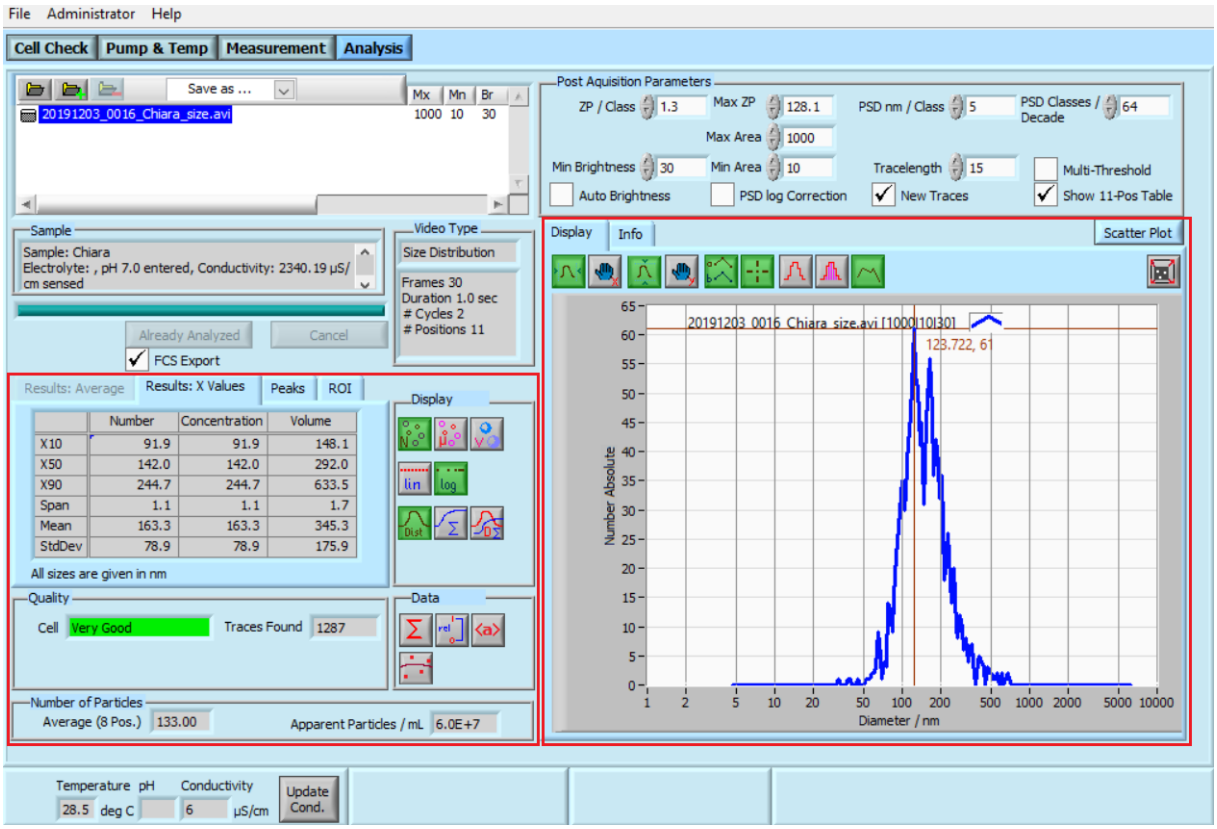


Figure 10-1: On the left side the percentiles, peaks, ROI, traces found and the measured particle concentration (Apparent Particles / ml) are shown (red frame on the left). The histogram (or the cumulative distribution) of the particle size distribution (PSD) is shown in the right part (red frame on the right)

10.1 Number-weighted, concentration-weighted, volume-weighted distribution

The ZetaView® software can display the histogram in three different distributions. With the **number-weighted distribution** the absolute number of particles is plotted against the particle size, with the **concentration-weighted distribution** the measured particle concentration is plotted against the particle size and with the **volume-weighted distribution** the volume of the particles is plotted against the particle size.

The ZetaView® software enables fast switching between the three types of distributions. The corresponding buttons are then marked green in the ZetaView® software.

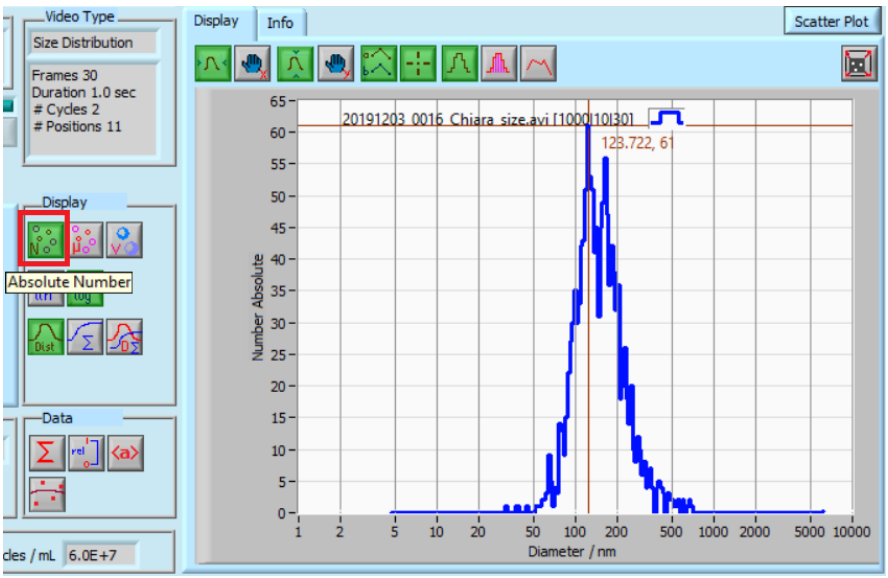


Figure 10-2: Number-weighted distribution.

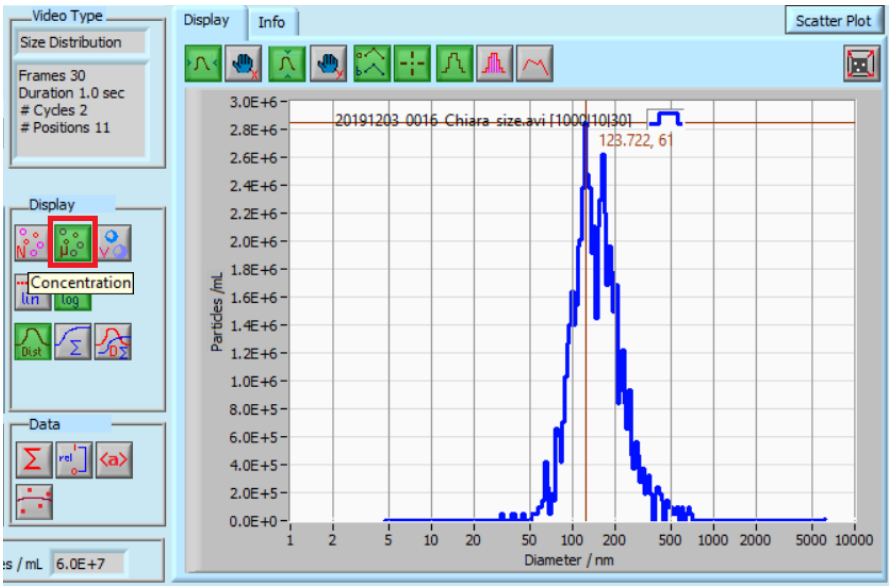


Figure 10-3: Concentration-weighted distribution

The particle concentration determined by the ZetaView® is based on the measured number of particles. Therefore number-weighted distribution and concentration-weighted distribution correlate to each other. Due to this correlation, the percentiles as well as the values for span, mean and standard deviation are identical.

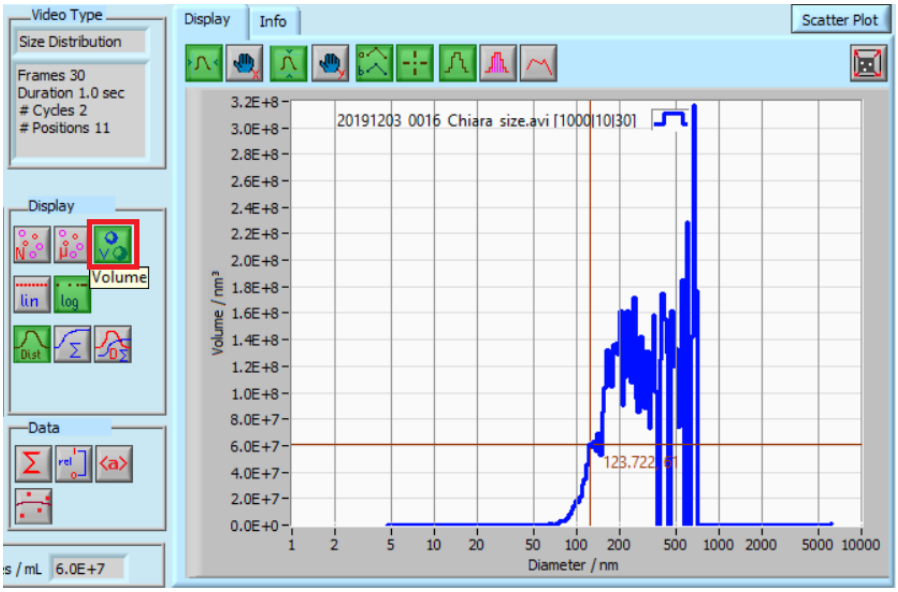


Figure 10-4: Volume-weighted distribution.

With volume-weighted distribution, the volume of the particles is calculated based on ideal spherical particles and the spherical formula.

$$\text{Number}(ø) \cdot \frac{4}{3}\pi \cdot \left(\frac{ø}{2}\right)^3$$

Number (ø) is the number of (traced) particles as a histogram. This represents to the number of particles that are assigned to the diameter ø in the corresponding histogram bin class.

10.2 Linear and logarithmic plotting of the histogram

The histogram can be displayed in a linear as well as in a logarithmic plot. Both are based on the corresponding linear and logarithmic data records, which are stored in the text file (see chapter 11). It should be noted that both, the peaks and the percentiles can have slightly different values for the linear and logarithmic display. This is normal and strongly depends on how the bin classes “PSD nm / Class” (see section 11.1) in the linear plot are selected. The two figures below show a linear and a logarithmic plot of the histogram of the same measurement.

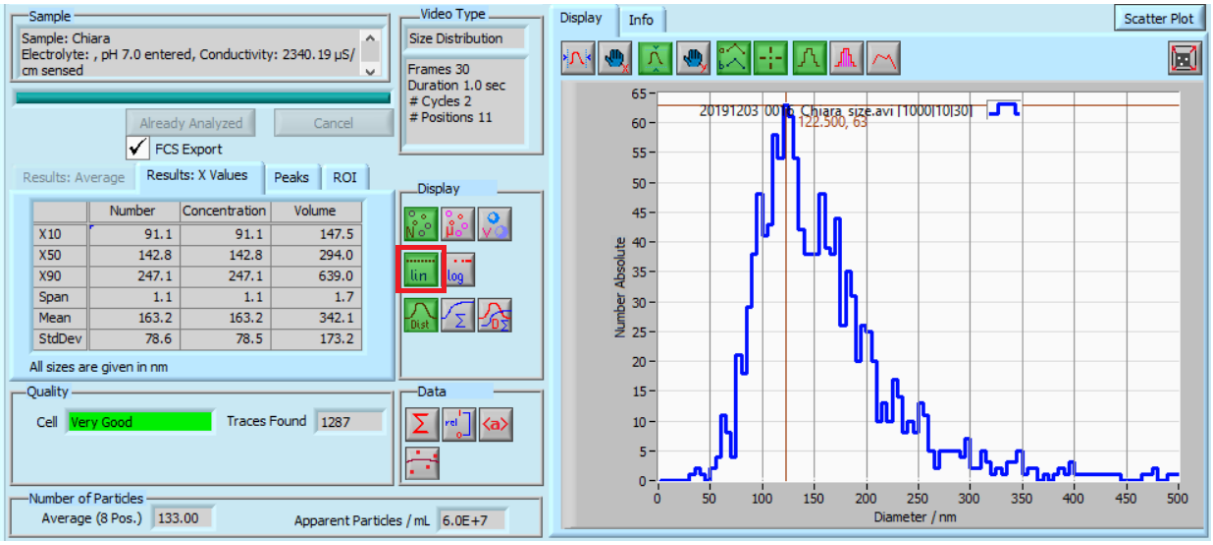


Figure 10-5: The histogram in a linear plot. The bin class "PSD nm / Class" was set to 5 here.

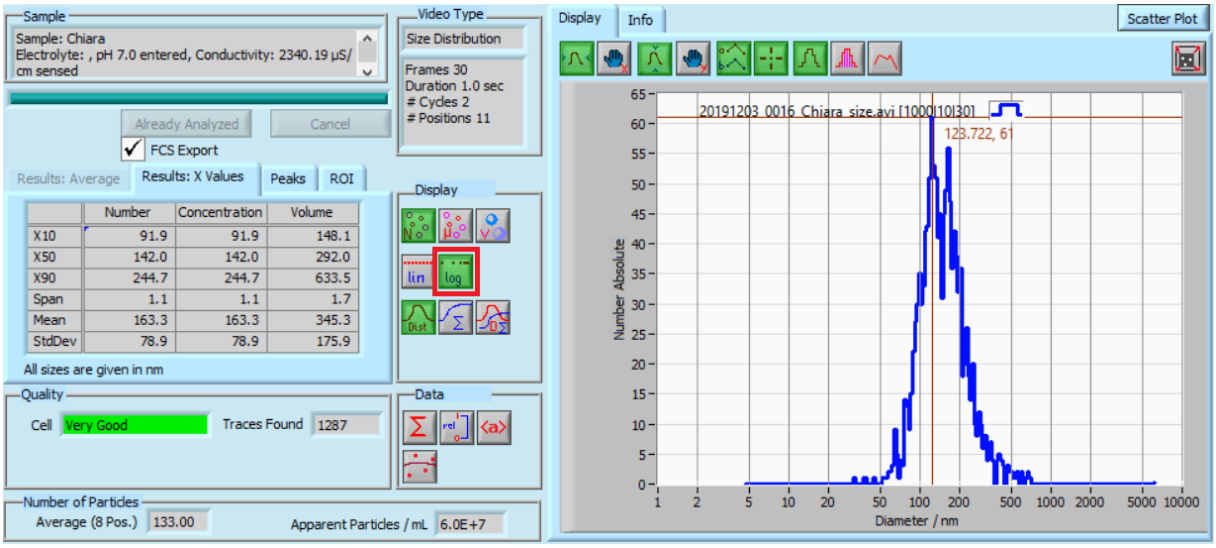


Figure 10-6: The histogram in a logarithmic plot.

The smaller the bin classes for the linear values (see chapter 11), the closer the percentiles and the values for span, mean and standard deviation are to those of the logarithmic plot. The differences in these values between linear and logarithmic representation become greater the larger the bin classes are adjusted in the linear plot.

10.3 Distributive and cumulative plotting of the histogram

The ZetaView® software can present the measurements distributively, cumulatively or distributively and cumulatively in a diagram. The green color of the corresponding button indicates which display is currently active.

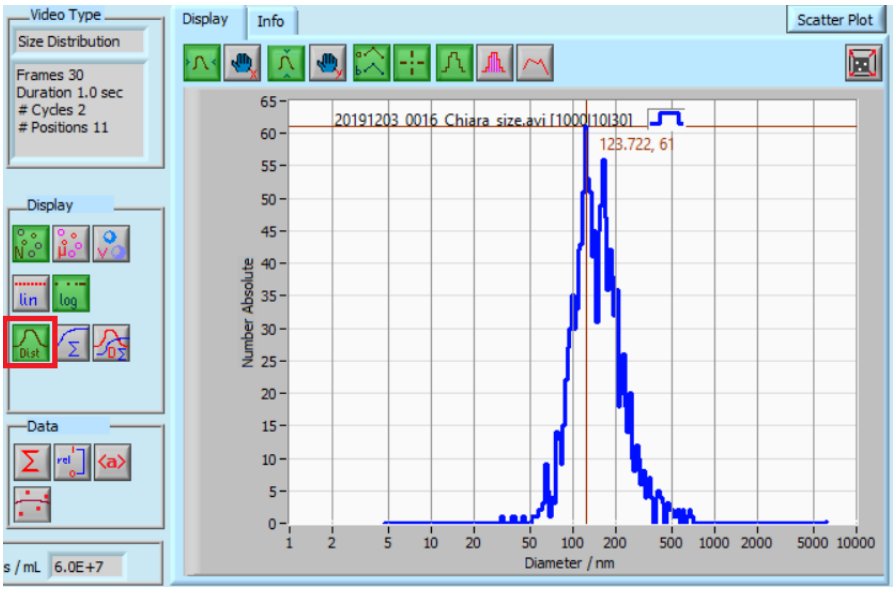


Figure 10-7: The histogram in the distributive plot.

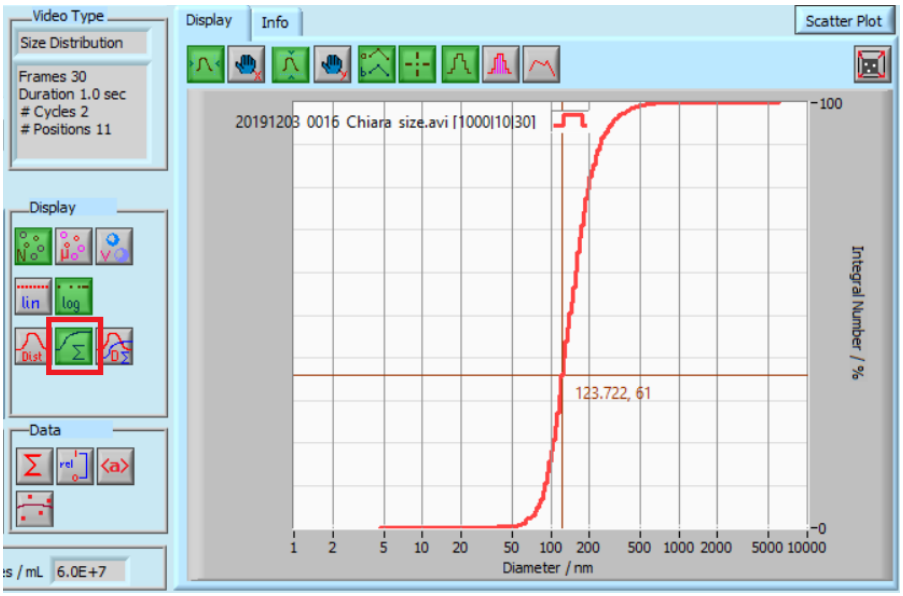


Figure 10-8: The cumulative plot is shown.

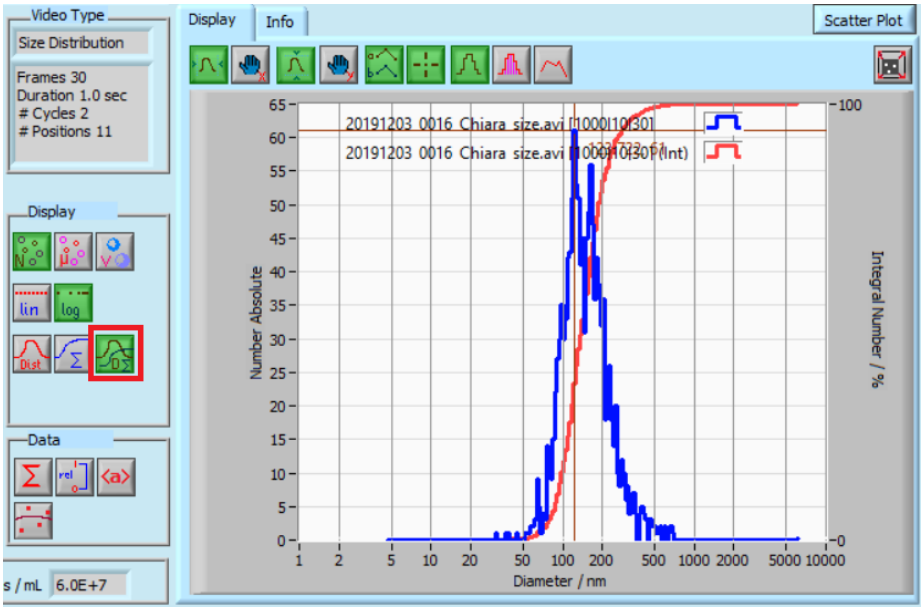


Figure 10-9: Distributive and cumulative plot.

10.4 Summation, normalization, averaging and smoothing of the measurement data

Summation:

The ZetaView® software can sum up result data of several measurements. To use the function, two or more results must be plotted in the diagram window at the same time. Please note that all histograms that are to be summed must have the same settings for "PSD nm / Class" and "Classes / Decade". In the following two plots, two measurements are shown, before and after the histogram data is summed. The accumulation works for number-weighted distribution, concentration-weighted and volume-weighted display, as well as for distributive and cumulative plots.

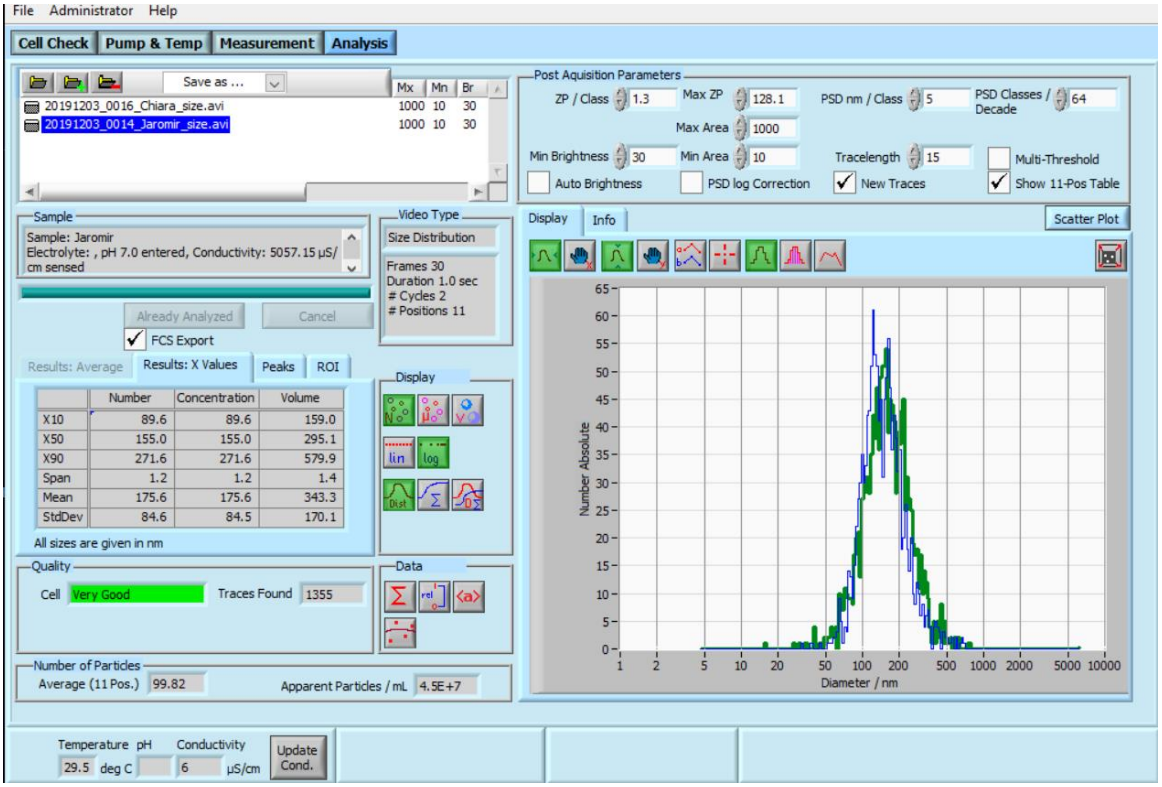


Figure 10-10: The diagram shows the histograms of two measurements that have not yet been summed.

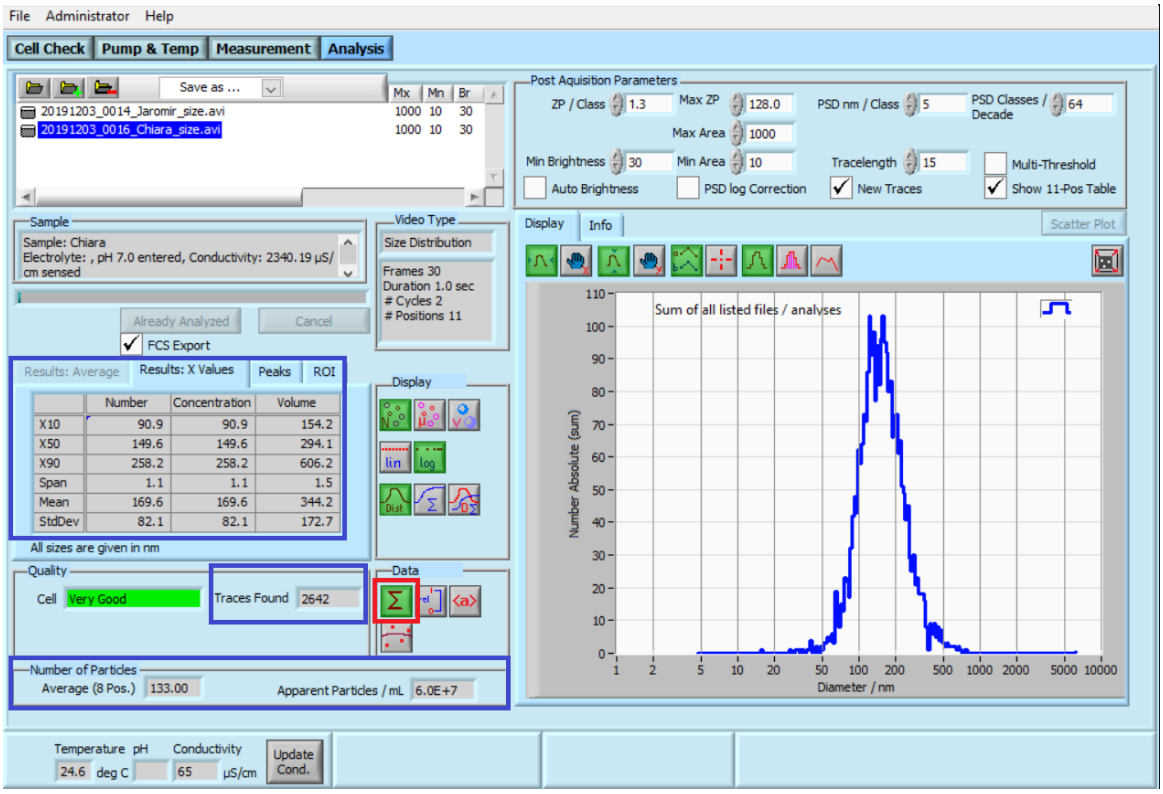


Figure 10-11: Result after summation of the histogram data derived from both measurements. To sum, the Σ button is to be clicked.

It should be noted that after the histogram data of all measurements have been summed, new results for the percentiles, peaks, Traces Found and number of particles are reported (highlighted in blue in the above screenshot). The reason for this is that the histogram data of all measurements are taken into account for the summation.

The new results and the new histogram cannot be saved as a new pdf report or new text file but can be saved as a screen shot.

Normalization:

Normalization can only be carried out with at least two or more data records (histograms). Normalization displays the histogram data in relative scale on the graph. The peaks of the histograms are set to 1 or 100%, respectively.

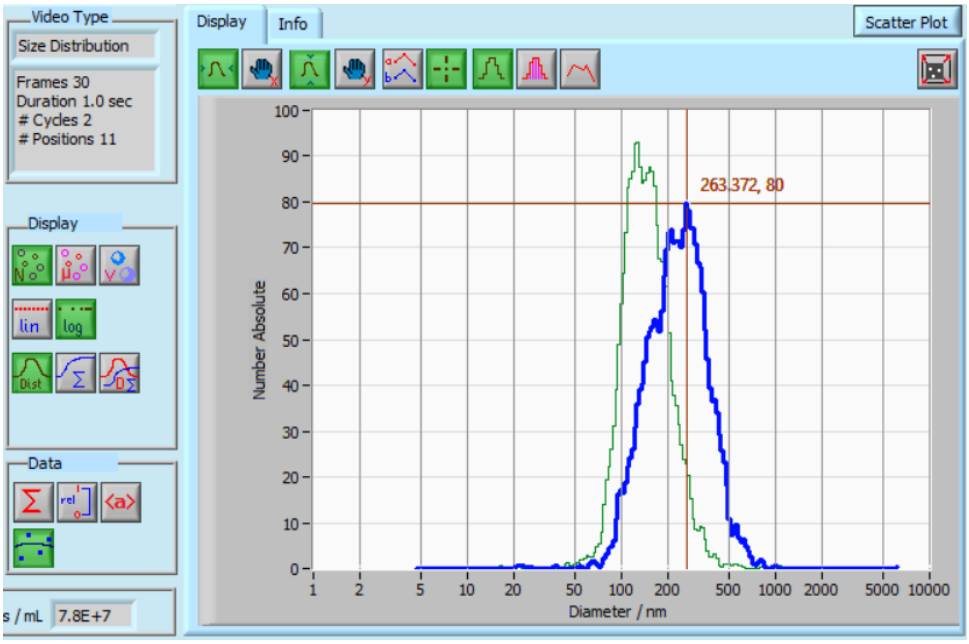


Figure 10-12: The graph shows the histograms of two measurements that are not yet normalized.

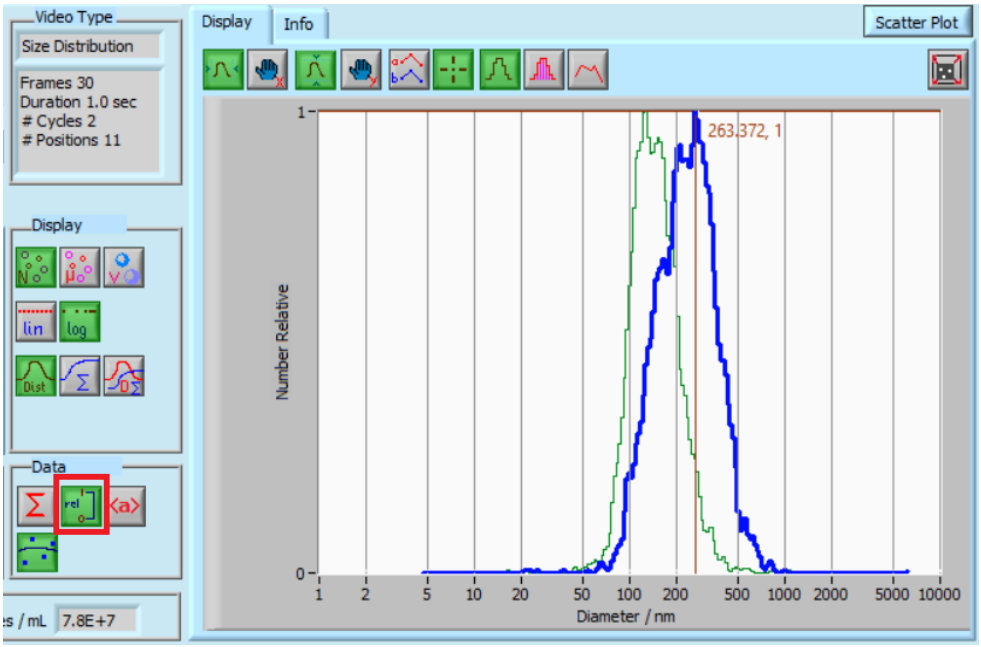


Figure 10-13: The diagram shows the histograms of two measurements, after normalization.

Please note that both new histograms cannot be saved as a new pdf report or new text file but can be saved as a screen shot.

Averaging:

The ZetaView® software allows average calculation of the histograms of at least two or more measurements. All histograms from which an average is to be calculated must have the same settings for "PSD nm / Class" and "Classes / Decade". The two screenshots below show two measurements before and after the average calculation of the histogram data. The average calculation works for number-weighted distribution, concentration-weighted and volume-weighted distribution as well as in distributive and cumulative plotting.

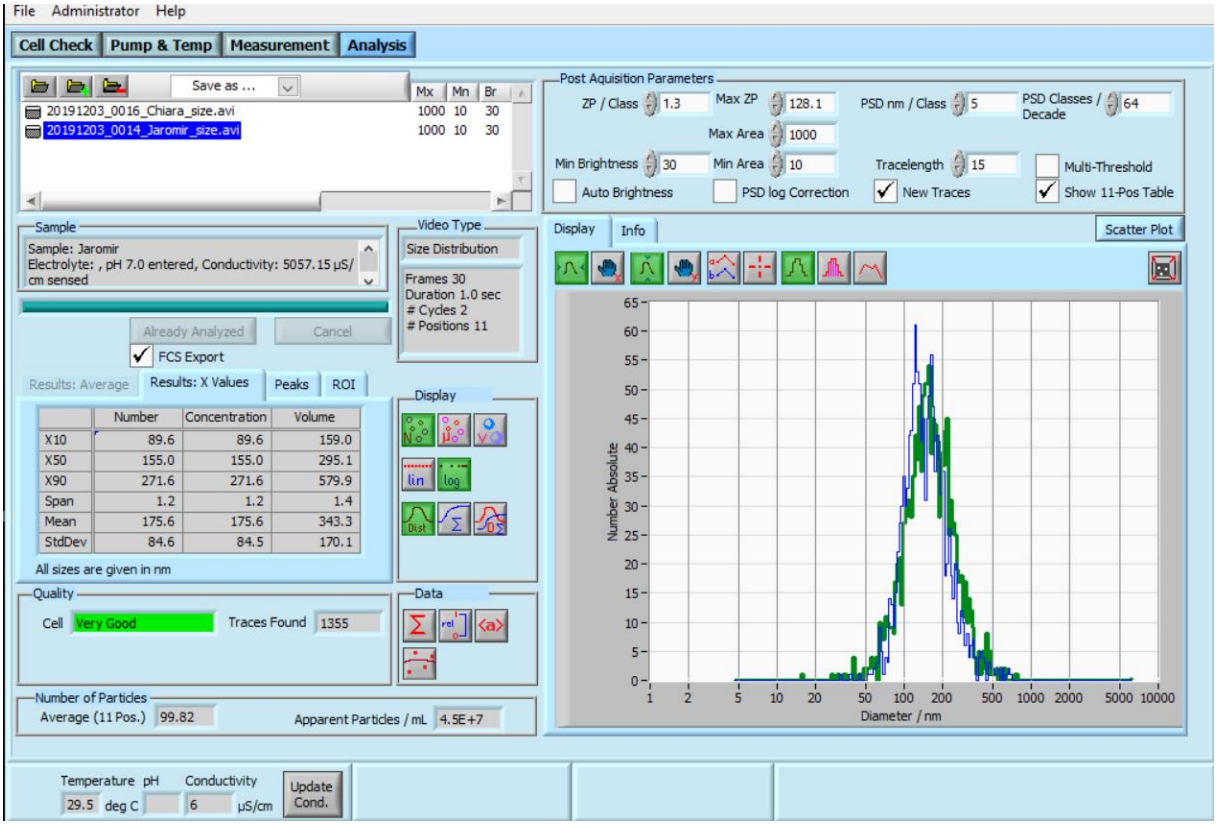


Figure 10-14: The diagram shows the histograms without averaging.

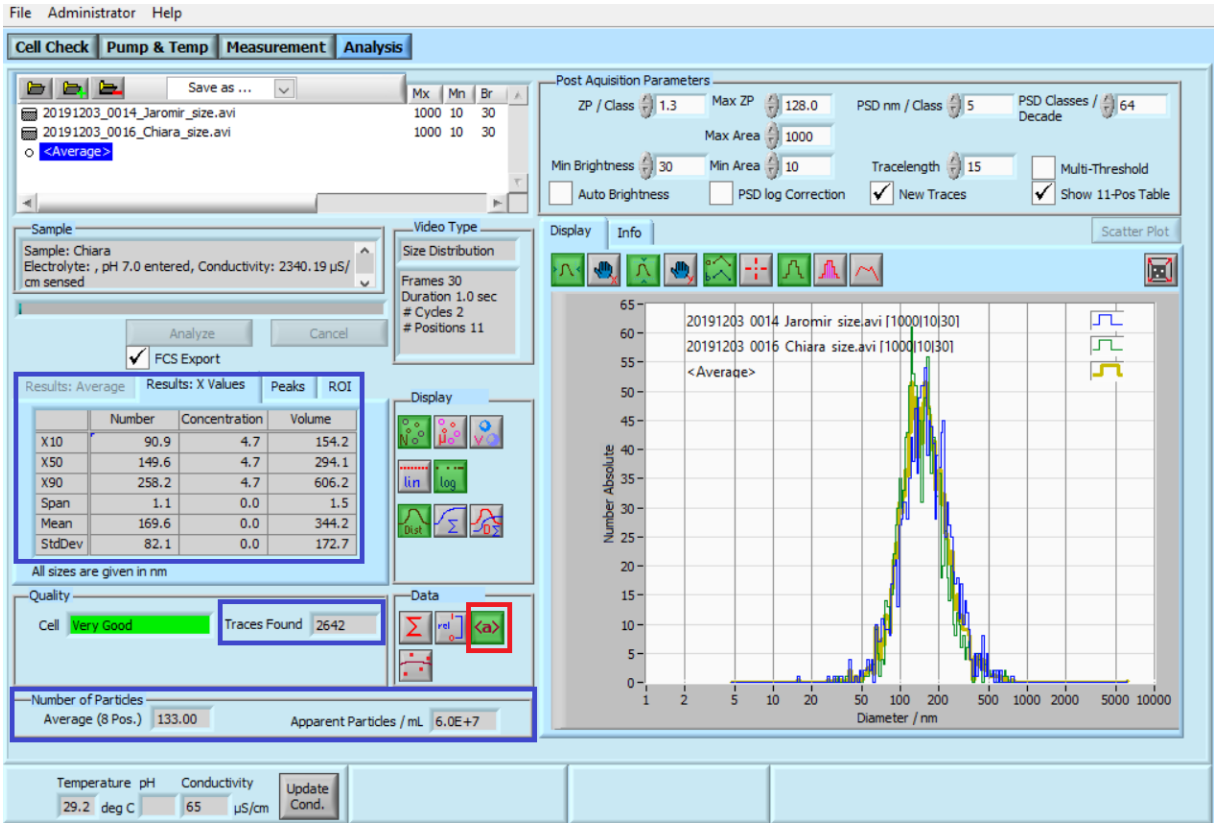


Figure 10-15: Two histograms have been averaged. In addition to the original two histograms, the diagram shows an additional histogram after average calculation.

After the histogram data have been calculated on average across all measurements, new results are obtained for the percentiles, peaks, Traces Found and number of particles (highlighted in blue in the screenshot above). The reason for this is that the histogram data of all measurements are considered for averaging.

The new results and the new histograms cannot be saved as a new pdf report or new text file but can be saved as a screen shot.

Smoothing:

The smoothing function is only an embellishment of the distribution in the ZetaView® software. It has no effect on the percentiles or other measurement results and does not change the data in the text file. However, after the graph has been smoothed, it can be saved as a new pdf report unless there is more than one graph displayed in the diagram.

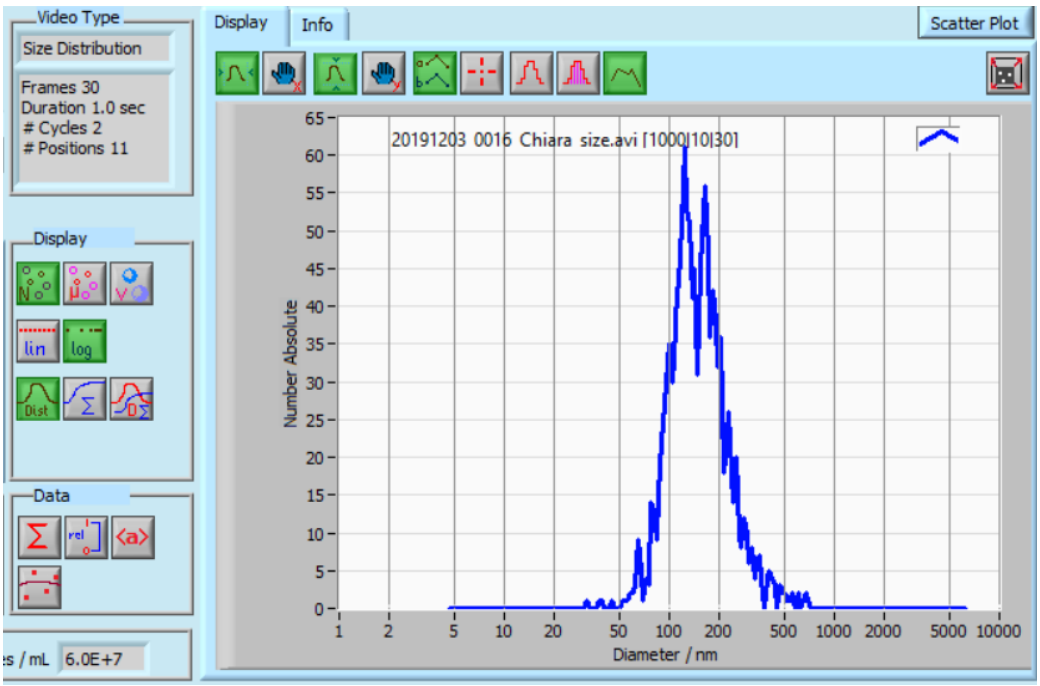


Figure 10-16: The diagram shows an unsmoothed histogram.

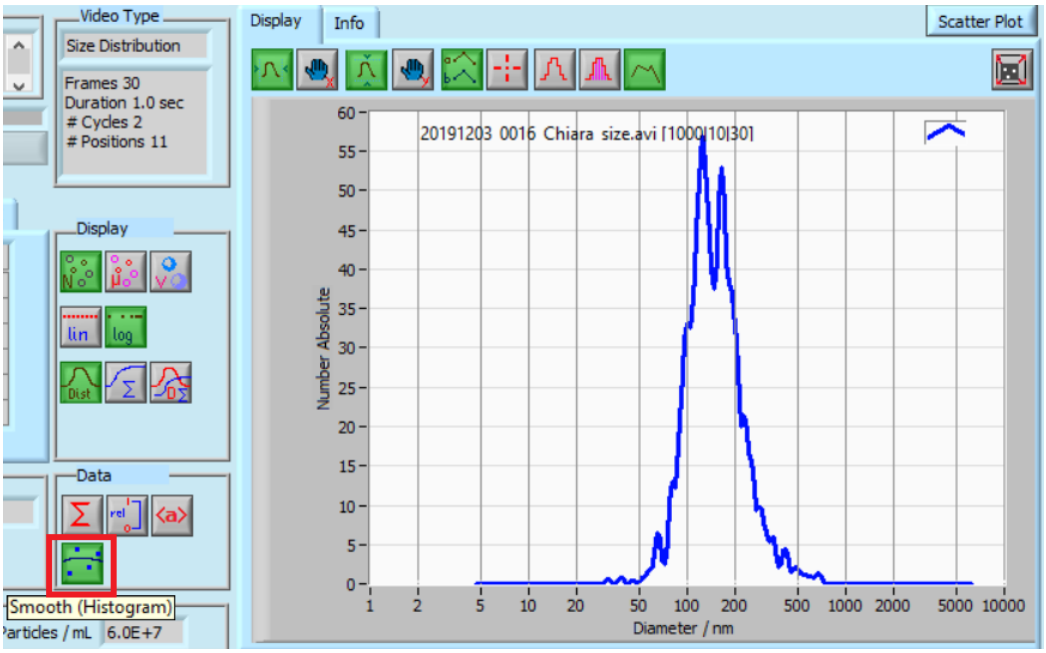
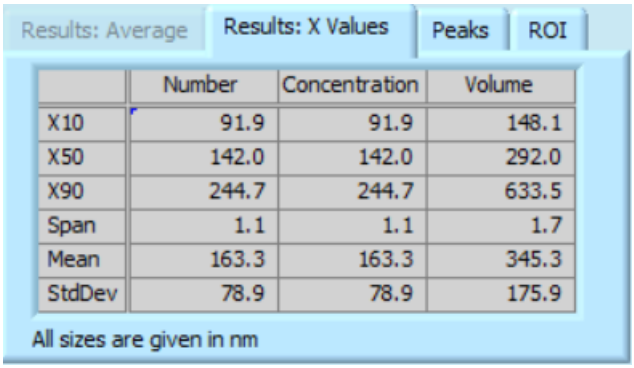


Figure 10-17: The diagram after smoothing.

10.5 Percentiles, Peaks, Region of Interest (ROI)

For each size measurement in scatter and fluorescence mode, the ZetaView® software shows statistical values in the left part of the software interface. Percentiles, span, mean and standard deviation are listed in the "X-Values" tab. For more details on those statistical values, the reader is referred to the corresponding literature.



	Number	Concentration	Volume
X10	91.9	91.9	148.1
X50	142.0	142.0	292.0
X90	244.7	244.7	633.5
Span	1.1	1.1	1.7
Mean	163.3	163.3	345.3
StdDev	78.9	78.9	175.9

Figure 10-18: Example of a result table for the X-Values from a size measurement.

The percentiles as well as the span, mean and standard deviation are listed for the number-weighted distribution ("Number"), concentration-weighted distribution ("Concentration") and for the volume-weighted distribution ("Volume").

The particle concentration is based on the number of particles that is determined in a measurement. Therefore number-weighted distribution and concentration-weighted distribution correlate to each other. The histograms and thus all percentiles as well as span, mean and standard deviation are identical for the number-weighted and for the concentration-weighted distribution.

If the measurement data of several histograms are summed or an average calculation is carried out, the values in the X values tab change. The reason is that there is no longer only one histogram that is taken into account, but there are more histograms selected by the operator.

The peaks of the histograms are reported in the "Peaks" tab. Depending on how many peaks the algorithm detects in the histogram, these are written in the table.

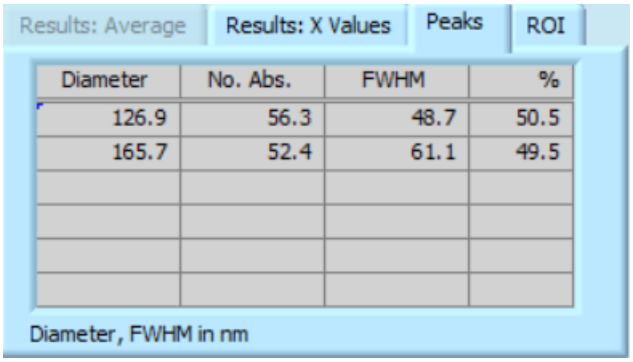


Figure 10-19: Example of a result table for the "Peaks" tab derived from a size measurement.

The first column gives the "diameter" of the particles included in the corresponding peak. This value corresponds to the center of mass of the peak and is the result of an interpolation. Therefore, the diameter value deviates slightly from the peak value showed in the histogram (see section 10.6).

In the second column, "No. Abs "indicates the absolute number of particles associated with the corresponding peak. This value is also the result of an interpolation and differs slightly from the number value showed in the histogram (see section 10.6).

The third column shows the width of the entire distribution as **F**ull **W**idth at **H**alf **M**aximum (FWHM). It denotes 2.4 times the standard deviation for a normal distribution and covers 68% of the area under the graph.

FWHM = 2.4 x STDEV = 68% of the area under the graph.

In the fourth column, after the measurement result has been smoothed and the peak analysis done, the percentage of particles assigned to a peak is calculated.

If you are interested in the particle concentration within a certain size range, you can check the tab "ROI" (Region of Interest).

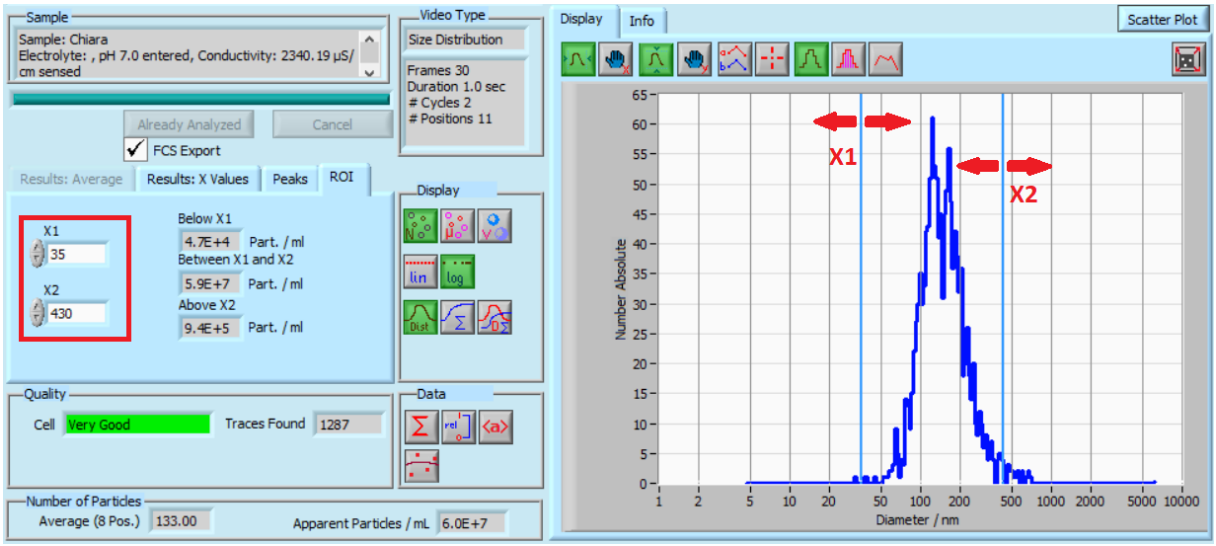


Figure 10-20: Example of concentration determination of the measured particles in a size range between 35 nm and 430 nm.

To adjust the size range in which the corresponding particle concentration is to be displayed, the vertical lines X1 and X2 can be moved in the histogram. However, it is much more precise to enter the minimum particle size for X1 and the maximum particle size for X2. The obtained results are particle concentrations below X1, between X1 and X2 and above X2.

This concentration information is based on the number of particles measured and therefore does not take the dilution factor into account. Therefore, the dilution factor must be considered if the original concentration in the desired size range becomes important.

10.6 Presentation and scaling of the diagram

Presentation

The histogram can be displayed as a step plot, bar plot and line plot.

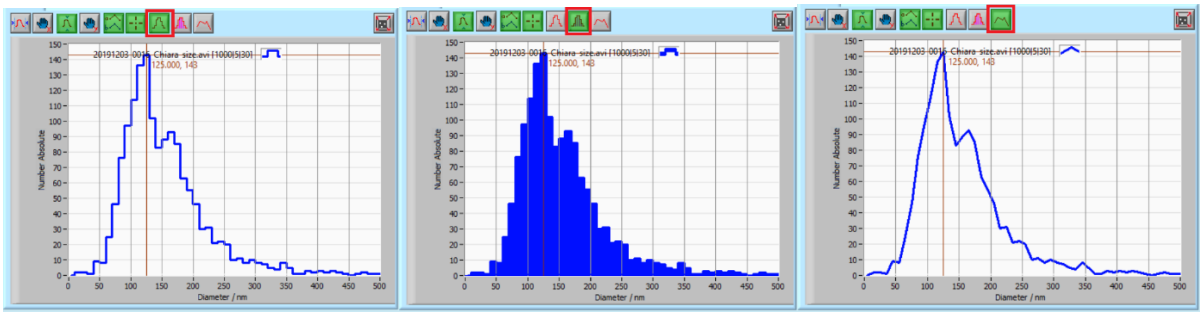


Figure 10-21: From left to right: step plot, bar plot, line plot. The type of plotting has no effect on the data in the text file.

Legend

The legend can be shown and hidden in the histogram. The legend contains the date of the measurement (YYYYMMDD) as well as the four-digit experiment ID, the name, the type of measurement (size, SL, prof), the extension (avi) and the post-acquisition parameters.

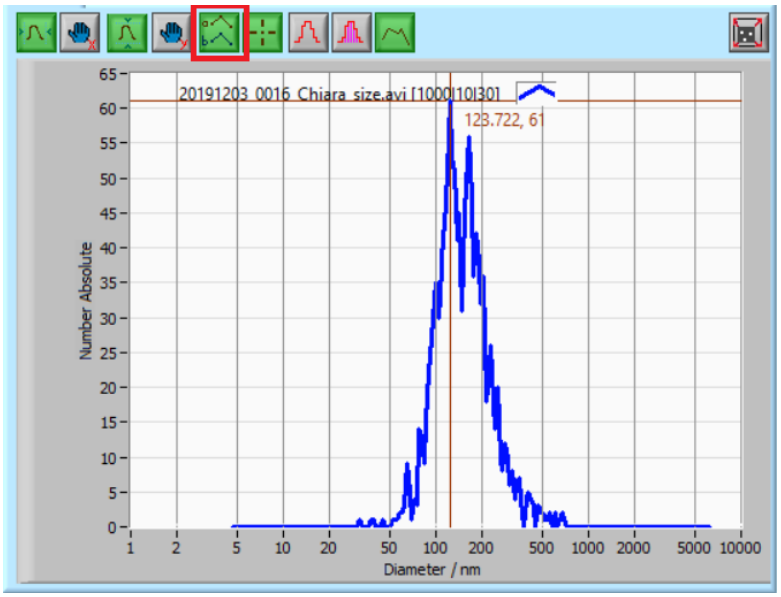


Figure 10-22: Example of an activated legend. In this example, it contains the date of the measurement (20191203), the four-digit experiment ID (0016), the name (Chiara), the type of measurement (size), the extension (avi) and the post-acquisition parameters (MaxArea1000; MinArea10; MinBrightness30)

Cursor

The cursor can be shown and hidden in the plot. If the cursor is displayed, it can be moved on the horizontal or vertical line over the plot. The intersection of both lines shows two values. The first value is the X value and corresponds to the respective bin class (see chapter 11). The second value is the Y value. Depending on the plot of the Y axis, the Y value corresponds to the absolute number of particles, the particle concentration or the particle volume that is assigned to the respective X value (bin class).

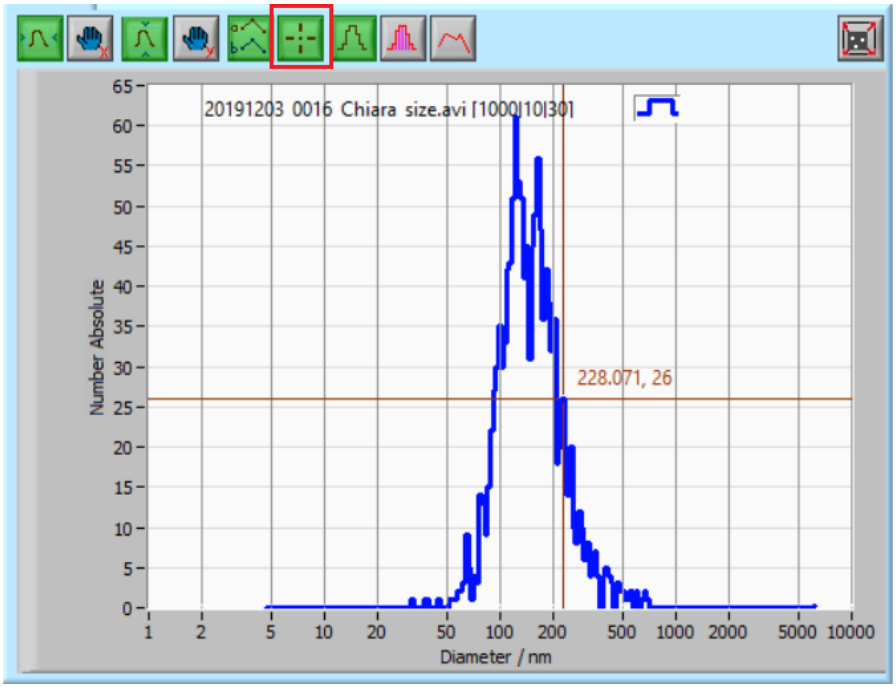


Figure 10-23: Cursor in a logarithmically plotted distribution in step plot. The cursor shows the pair of values 228.071; 26. This means that 26 particles were found that were assigned to the bin class 228.071 due to their size.

Scaling of X-axis and Y-axis

The X- and Y-axis can be scaled manually or automatically. With manual scaling you can enter the value range manually.

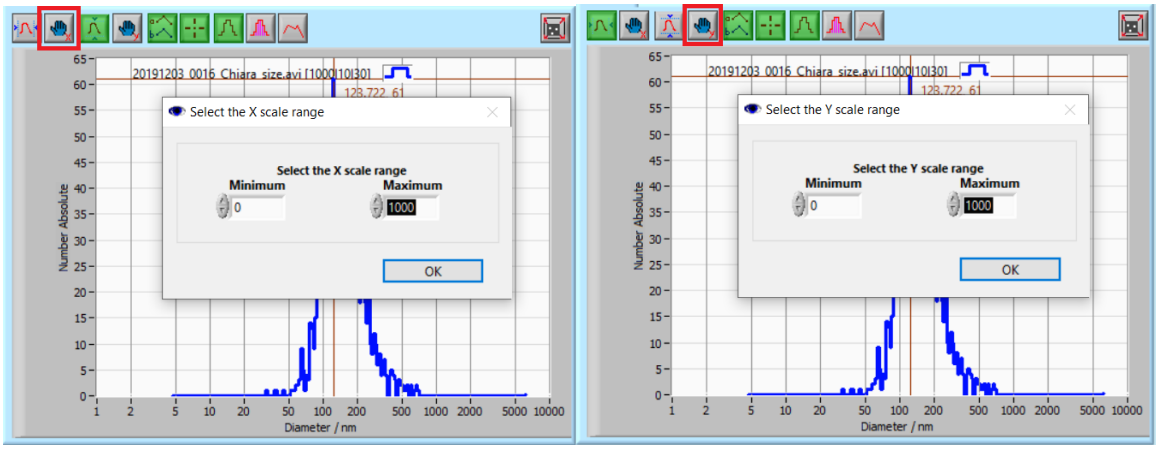


Figure 10-24: Manual scaling for the X- and Y-axes.

If the X- and Y-axes are to be scaled automatically, the buttons shown below must be clicked.

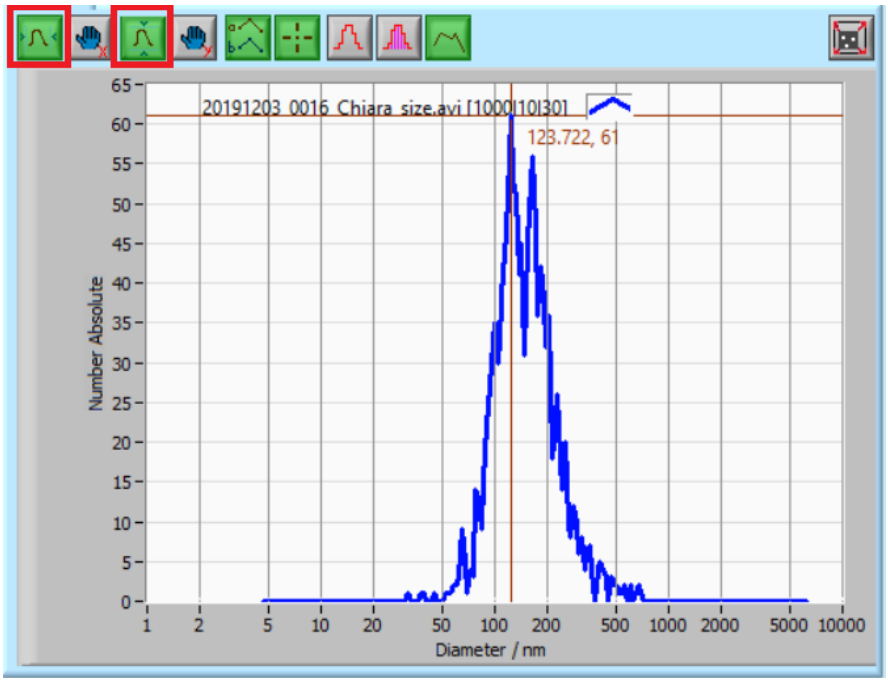


Figure 10-25: Automatic scaling of the X- and Y-axis.

10.7 Re-analysis of an existing size measurement

The ZetaView® software can re-analyze an existing measurement with altered post-acquisition parameters. The precondition for this is that the corresponding video file of this measurement is available since it contains all raw data for a new analysis. If there is no video file, a re-analysis is not possible.

10.7.1 Load a video for re-analysis

For a re-analysis, the video file of any desired measurement can be (re-) loaded in the Analysis tab via the button "Load Files / Analyses (Clear List)". The corresponding procedure is shown in the figure below.

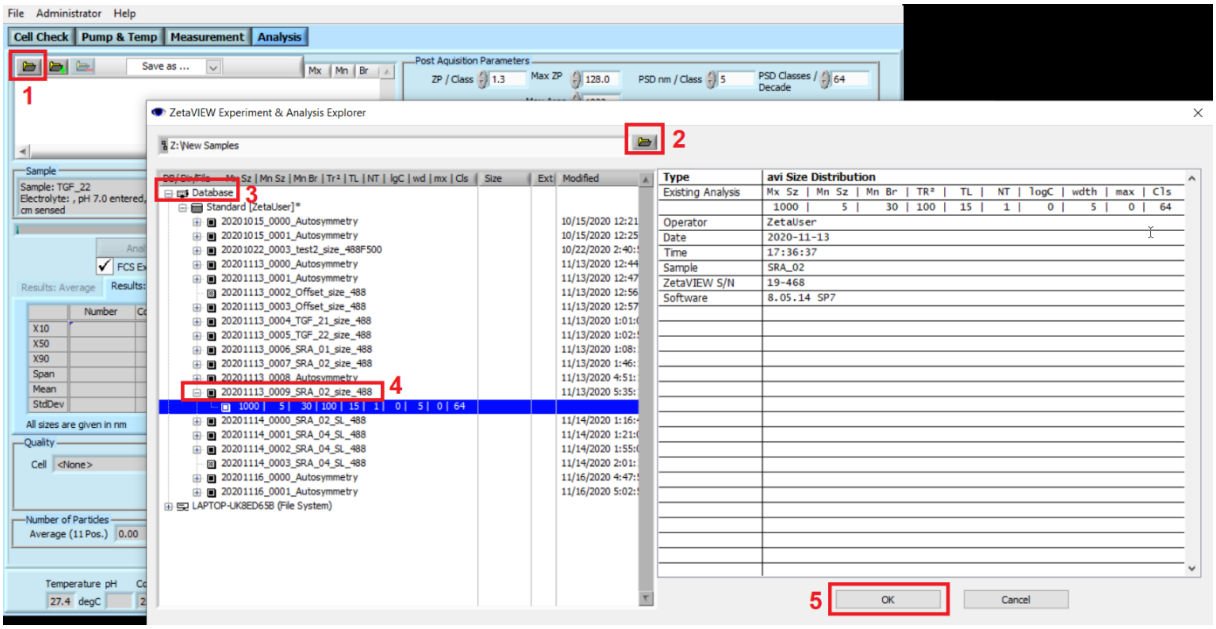


Figure 10-26: Loading the video of an existing size measurement.

1. Click on "Load Files / Analyzes (Clear List)"
2. Browse to the desired storage folder
3. Open the database
4. Select the desired measurement for (re-) analysis. The sub-item highlighted in blue in this example already contains parameters from the first analysis
5. Load the histogram with “OK”

A dialogue opens and asks whether an existing analysis should be loaded or a new analysis should be carried out with the current post-acquisition parameters.

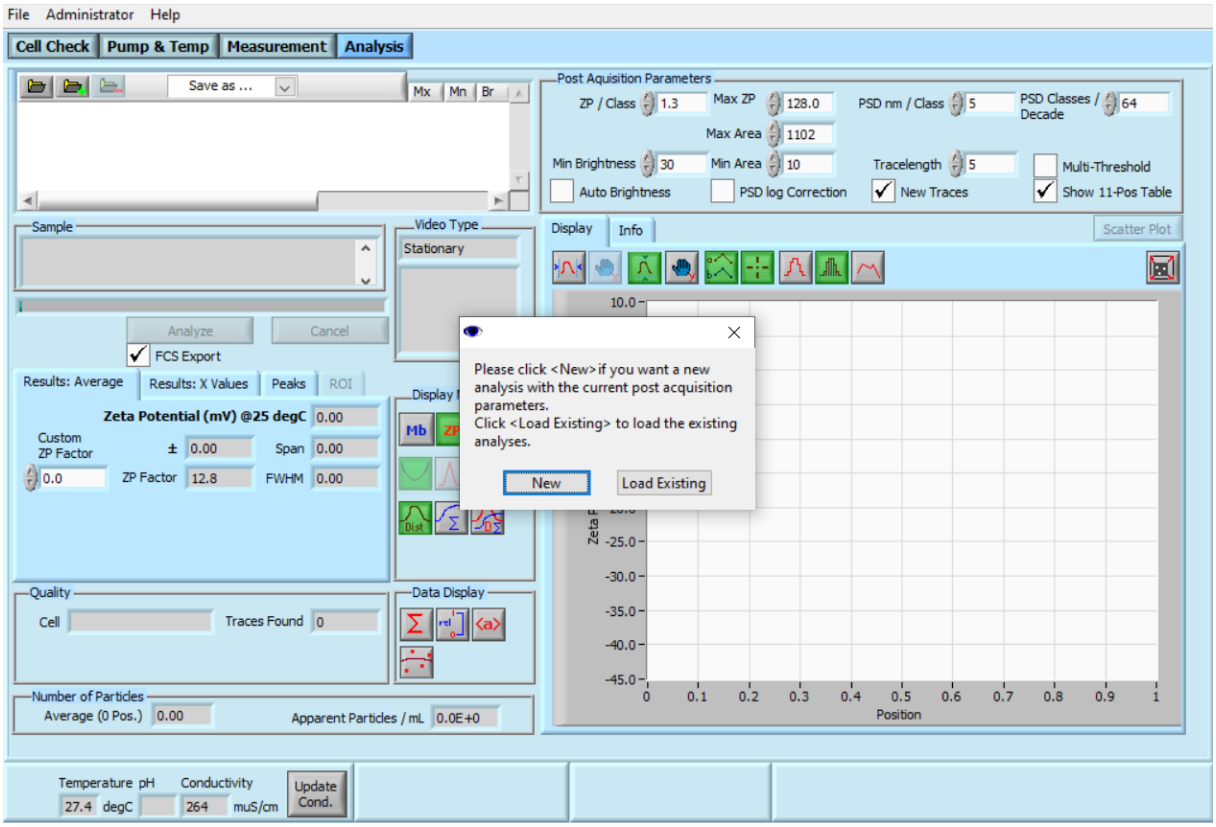
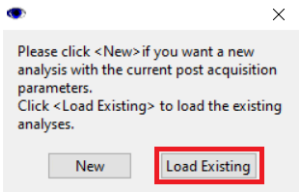


Figure 10-27: You can select whether an existing analysis is to be loaded or a new analysis should be carried out with the current post-acquisition parameters.

After clicking “Load Existing”,



the software loads an already existing analysis that was previously analyzed with the post-acquisition parameters set at the time.

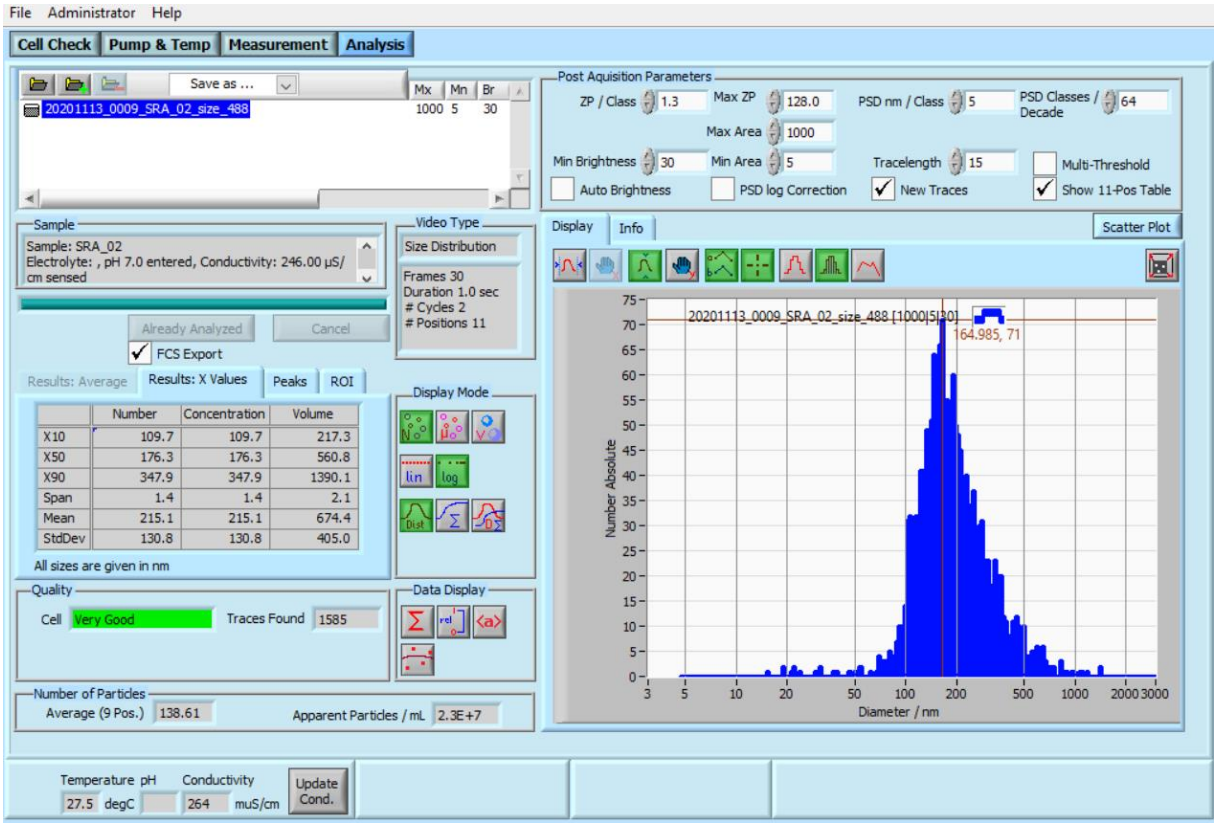
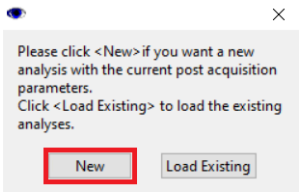


Figure 10-28: An already existing analysis has been loaded.

After clicking “New”,



a re-analysis starts with the currently set post-acquisition parameters.

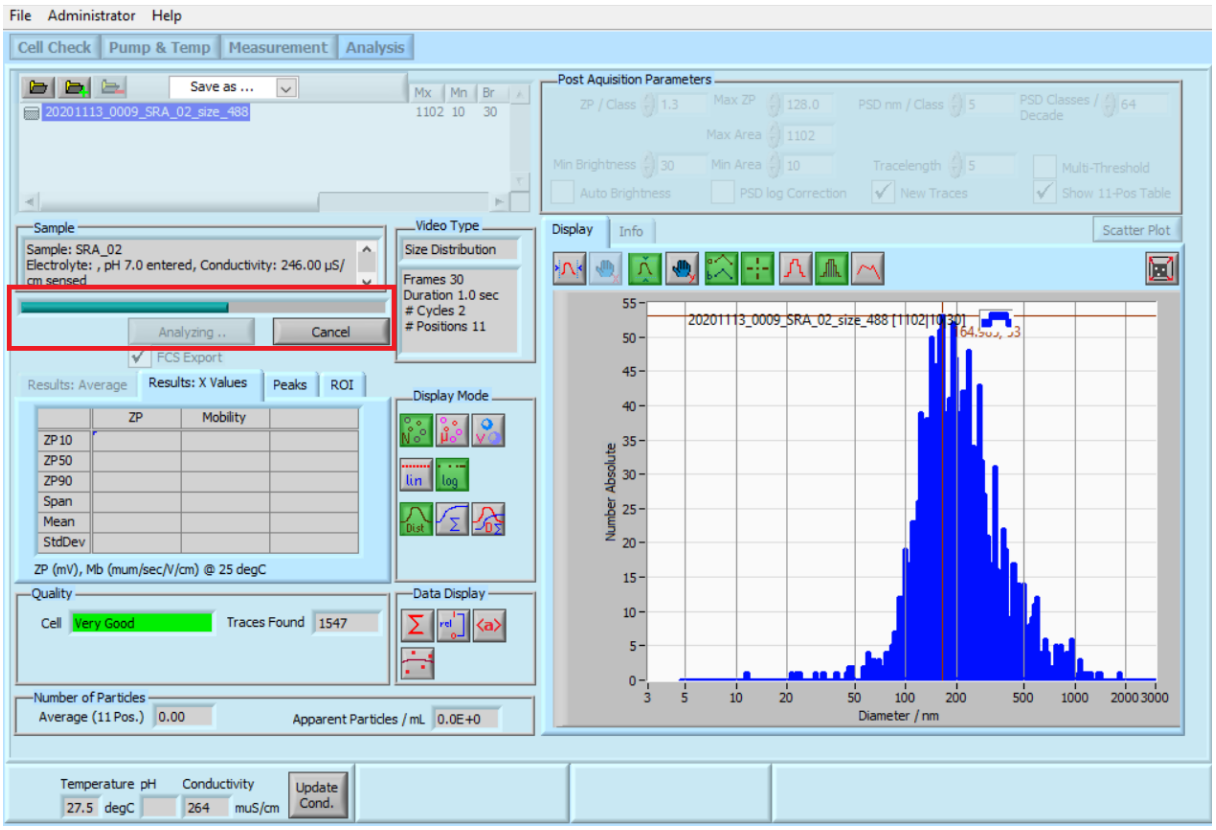


Figure 10-29: Re-analysis of an existing measurement with the current post-acquisition parameters. Re-analysis is indicated by the progress bar, it can also be stopped at any time.

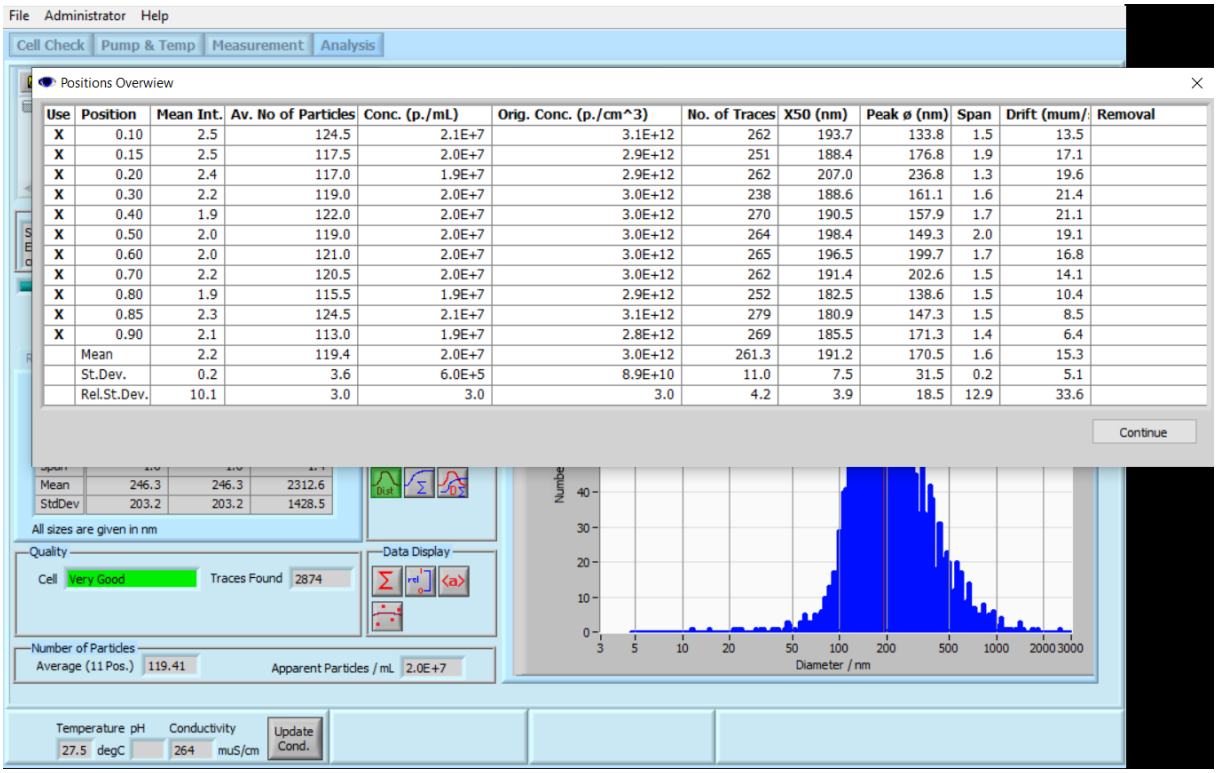


Figure 10-30: Completed re-analysis. In this example, the original video contained an 11-position measurement.

All newly performed analyses are saved and displayed as sub-items under the corresponding sample / measurement name. If a measurement has been re-analyzed several times with different post-acquisition parameters, all analyses performed appear as a compilation of the post-acquisition parameters used. An example of a measurement that was re-analyzed three times with different parameters is shown in the following figure.

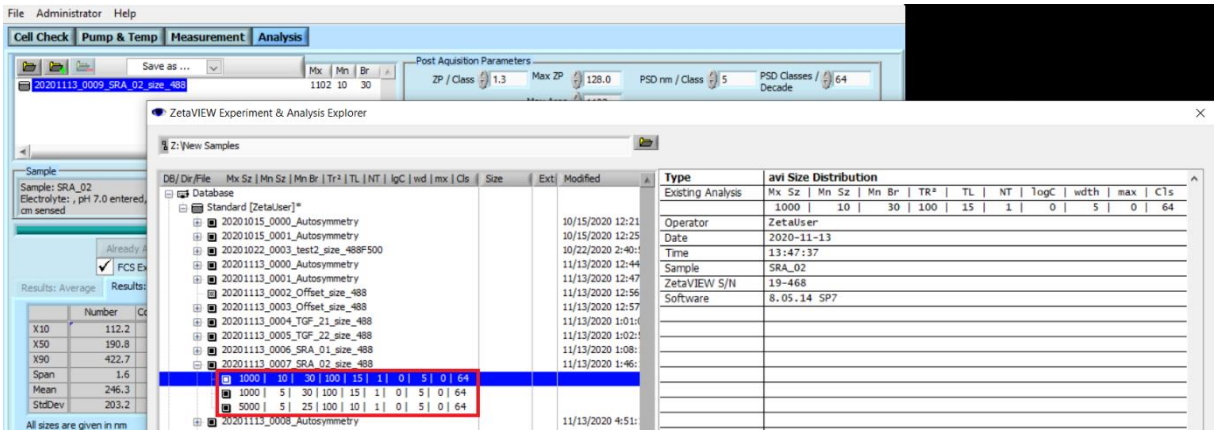


Figure 10-31: 3 analyses of the measurement "20201113_0007_SRA_02_size_488" are highlighted in red. The post-acquisition parameters used are listed in each of the three lines. The currently selected analysis is highlighted in blue.

10.7.2 Re-analysis of a measurement that has just been carried out

Starting from an already existing and analyzed histogram (video file), all post-acquisition parameters can now be changed according to the requirements of the operator. In the example below, the “Min Area” was reduced from 10 to 5 in order to include smaller particles in the new analysis.

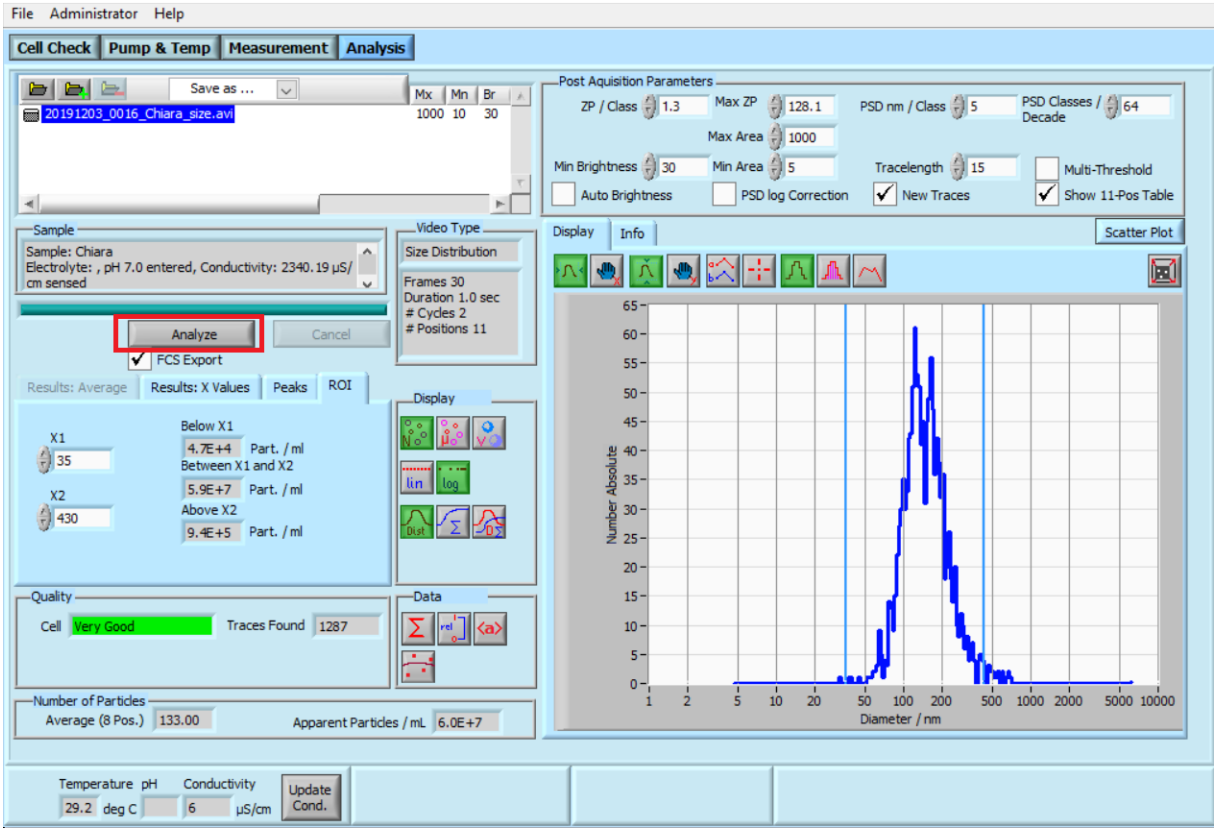


Figure 10-32: In this histogram, the “Min Area” has been reduced from 10 to 5. After changing at least one post-acquisition parameter, the new analysis can be started with the "Analyze" button. However, it is also possible to change more than one post-acquisition parameter simultaneously for re-analysis.

The duration of the re-analysis depends on which post-acquisition parameters have been changed and how much they have been changed.

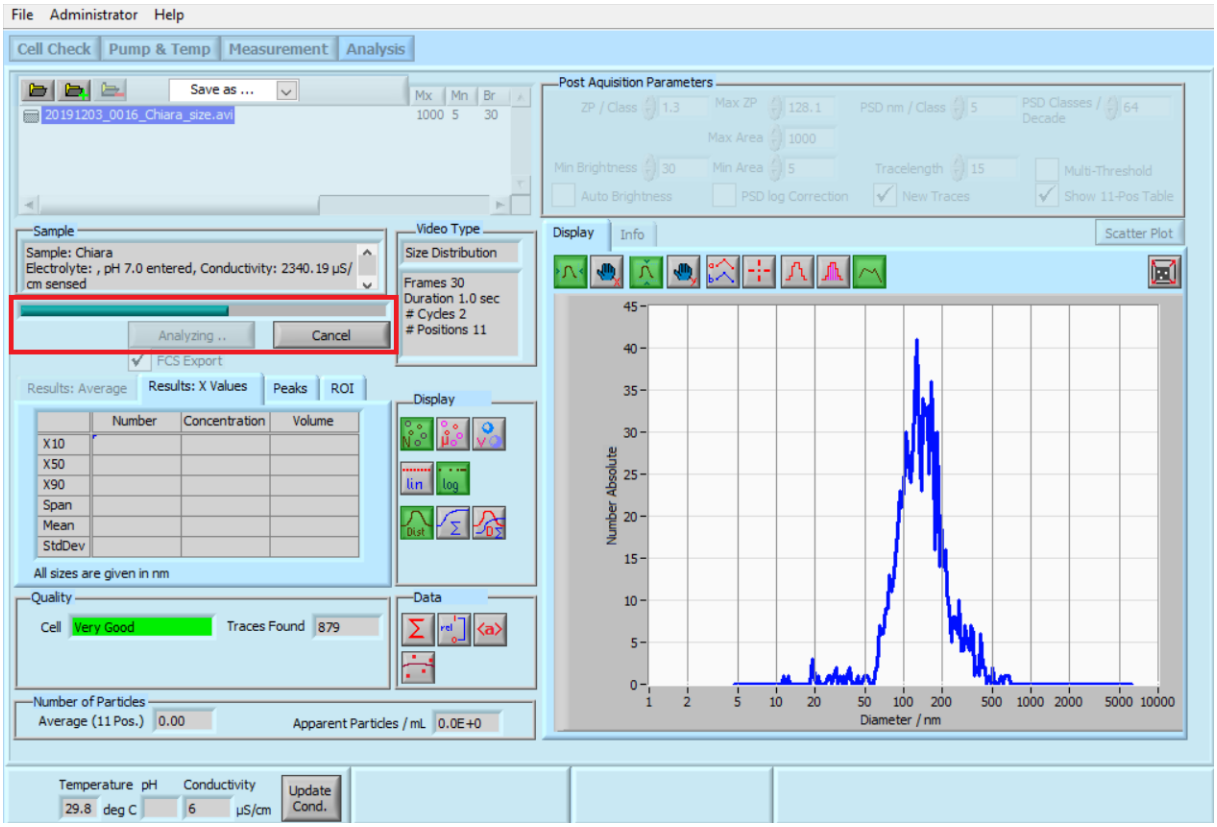


Figure 10-33: Again, the re-analysis can be observed on the progress bar, it can also be stopped at any time.

The following screenshot shows the completed re-analysis. If the original video contained an 11-position measurement, an 11-position table will be shown again after the re-analysis, provided that this option has been activated in the Analysis tab (see section 7.1). If the video contains only a 1- or 2-position measurement, no table is shown.

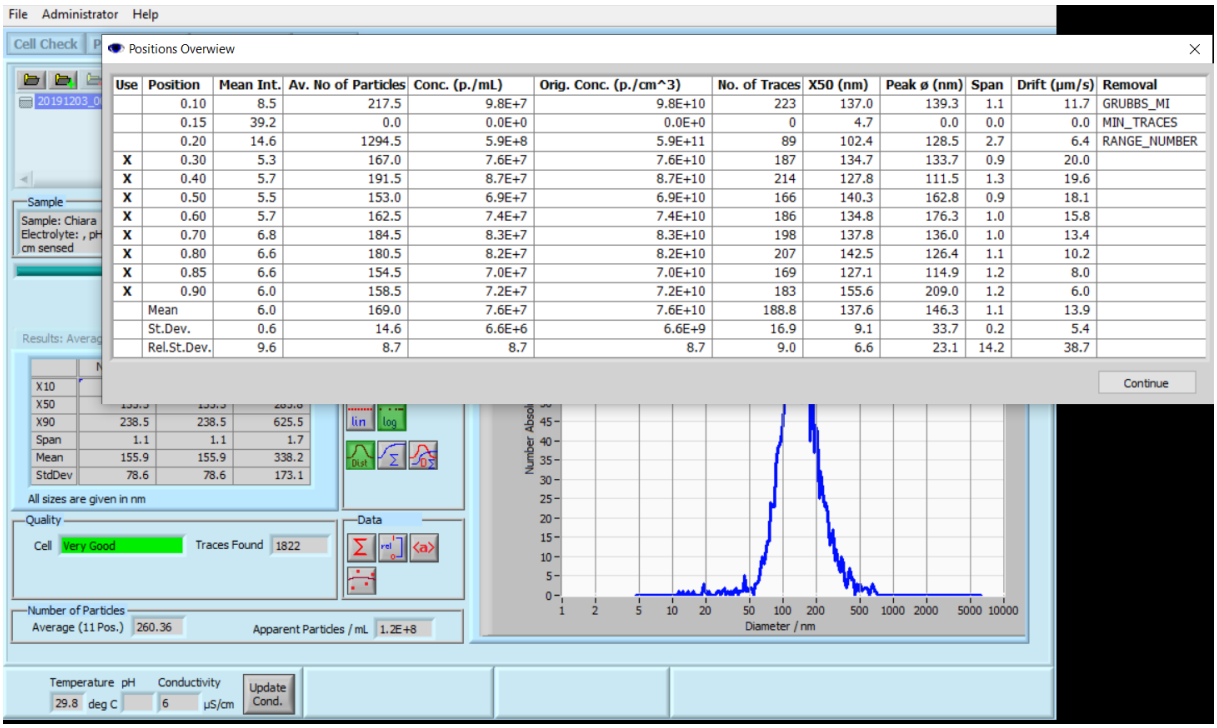


Figure 10-34: Completed re-analysis. In this example, the original video contained an 11-position measurement too.

Since a completed re-analysis is not automatically saved as a pdf report or as a text file, it is strongly recommended to save it manually. This is also important because the X values, peaks and all other values in the Analysis tab are changed after a re-analysis.

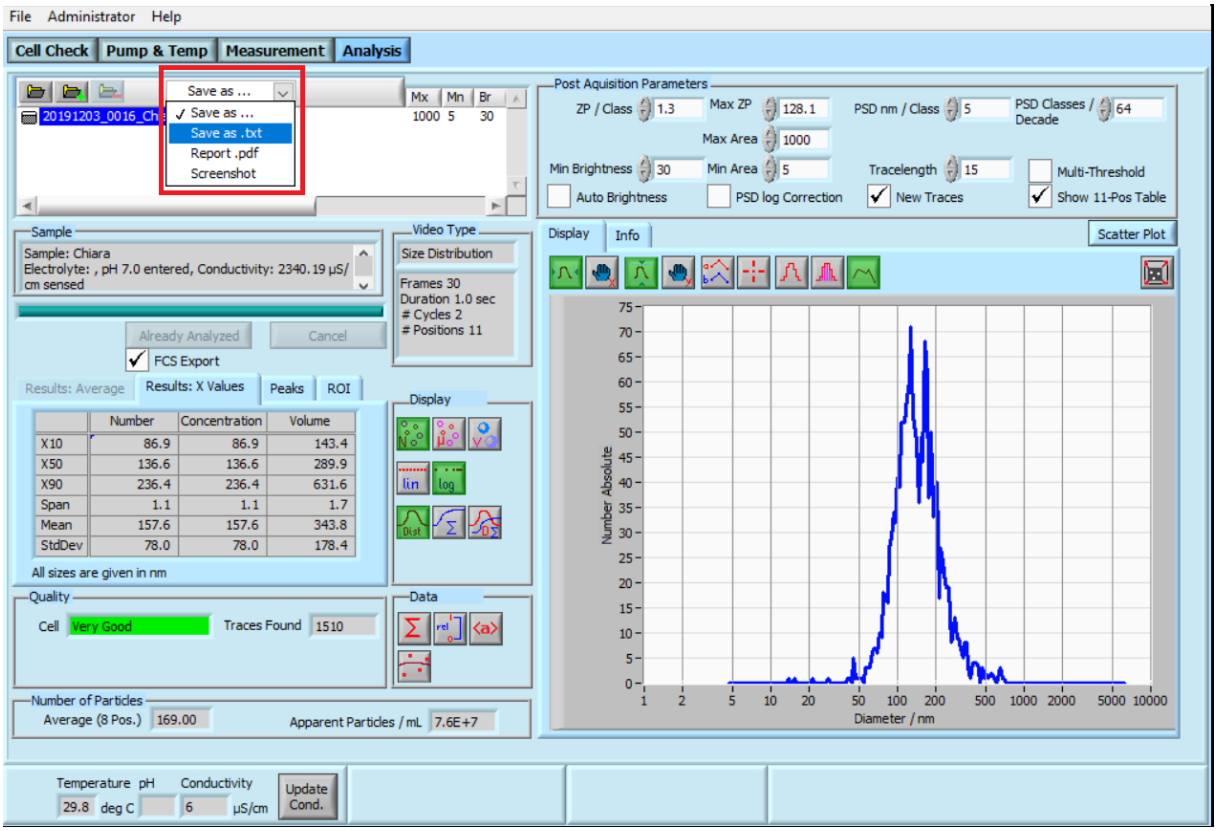


Figure 10-35:Manual saving of the analysis.

Scatter Plot

By clicking on "Scatter Plot" at the top right of the histogram, the measurement can also be displayed as a scatter plot. For this, an FCS file is required. The precondition for displaying the scatter plot immediately is that "FCS-Export" was switched on in the Analysis Menu before the corresponding measurement has started.

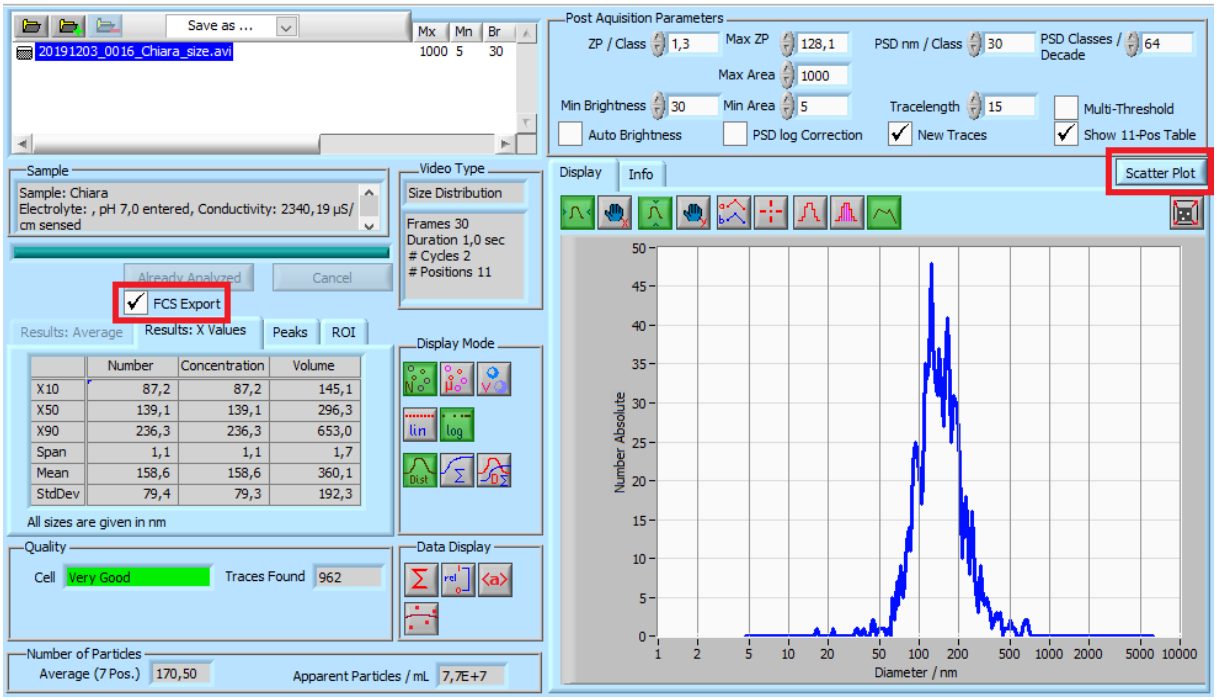


Figure 10-36: The scatter plot can be accessed by clicking the corresponding button right above the histogram. It is important that "FCS Export" was switched on before the corresponding measurement has started.

An example image of a scatter plot after an 11-position size measurement is shown below.

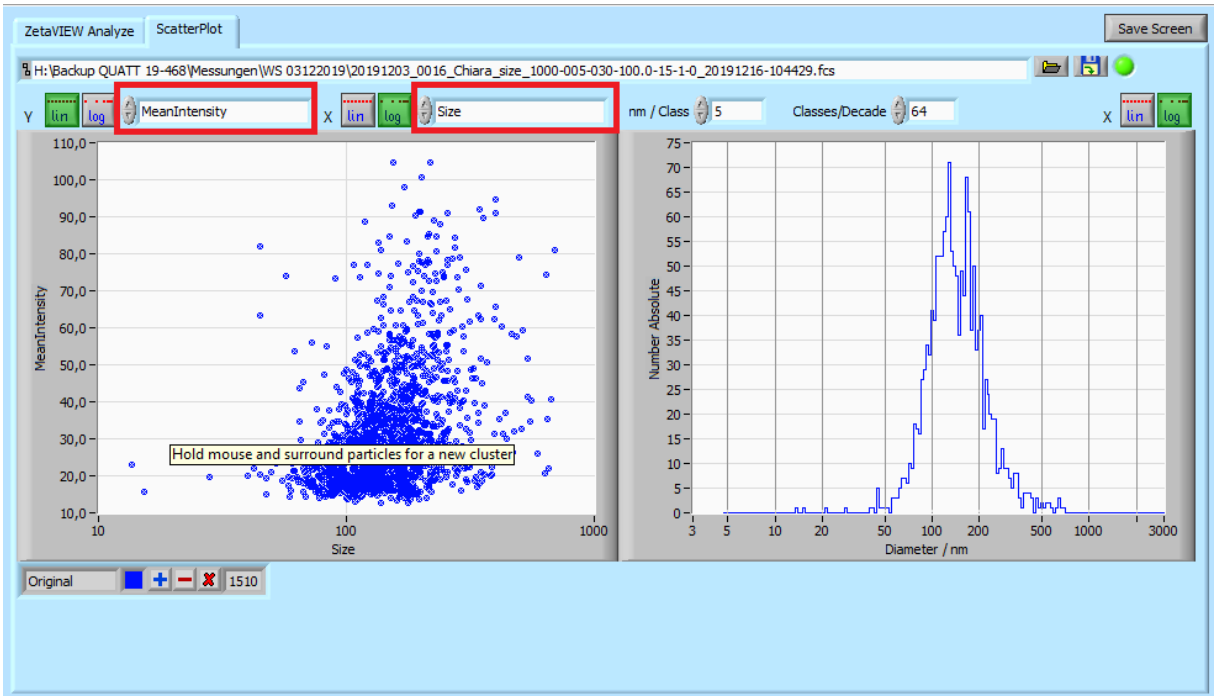


Figure 10-37: Scatter plot after an 11 position measurement. In this example, the Y-axis is plotted with the mean intensity and the X-axis with the size. However, other parameters can be plotted as well.

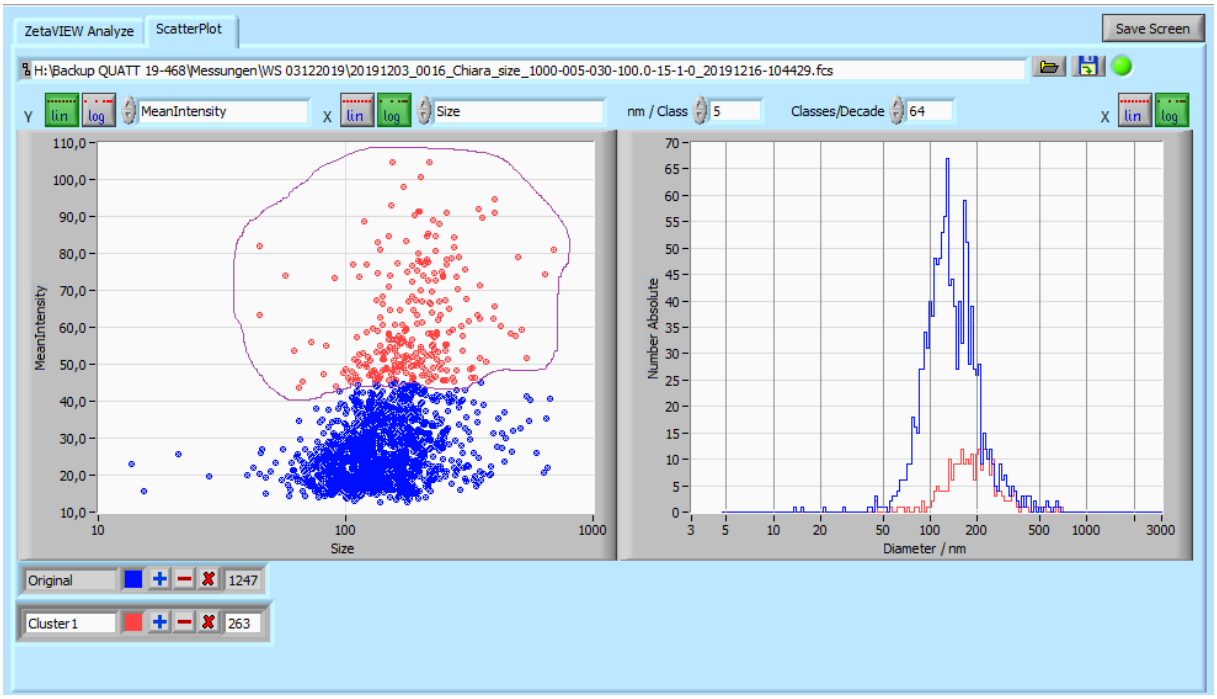


Figure 10-38: Particles in the scatter plot can be surrounded by the mouse to separate particle populations. New clusters are displayed under the scatter plot and in the corresponding histogram on the right. The number of particles of every cluster is displayed as well.

If “Scatter Plot” is clicked and no FCS file is available after the measurement, the software gives a message that no FCS file is available and asks for creating an FCS file for subsequent re-analysis.

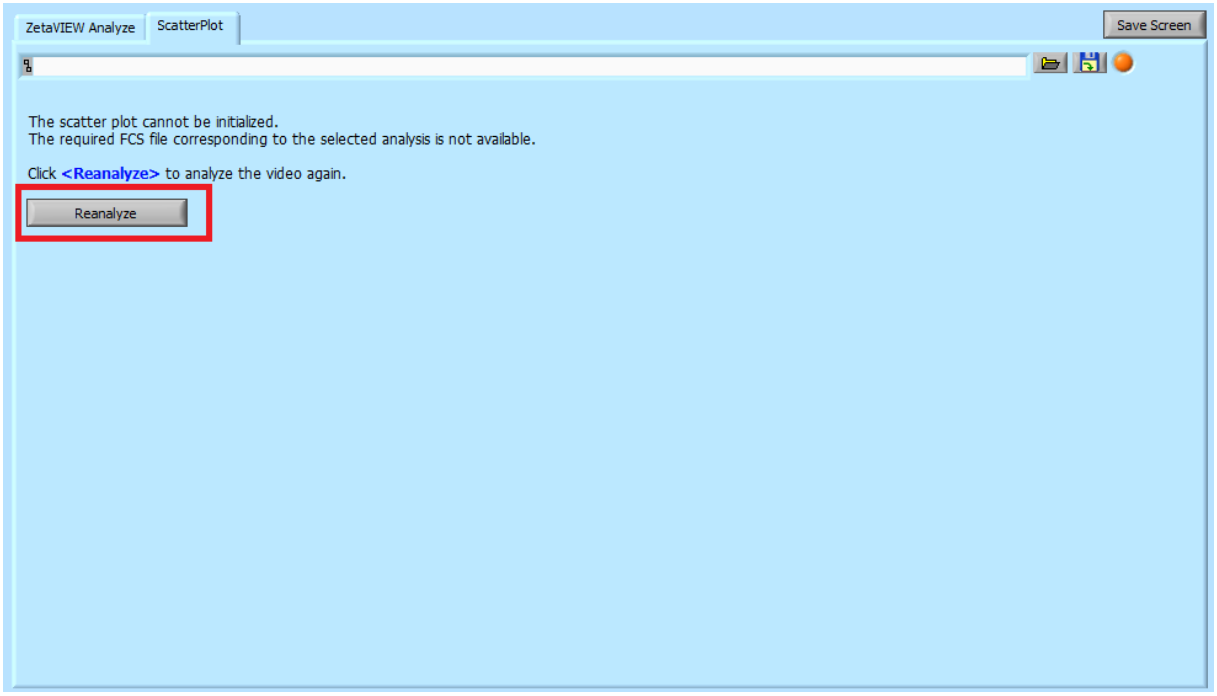


Figure 10-39: If an FCS file is not available for re-analysis, it can be created by clicking “Reanalyse” in the Scatter Plot tab.

11 Txt-file of size measurement

After a size measurement or a zeta potential measurement has been carried out and evaluated, a text file is stored in addition to a video file (which contains the actual raw data of the measurement) and the pdf report, regardless of whether the sample was measured in scatter mode or in fluorescence mode. Here, the text file created for a size measurement is explained in more detail.

Basically, the text file consists of 3 main parts:

The top part represents the header of the text file, and contains the name of the file, operator, software version, and the numerous parameters used to measure the sample. A section of this header is shown in the figure below.

```
Original File:  Z:\27092019\20190927_0011_Thylakoid_260919_Serie_F405_size.avi  Section:        0.1 [0]
Operator:       ZetaView PC
Experiment:     20190927_0011
ZetaVIEW S/N:   19-468
Software:       ZetaView (version 8.05.11)      Analyze:        8.05.11
Sample: Thylakoid_260919_Serie_F405
Electrolyte:
pH:     7.000000        entered
Conductivity:   98.923283       sensed
Temperature:    24.990000       sensed
Viscosity:      0.901484
Date:   2019-09-27
Time:   14:52:20
General Remarks:        PS100nm 1:250,000
```

Figure 11-1: Detail of the header of a text file from a size measurement.

The second part of the text file contains the linear measured values. These values start just below the header and are grouped in five columns.

```
Reference Electrode Distance:   5.150000
Voltage:        0.000000
Polarity:       0
Reference Voltage:      0.000000
Microscope Magnification:       x10
Laser Wavelength:       405.000000
ZP Factor:      12.828778 (Smoluchowski)Results (Pos 0.100004):
Median Number (D50):    214.787748
Median Concentration (D50):     214.787748
Median Volume (D50):    356.540467
Size Distribution
Size / nm       Number  Concentration / cm-3    Volume / nm^3   Area / nm^2
2.500E+0        0.000E+0        0.000E+0        0.000E+0        0.000E+0
7.500E+0        1.000E+0        4.698E+4        2.209E+2        4.418E+1
1.250E+1        2.000E+0        9.396E+4        2.045E+3        2.454E+2
1.750E+1        2.000E+0        9.396E+4        5.612E+3        4.811E+2
2.250E+1        1.000E+0        4.698E+4        5.964E+3        3.976E+2
2.750E+1        1.000E+0        4.698E+4        1.089E+4        5.940E+2
3.250E+1        2.000E+0        9.396E+4        3.595E+4        1.659E+3
3.750E+1        3.000E+0        1.409E+5        8.283E+4        3.313E+3
4.250E+1        3.000E+0        1.409E+5        1.206E+5        4.256E+3
4.750E+1        4.000E+0        1.879E+5        2.245E+5        7.088E+3
5.250E+1        4.000E+0        1.879E+5        3.031E+5        8.659E+3
5.750E+1        6.000E+0        2.819E+5        5.972E+5        1.558E+4
6.250E+1        2.000E+0        9.396E+4        2.557E+5        6.136E+3
6.750E+1        2.000E+0        9.396E+4        3.221E+5        7.157E+3
7.250E+1        4.000E+0        1.879E+5        7.981E+5        1.651E+4
7.750E+1        3.000E+0        1.409E+5        7.312E+5        1.415E+4
8.250E+1        6.000E+0        2.819E+5        1.764E+6        3.207E+4
8.750E+1        9.000E+0        4.228E+5        3.157E+6        5.412E+4
9.250E+1        6.000E+0        2.819E+5        2.486E+6        4.032E+4
9.750E+1        1.100E+1        5.168E+5        5.338E+6        8.213E+4
```

Header

Linear values

Figure 11-2: Extract from the linear value range of the text file from a size measurement.

Table 11.1: Description of the columns in the text file.

Column	Headline	Description
1st column	Size / nm	Bin class to which the particles are assigned according to their measured size. The bins can be adjusted in the post-acquisition parameters. This number represents the centre of each bin.
2nd column	Number	Number of analysed particles assigned to the corresponding bin (size class).
3rd column	Concentration /cm^{-3}	Concentration of the analysed particles assigned to the corresponding bin (size class). The dilution factor is not included.
4th column	Volume / nm^3	Volume of the analysed particles assigned to the corresponding bin (size class) in terms of number-weighted distribution.
5th column	Area / nm^2	Surface of the analysed particles assigned to the corresponding size class in relation to the number-weighted distribution.

The third part of the text file represents the logarithmic measured values for the adjusted size classes and is separated by the -1.000E+0 line from the linear measured values. This is shown in the following figure.

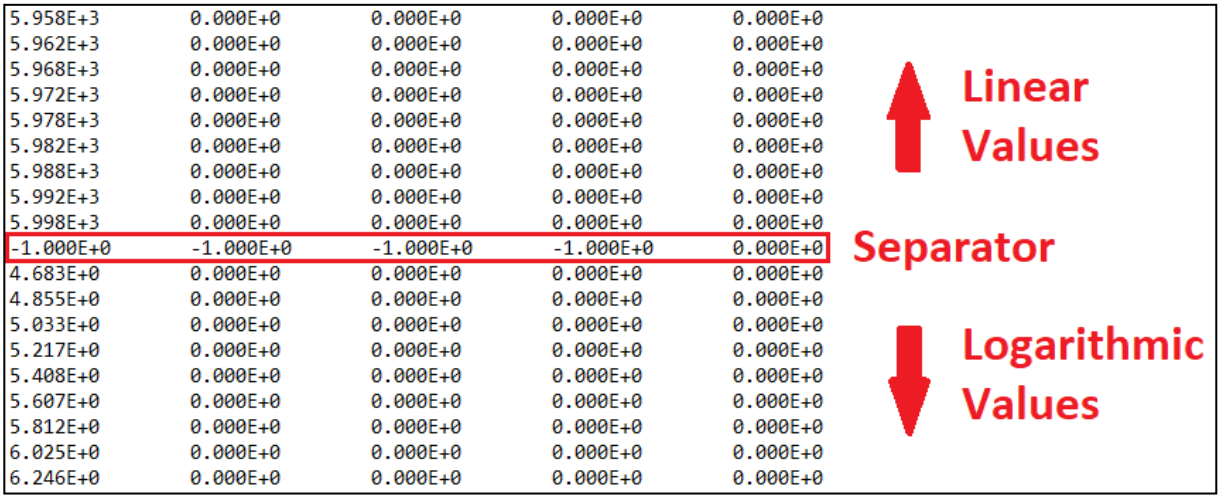


Figure 11-3: Linear and logarithmic values in the text file are separated by the -1.000E+0 line.

The linear and logarithmic values refer to the distribution of the bins (size classes):

In the linear value range, the size classes are distributed linearly. The parameter "nm / Class" is used for this purpose and can be found in the Measurement Menu as well as in the Analysis Menu.

In the logarithmic value range, the size classes are chosen such that they are equally far apart in a logarithmic representation (base 10).

The parameter "Classes per Decade", which can be found in the Measurement Menu as well as in the Analysis Menu can be defined here. This is shown in the figure below.

Figure 11-4: "nm / Class" applies only to the linear values. "Classes / Decade" applies only to the logarithmic values. Both can be adjusted in the Measurement Menu.

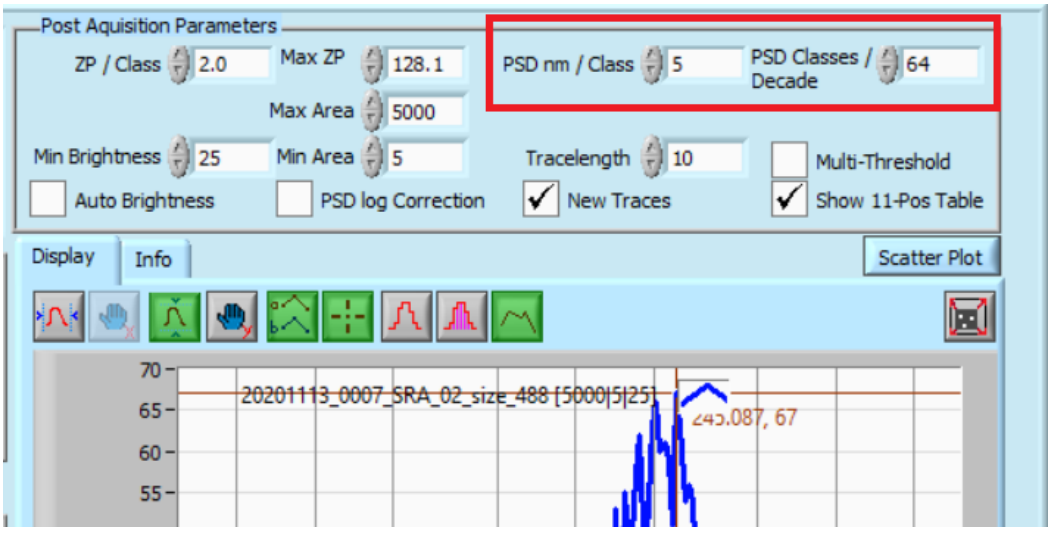


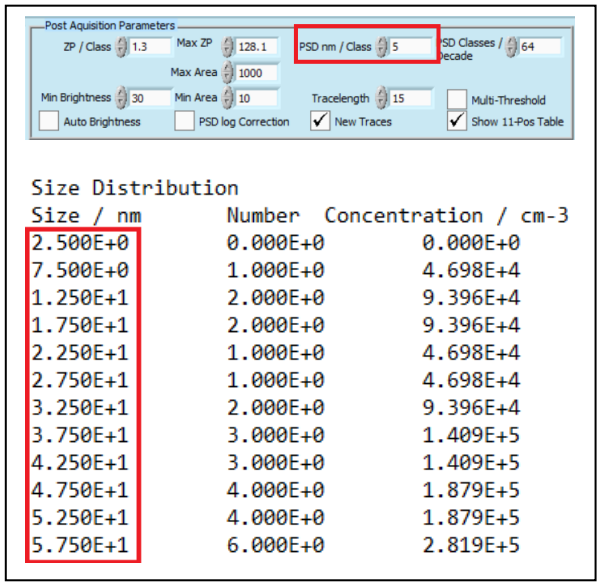
Figure 11-5: "nm / Class" and "Classes / Decade" can be adjusted in the Analysis Menu as well.

Based on the specified classes, the histograms are calculated.

It should be noted that the linear and logarithmic calculations are completely independent of each other. A histogram based on linear values cannot be converted into a histogram with logarithmic values.

11.1 Adjusting the class width

The class width can be adjusted and changed with the parameter "PSD nm / Class" for the linear value range. This function allows the user to modify and adjust the resolution of the displayed histogram according to his requirements. The smaller the class width, the higher the resolution of histogram, and the more classes are considered when displaying the histogram. In the first column "Size / nm" of the text files the corresponding values are written in scientific notation.



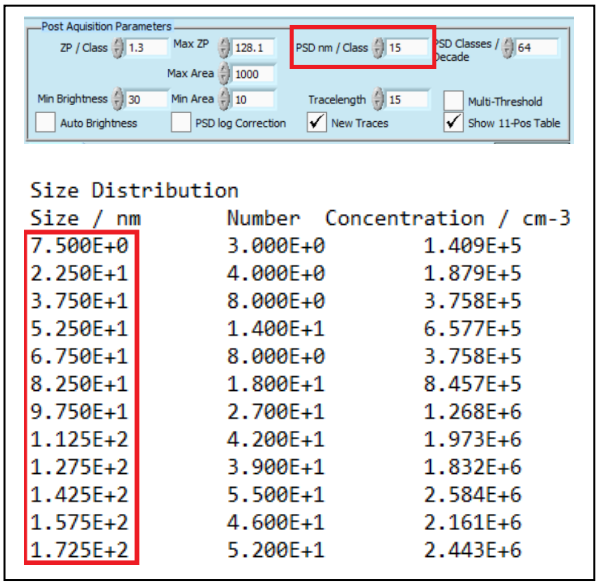


Figure 11-6: Extract from the text file, if "PSD nm / Class" were set to 5 (left) and 15 (right) in the post-acquisition parameters.

Each individual value in the "Size / nm"-column represents the center of the adjusted bin class. This is shown in the figure below.

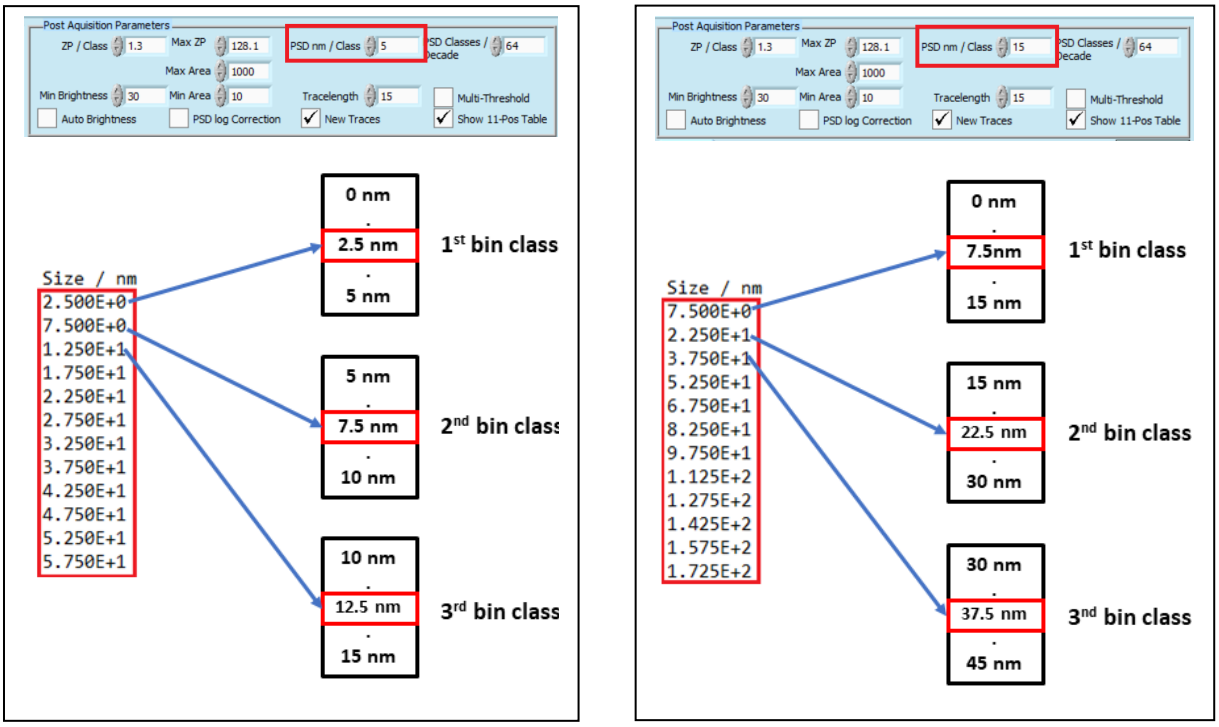


Figure 11-7: On the left side a bin class of 5 nm is adjusted. 2.500E+0 nm corresponds to the first bin class. It represents the centre of a class width ranging from 0 nm to 5 nm. 7.500E+0 nm corresponds to the second bin class, ranging from 5 nm to 10 nm, etc

The best possible histogram resolution is achieved with a bin class of 5 nm. A smaller class width is available but not recommended because the accuracy of the ZetaView® at 5 nm is maximized. For comparison, the figure below shows two histograms of the same measurement, one with class width of 5 (higher resolution) and the second with class width of 15 (lower resolution).

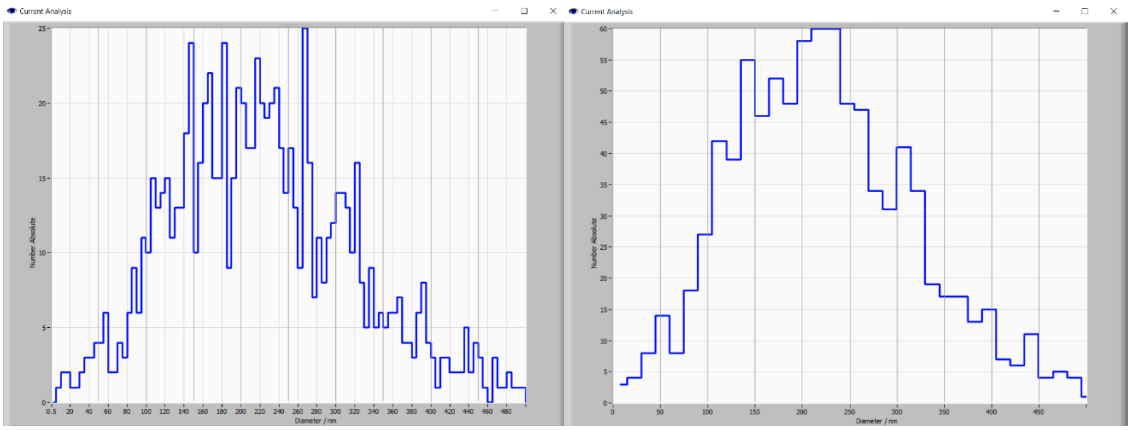


Figure 11-8: Left: Histogram created by using a bin class of 5. Right: Histogram created by using a bin class of 15. Both apply for the linear values of the text file.

It is important to note that the mode of the histograms may differs depending on how the bin classes are adjusted.

11.2 Adjusting the Classes per Decade

The number of the classes per decade can be adjusted and changed with the parameter "PSD Classes per Decade" for the logarithmic values. Like the linear values, this function allows modification and adjustment of the resolution of the displayed histogram according to the user's needs. The smaller the number of the classes per decade, the lower the histogram is resolved, and fewer classes are considered when displaying the histogram. The image below shows 2 screenshots comparing 20 classes per decade versus 64 classes per decade.

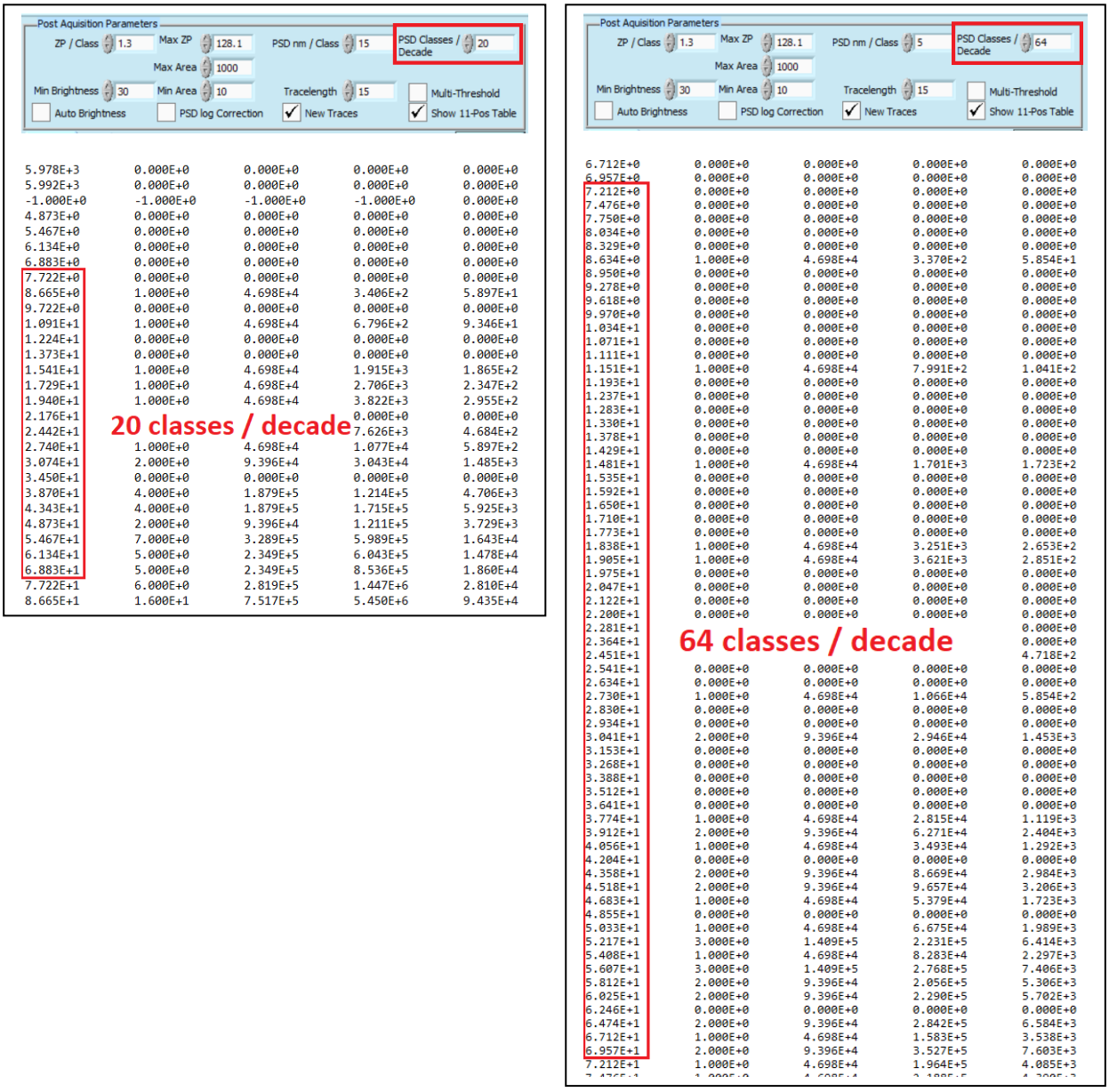


Figure 11-9: Extract from the text file, if "PSD Classes / Decade" were set to 20 (left) and 64 (right) in the post-acquisition parameters. These settings only apply to the logarithmic value range. Again, the mode of the histograms may differ depending on how many classes per decade are selected.

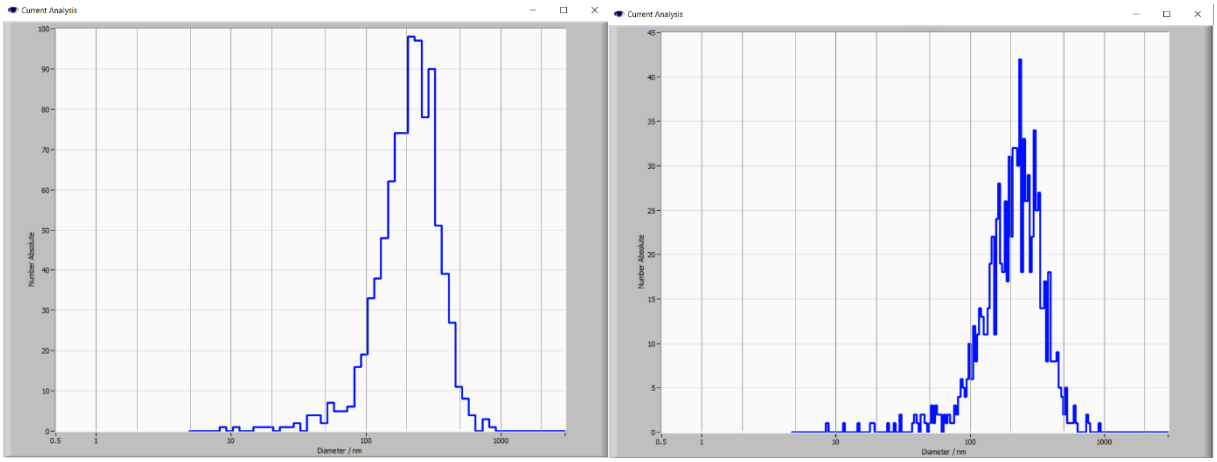


Figure 11-10: Left: Histogram created by using a 20 classes/decade. Right: Histogram created by using 64 classes per decade. Both apply for the logarithmic values of the text file.

12 Fluorescence

If light interacts with for example a molecule, the photons are transformed. In general, the wavelength of the incoming light (excitation) is transformed to light with a longer wavelength (emission). Many crystals such as $CaCO_3$ show fluorescence. Fluorescence also depends on the purity and crystalline structure. One of the most common fluorescent organic molecules is fluorescein, an orange-green powder.

In order to excite molecules, there are several preconditions to be met:

1. Electromagnetic wave of certain frequency and intensity (excitation light).
2. Molecule structure allowing transformation of the incoming electromagnetic wave (absorption and emission of light).
3. Absence of any kind of molecules absorbing the generated fluorescence (self-absorbing) or preventing fluorescence generation (quenching).

For more details on fluorescence, the reader is referred to the corresponding literature.

12.1 Fluorescence Nanoparticle Tracking Analysis (F-NTA)

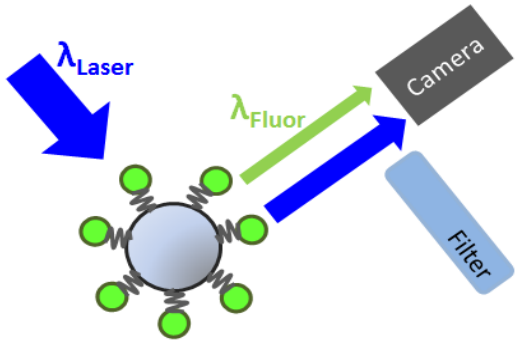
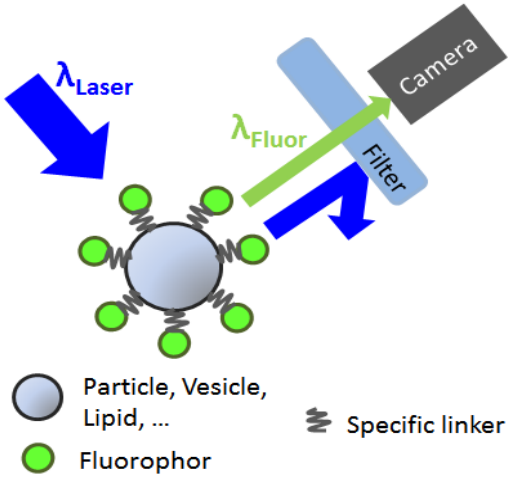
With Nanoparticle Tracking, particles can be detected, however as it is a physical principle of detection of scattered light; a chemical discrimination is not given. Precipitates from buffer, impurities or bio-nanoparticles of interest are detected with no differentiation, resulting in higher concentration levels than expected. In order to add specificity, nanoparticles such as extracellular vesicles (EVs) can be labeled with either appropriate membrane dyes or with specific fluorescently labelled antibodies directed against a particular surface protein. The ZetaView® instrument is designed in that way such that by using a filter, the scattered light coming from the laser is blocked and only the generated fluorescence light is transmitted to the camera.

Depending on the antibody and/or (membrane-) dye, non-target material can be discriminated from the bio-nanoparticles of interest. This enables NTA technology for the specific detection and quantification of nanomaterial, which is termed Fluorescence Nanoparticle Tracking Analysis, F-NTA.

12.2 F-NTA detection principle

The ZetaView® instruments works in two modes, termed “scattering mode” and “fluorescent mode”. In the scattering mode, each particle is counted, regardless of whether it is fluorescently labelled or not. In the fluorescent mode, the scattered light (same wavelength as the laser) is blocked, and only particles emitting fluorescence (usually at longer wavelengths compared to the excitation wavelength) are detected. The table below illustrates the differences in principle and what both modes detect.

Table 12.1: Scattering and Fluorescent mode.

Scattering mode (fluorescence filter is out)	**Fluorescent mode (fluorescence filter is in)**
	
Measured concentration c: $c = c_{scatter} + c_{fluor}$	Measured concentration c: $c = c_{fluor}$
Note: Both wavelengths reach the camera; scattering from laser AND fluorescence (if present). Hence the measured concentration (c) is the sum of both particle types, unlabeled particles (scattering; $c_{scatter}$) and labeled particles (the fluorescent ones; c_{fluor}). This is the total particle count.	Note: The filter blocks light with wavelengths smaller than the cut-off wavelength, which is typically 10-20 nm larger than the laser wavelength. Scattered light from unlabeled particles cannot reach the camera, only light generated by fluorescence, i.e. $\lambda_{Fluorescence} > \lambda_{Laser}$ will be transmitted. The camera only detects fluorescent particles. In real world samples, the concentration (c_{fluor}) of fluorescently labelled EVs is typically smaller than the total particle count (that is the concentration measured in scattering mode).

12.3 Selection of Fluorophores

As there is a large variety of fluorophores, the choice should be made according to:

1. Excitation wavelengths 405, 488, 520 and 640/660 nm, depending on the laser or lasers equipped in the instrument.
2. High photo stability of the fluorophore.
3. High brightness of the fluorophore (also when suspended in buffer).

The table below lists fluorophores, which have shown sufficient characteristics for detection when used with F-NTA:

Table 12.2: Fluorophores used with F-NTA, +++ = very good, ++ = good, + = sufficient.

Fluorophores	**Photostability**
Alexa488® Atto488® Atto 647®N Quantum dots (λ_{ex} = 405 nm) CMO CMG CMDR	+++
RhodamineB DIL PKH67 Phycoerythrin Neon-GFP DIO DiL	++
GFP, eGFP	+

12.4 How to switch between scatter and fluorescence mode

Switching between the scatter and fluorescence mode is always performed via the drop-down menu with all ZetaView® devices.

12.4.1 Instruments equipped with a manual fluorescence filter

Click on the drop-down menu and select the fluorescence channel. The software prompts you to insert the manual fluorescence filter.

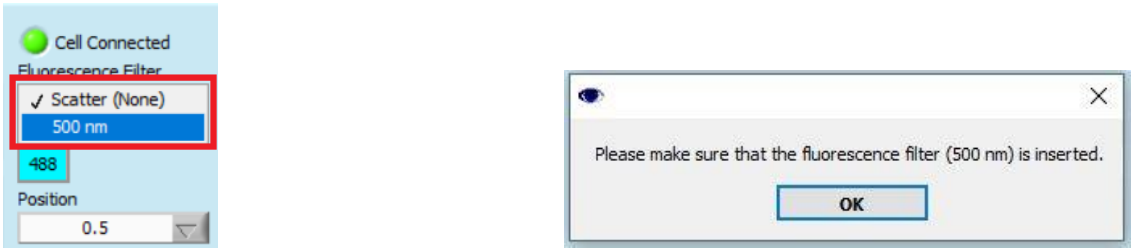


Figure 12-1: Switching from scatter- to fluorescence mode on a monolaser instrument equipped with a manual fluorescence filter. The software prompts you to insert the fluorescence filter.

Move the manual fluorescence filter up one notch. Make sure that the filter is placed in the corresponding end position as shown in the figure below (middle). The filter locks into place with a click. Do not push the filter all the way up, as this can block the microscope's optical path.

Figure 12-2: Positions of the manual fluorescence filter in scatter- (left) and in fluorescence mode (middle). Make sure not to push the filter to the top position (right), as this will block the microscope's beam path.

12.4.2 Instruments equipped with an automatic fluorescence filter changer

Click on the drop-down menu and select the fluorescence channel.

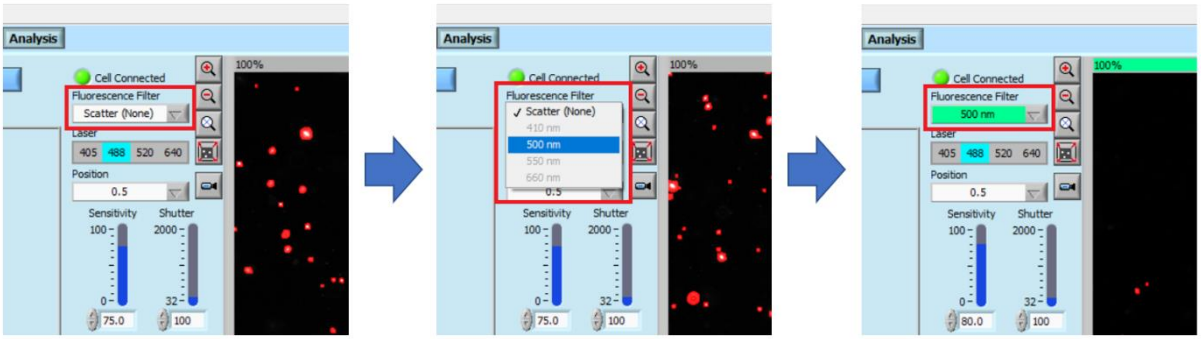


Figure 12-3: Switching between scatter mode and fluorescent mode is performed by clicking on the dropdown menu. In addition, the laser wavelength can be selected in both modes.

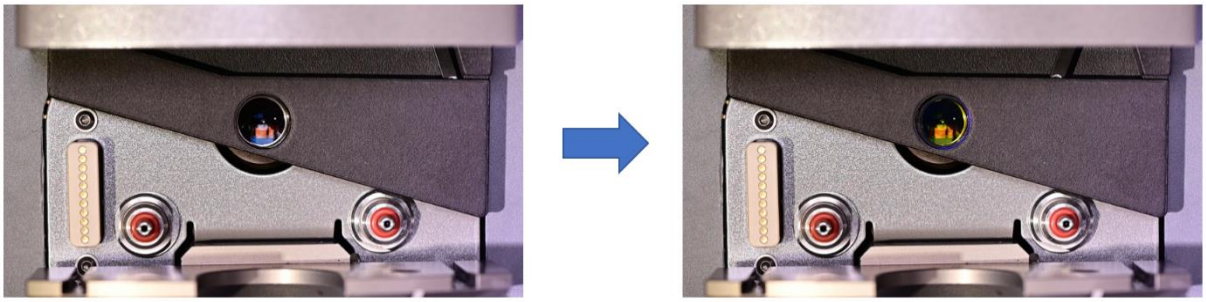


Figure 12-4: Switching from the scatter mode to the green fluorescence channel on a multilaser instrument.

Depending on the laser wavelengths the ZetaView® instrument is equipped with, the corresponding fluorescence filters are displayed.

Figure 12-5: Laser wavelengths and corresponding fluorescence filters.

Table 12.3: ZetaView® parameter settings for scatter and fluorescent mode on a ZetaView® QUATT instrument. However, these parameters can also be used for ZetaView® instruments equipped with one or two lasers. However, depending on laser power and tolerances, these parameters should be used as a guideline and may vary a bit.

	Scatter mode	Fluorescence mode			
Parameter	**YG-488**	**YG-488**	**YG-405**	**YO-520**	**DR-640**
Sensitivity	65	80	88	85	75
Shutter	100	100	200	200	300
Minimum Brightness	30	30	30	30	30
Min Area	10	10	10	10	10
Max Area	1000	1000	1000	1000	1000
Tracelength	15	15	15	15	15
Frame Rate	30	30	30	30	7.5

Note that the above settings are exemplary and can be used as a guideline to make the following descriptions easier to understand.

It is important to mention here that for TWIN and QUATT ZetaView® instruments the 488 nm laser is always considered to be the reference laser for measuring particle concentration in the scatter mode. Therefore, scatter mode parameters for the 405, 520 and 640 nm lasers are not shown.

For instruments that have only one laser (mono-laser instruments), the above statement does not apply. With mono-laser devices, the respective laser wavelength is always used for concentration measurements in scatter mode.

12.5 Guideline for concentration calibration in the fluorescence mode

Concentration calibration in the fluorescence mode is useful when a sample contains a mixture of fluorescent and non-fluorescent particles and if you want to know the content and/or the number of fluorescent and non-fluorescent particles.

Assuming a sample in which 100% of all particles are fluorescent, the measured number and concentration of the particles should be the same in the scatter mode and in the fluorescence mode. In reality, this is not often the case. By using identical measuring parameters, a higher number is usually measured in the scatter mode than in fluorescence mode. The reason for this is that the fluorescence filter represents an additional element in the ZetaView® optics. Smaller and weakly fluorescent particles are no longer displayed in the live view and are lost. To compensate for this, the sensitivity in fluorescence mode must be increased.

Concentration calibration in fluorescence mode takes this into account with the calculation of a correction factor. This correction factor can be different for different sensitivity values as well as for fluorescent dyes.

Below, typical results from an actual example are shown. Data were governed on an instrument equipped with a 488 nm laser and a 500 nm cut-off filter and fluorescent polystyrene standard YG-488.

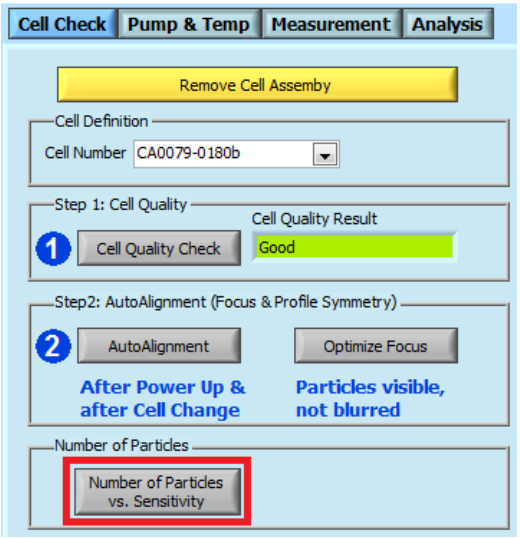
12.5.1 Prepare fluorescently labelled standard

1. Prepare the fluorescence standard (refer to the documents of the fluorescent standard beads).
2. Adjust the instrument to scattering mode (filter position “out”). Refer to section 12.4.
3. Inject the diluted fluorescence standard (from step 1).
4. Adjust the parameters of the ZetaView® instrument according to table 12.3.
5. Adjust the concentration in the scatter mode if necessary; the column “No. of Detected Particles” should be green.

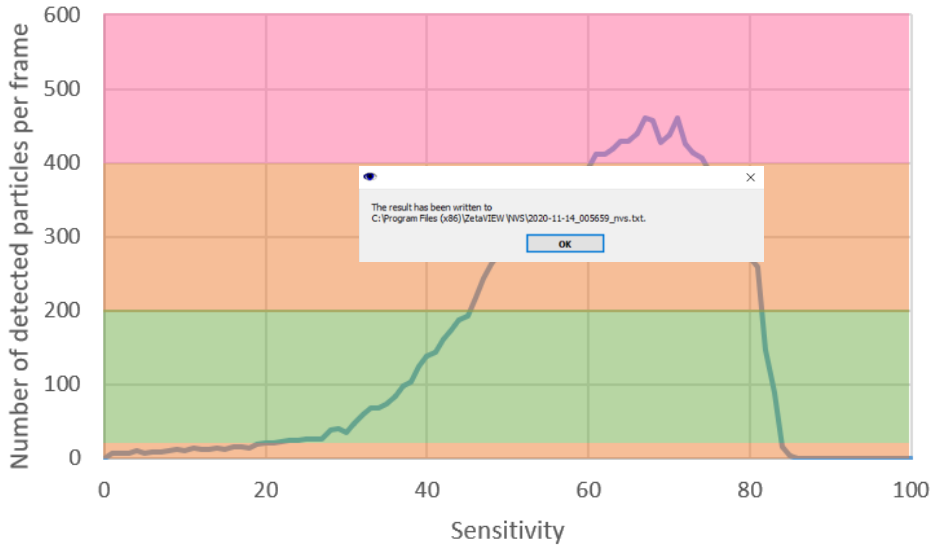
12.5.2 NvS Method

12.5.2.1 Acquire data in scatter mode (total count)

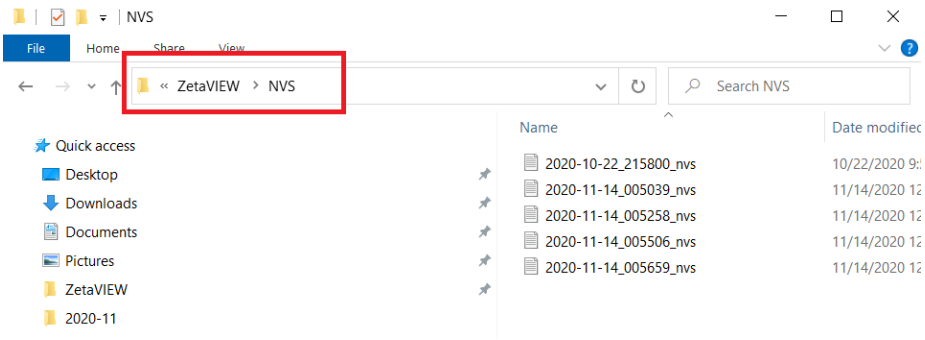
1. Click “Number of Particles vs. Sensitivity” (NvS)” (for example at position 0.5).



2. You should see a result similar to that shown below.

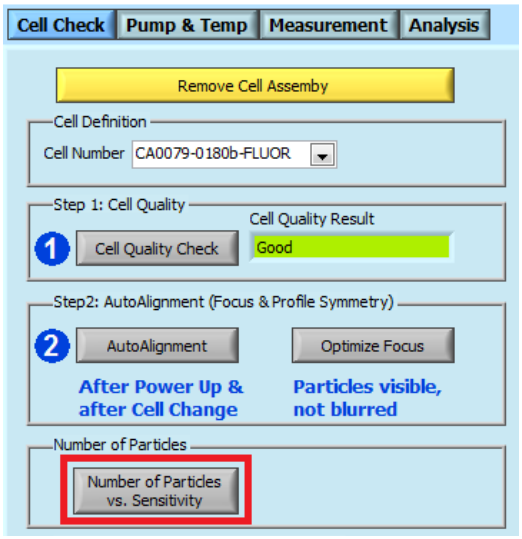


3. The result of the NvS measurement is saved in the NVS folder.

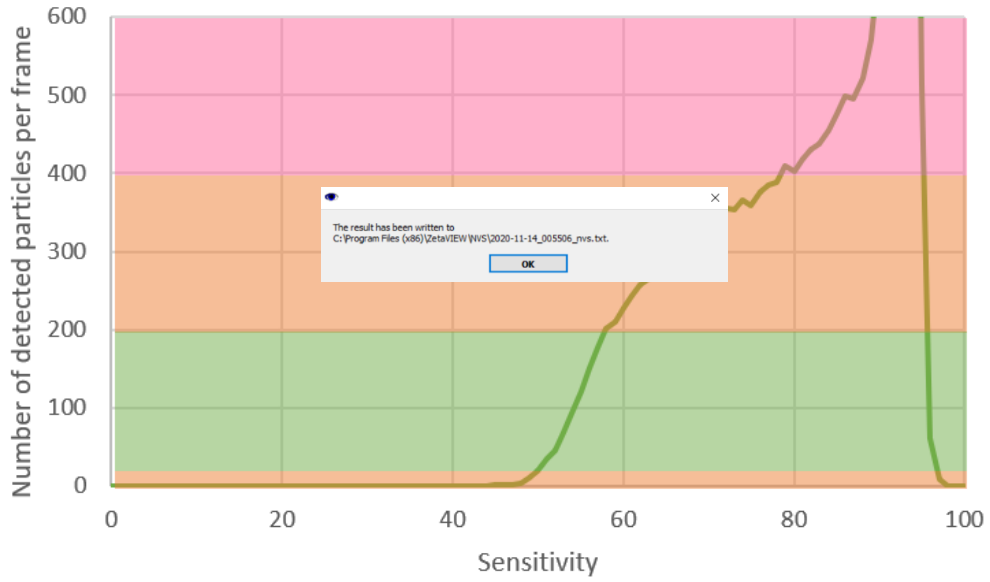


12.5.2.2 Acquire data in fluorescent mode (fluorescent count)

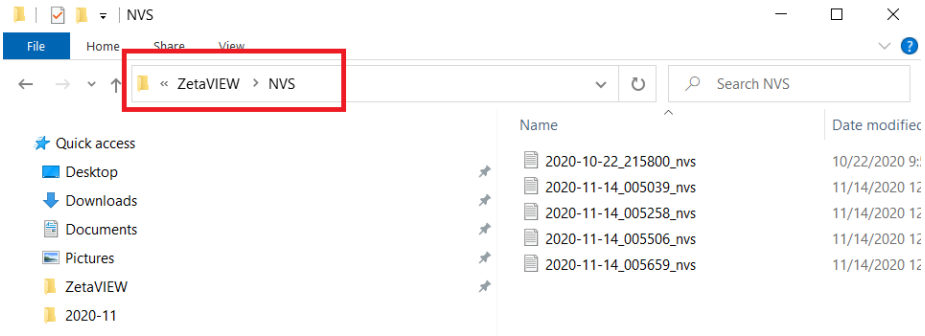
1. Keep the fluorescent standard beads in the instrument and switch to the fluorescent mode.
2. Check the focus and run “Optimize Focus” if necessary.
3. Click “Number of Particles vs. Sensitivity (NvS)” again.



4. You should see a result similar to that shown below.



5. The result of the NvS measurement is saved in the NVS folder



12.5.2.3 Calculate the relative concentration factor

Plot the data of both NvS files in a diagram. The NvS files are text files that can be easily copied into a spreadsheet software. You will obtain two curves, one for the scatter mode and one for the fluorescent mode (see an example below).

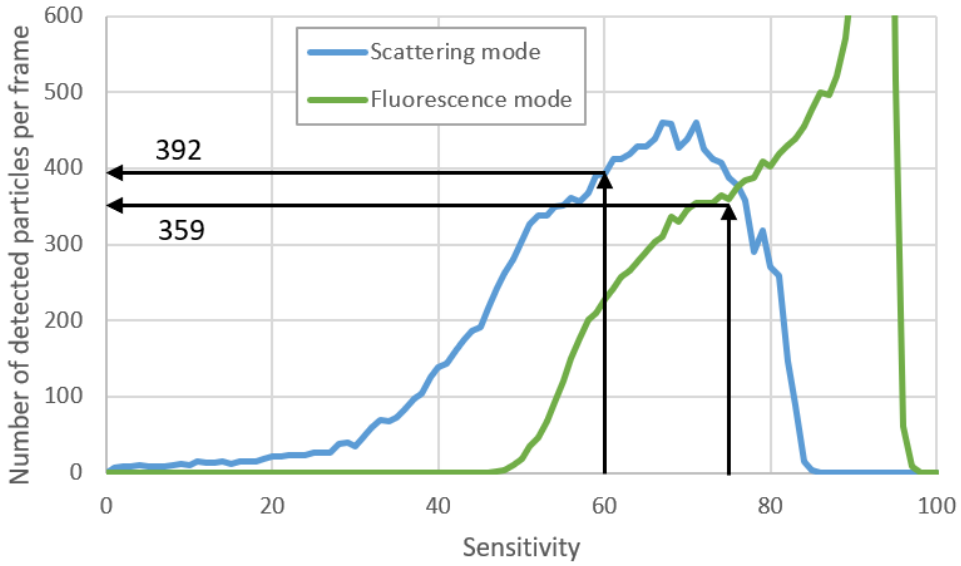


Figure 12-6: Typical result of NvS, plotted from two NvS text files (488 nm laser, YG-488 fluorescent bead standard). In this example, the sensitivity values of 100 nm fluorescent particles measured in scatter (blue) and fluorescent mode (green) were 60 and 75, respectively.

When taking the count at the sensitivity settings for scatter and fluorescent mode (figure 12-7) the count of fluorescent particles results in 392 particles in scatter mode and 359 particles in fluorescent mode. If we take 392 particles in the scatter mode to be 100% it means that in the fluorescent sample the concentration of fluorescent particles is underestimated by ≈ 8%. To calculate the concentration in fluorescent mode (c_F), the relative calibration factor K_f is introduced:

$$c_F = K_f * c_{F,measured}$$

Based on the measurements described above the correction factor (K_f) calculates to

$$K_f = \frac{n_{det}(S, sens = 60)}{n_{det}(F, sens = 75)} = \frac{392}{359} = 1.09$$

A real sample typically contains a mixture of fluorescent and non-fluorescent particles. First, find the optimum camera settings for scatter and fluorescent mode and note the sensitivity values respectively. Second, determine K_f, use the NvS data of your fluorescent standard, see procedure above. You may want to repeat the NvS measurements of a fluorescence standard from time to time (within 6-12 months or earlier). Finally, multiply the concentration value (fluorescent mode only) from your sample with the correction factor K_f (e.g. 1.09) to obtain the concentration values.

In order to avoid having to manually multiply the concentration (only in fluorescent mode) with the correction factor K_f, you can enter the correction factor in the SOP menu before the corresponding fluorescence measurement of your samples is started.

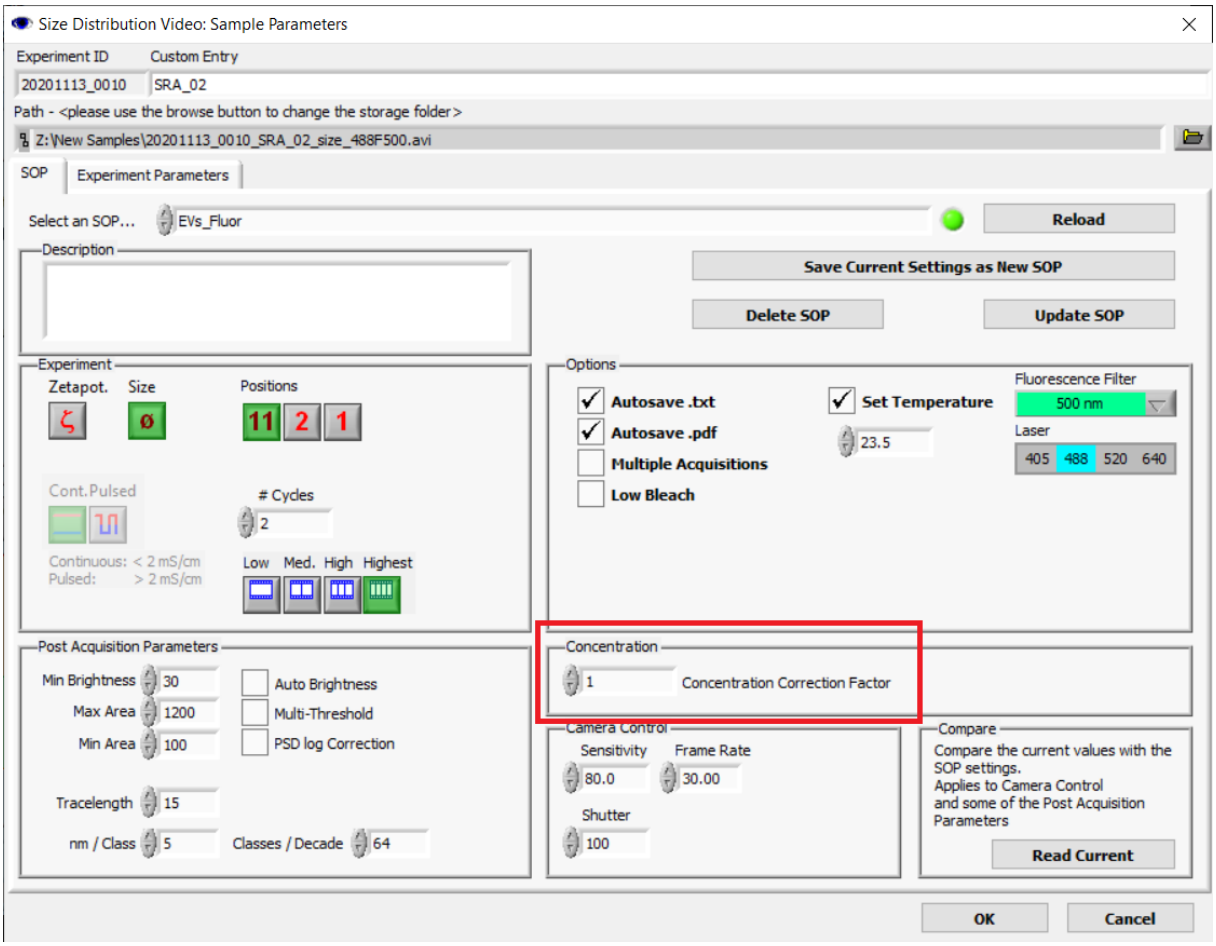


Figure 12-7: Enter the concentration correction factor.

The correction factor is then taken into account during the fluorescence measurement, so that the corrected particle concentration is obtained at the end of the measurement. A manual multiplication of the correction factor with the particle concentration obtained after the fluorescence measurement is then no longer necessary.

12.5.3 Manual method

For a full response curve, an example for the determination of the correction factor in the fluorescence mode is given. Determination is performed with fluorescence particles. The example below was conducted with a ZetaView® instrument equipped with a 488 nm laser using YG488 fluorescent particles.

In the table below **Δs** is the delta sensitivity, that is the difference of sensitivity in the scattering and fluorescence mode. **n** represents the number of detected particles (readout from ZetaView® software). The average, standard deviation, and relative standard deviation is calculated from three replicates. Please verify that the relative standard deviation is less than 15%. When your data shows larger variance, remove outliers and repeat the measurements. Finally, the correction factor for the fluorescence mode is calculated by the ratio of number of detected particles in the scatter mode n(S) and in the fluorescence mode n(F).

The table below contains data from a typical calibration procedure. Ideally, measurements should result in a delta sensitivity of 16. Then, the instrument performance in scatter mode and fluorescence mode is equal. In case delta sensitivity of 16 is not achieved, e.g. when there is background interference, reduce sensitivity to reduce background. Correct the concentration with the help of the correction factor for the delta sensitivity you are measuring. For same instrument performance, delta sensitivity is typically 15 ± 5.

Please note: the correction factor is a function of delta sensitivity and is independent from the sensitivity you measure. For EVs, you typically perform measurements in scatter mode s(S) = 80, thus the sensitivity in fluorescence s(F) = 95.

Table 12.4: Typical result for calibration of fluorescence mode.

Δs = Delta sensitivity, n_{det} = number of detected particles, K_F = correction factor, n(S) = number of detected particles in the scatter mode, n(F) = number of detected particles in the fluorescence mode

	Scatter	Fluorescence			
Sensitivity	60	65	70	75	80
Δs = sS - sF		5	10	15	20
n_{det} run 1	124	87	114	146	158
n_{det} run 2	148	83	109	120	169
n_{det} run 3	126	81	105	112	162
average	135	84	109	126	163
stdev	13	3	5	18	6
Rel. stdev	10%	4%	4%	14%	3%
Correction factor K_F = n(S)/n(F)		1.61	1.23	1.07	0.83

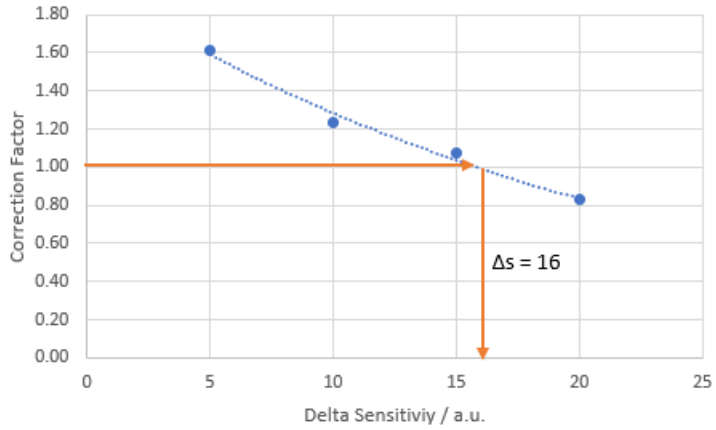


Figure 12-8: Graphical representation of the correction factor as a function of delta sensitivity from the table above.

12.5.4 Worked example

Worked example for fluorescently labeled sample:

1. Sample diluted and measured in scatter mode at sensitivity 80 *n*(sens=80, scattering) = 300
2. Sample measured at same dilution in fluorescence mode at sensitivity 85 n(sens=85, fluorescence) = 66, Δs = 5
3. Determine correction factor with manual method (see section 12.5.3). From table above K_F = 1.61
4. Correct concentration and/or count of fluorescence mode: 66 * 1.61 = 72
5. Calculate ratio of the fluorescent particles in the sample:

$$fluorescent\ ratio = \frac{n_F * K_F}{n_S} = \frac{72 * 1.61}{300} x100\% = 35\%$$

12.5.5 Assumptions

For optimum results, the properties of fluorescent standard beads and sample should be as close as possible. The correction of fluorescence concentration in the above example was performed under the assumption that the properties of the fluorescence calibration beads are similar to the sample. In detail, the assumptions are as follows:

1. Size of fluorescence standard (100 nm) and sample (e.g. 60 – 200 nm) is similar.

2. The fluorescent dye on standard and sample is similar (ideally same). Excitation and emission properties should be as close as possible.

3. For optimum results, the dyes should be photostable to avoid effects of bleaching, i.e. reduction of concentration over measurement time. However, due to the fast acquisition (<1 sec. per position), even dyes showing bleaching may be used.

4. The coverage of sample particles with fluorescent molecules is sufficiently high to avoid quenching and bleaching effects. For particle detection, the number of fluorescence photons per second per particle needs to be sufficiently high.

12.6 Low Bleach

The “Low Bleach” function is suitable for the measurement of fluorescent dyes that tend to quickly bleach under strong laser light. Excessive bleaching of fluorescent dyes, e.g. those which are conjugated to biological particles (e.g. extracellular vesicles) for example by antibodies, can lead to a major problem when measuring the particle concentration. If the duration of the bleaching process is shorter than the duration of the video recording at a measuring position, the concentration of the fluorescent particles is underestimated. The exposure of the particles to the laser should therefore be as short as possible. In addition, several measurements of the same sample should measure particles that have not previously been exposed to the laser.

The “Low Bleach” function takes these considerations into account. It can therefore reduce, but not prevent, the effects of fluorescence bleaching. In the ZetaView® instrument, bleaching is reduced by an optimized laser control, in combination with the “Dose Sub Volume” function. “Low Bleach” can be operated either with or without the "Multiple Acquisitions" function.

12.6.1 “Low Bleach” without “Multiple Acquisitions”

After activating “Low Bleach”, the "Dose Sub Volume" function is always activated at the same time. With “Dose Sub Volume” you select the pump that is connected to the reservoir that contains the liquid that is also used for the dilution of the sample particles. Then the volume for the "Dose Sub Volume" function can be adjusted. An example is shown in the screenshot below.

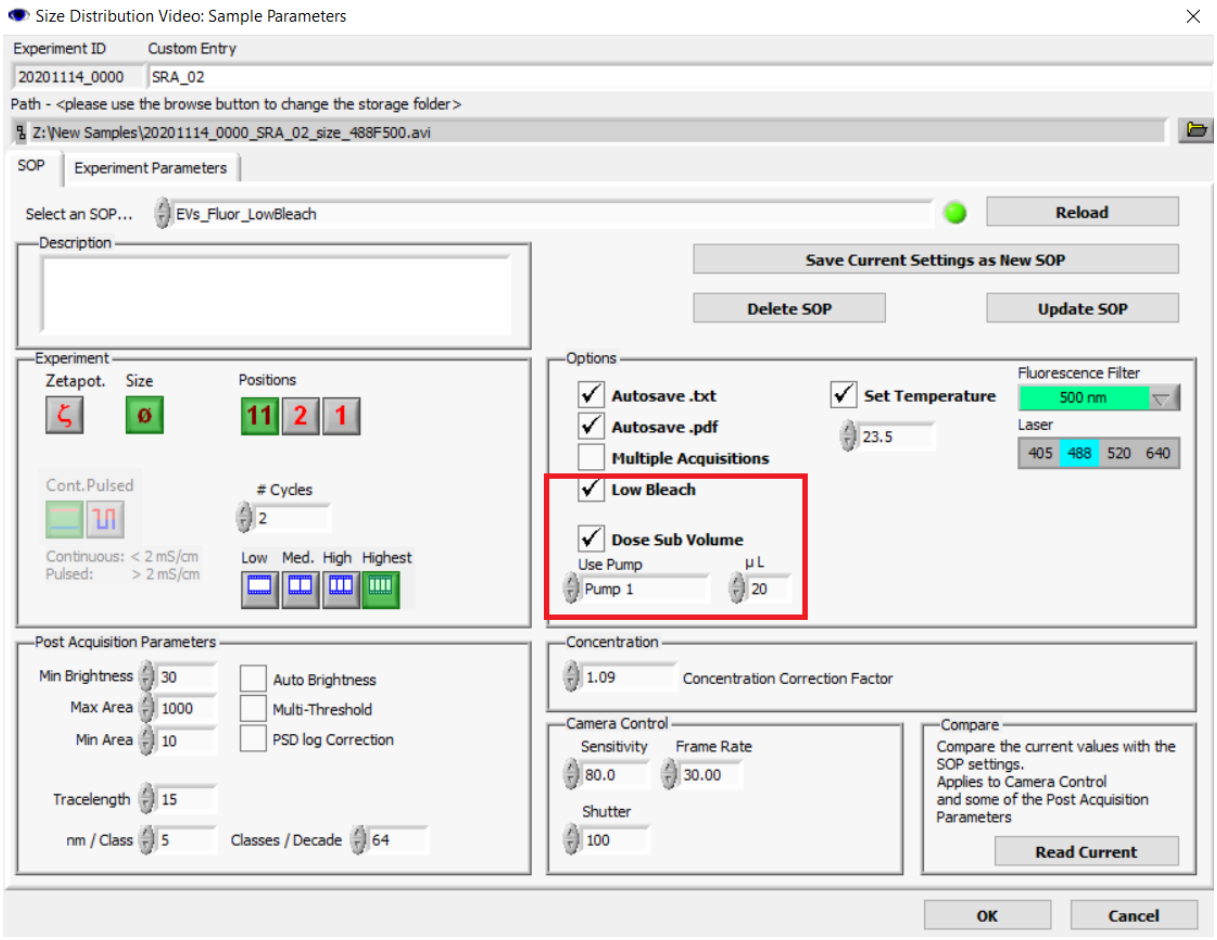


Figure 12-9: By activating "Low Bleach" function, "Dose Sub Volume" is also activated by default. You need to adjust the volume by which the particles are moved through the measuring cell immediately before the actual measurement starts in order to move unbleached particles in the field of view.

After confirming the measurement with OK, the selected pump is used to move the sample by the adjusted volume (here: 20µl) through the measuring cell. This brings particles into the field of view that were not previously exposed to the laser. The measurement starts immediately afterwards. In combination with an optimized laser control, bleaching of the fluorescence can be reduced in this way.

12.6.2 “Low Bleach” combined with “Multiple Acquisitions”

The "Low Bleach” function" can be expanded with the "Multiple Acquisitions" function (see section 7.2). The corresponding settings are shown in the figure below as an example.

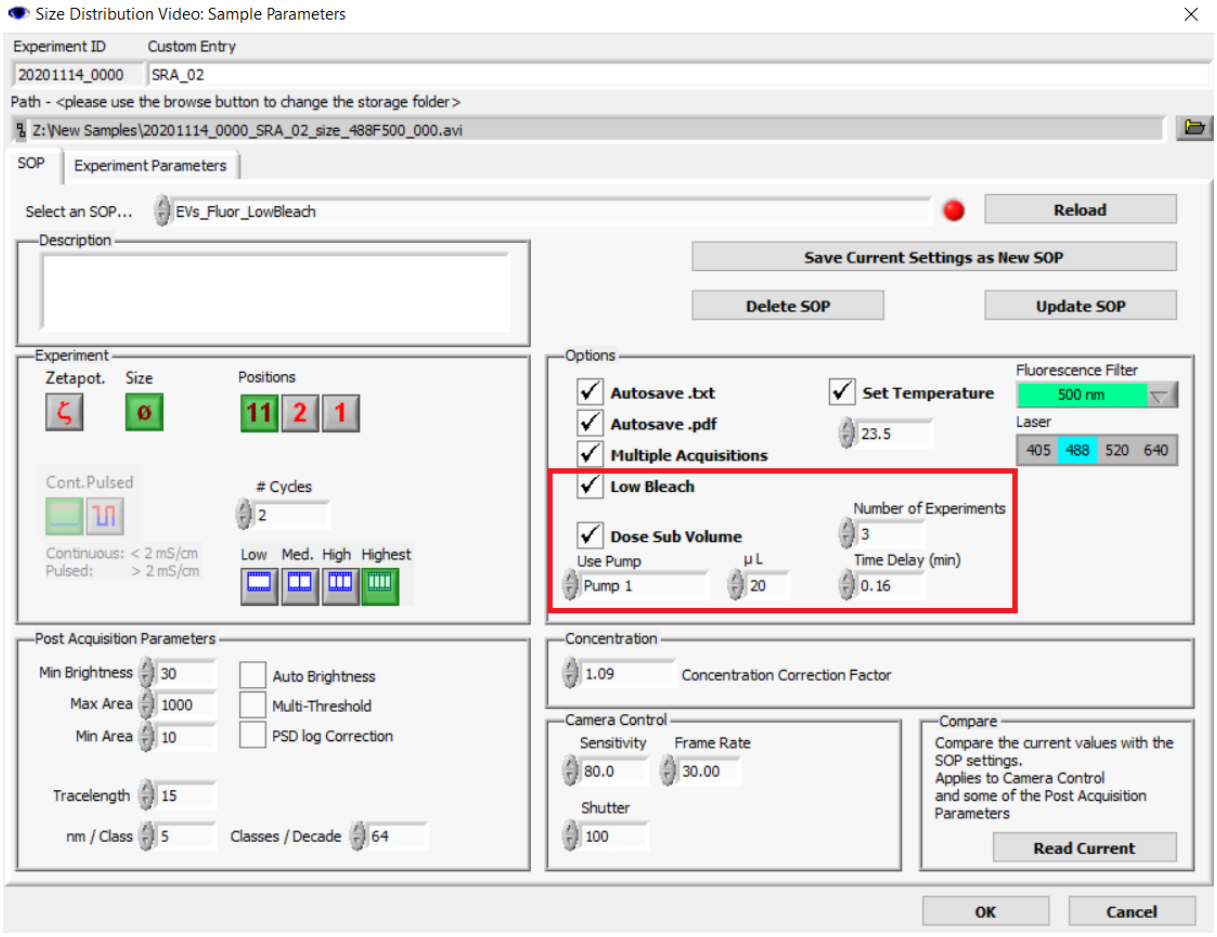


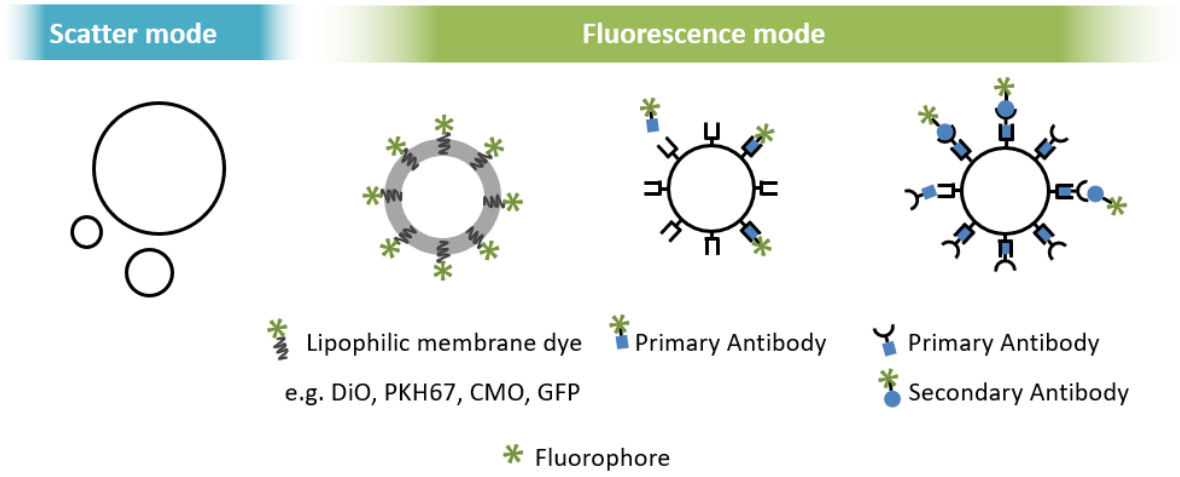
Figure 12-10: Activated “Low Bleach” function combined with "Multiple Acquisitions".

As already described above, the adjusted volume is first moved through the measuring cell immediately prior to the start of a measurement. The ZetaView® instrument pump moves the sample forward by the adjusted volume (here 20µl) with the selected pump1 through the measuring cell. This brings particles into the field of view that were not previously exposed to the laser. Immediately afterwards, the first of three measurements starts.

As already described in section 7.2, after a pause (here 0.16 minutes = around 10 seconds) the sample is moved by a further 20µl after the first measurement in order to move new particles into the field of view for the subsequent second measurement.

Between the individual measurements, new particles are always brought into the field of view by the adjusted volume. This prevents particles that have already bleached from being measured again in a subsequent measurement. At the same time, measurement of "fresh" and unbleached particles in the same sample significantly increases the statistics of the analysis.

12.7 Strategies for labeling



1. Lipophilic membrane dye

   The dye molecules (organic) interact with vesicles, in most cases the dye intercalates into the membrane. Example: Cell Mask membrane dyes.

2. Directly labelled Antibody

   On the antibody, a dye molecule (e.g. Alexa®Fluor488) is directly covalently bound. The antibody attaches specifically on the antigen. Example: Tetraspanin detection CD9/63/81.

3. Secondary Antibody

   An antibody directed against another antibody that already binds to an antigen. The secondary antibody carries a fluorophore.

4. Surface chemistry (not shown)

   In some cases, instead of antibodies a fluorophore is directly linked to the surface.

Wash and no-wash strategies:

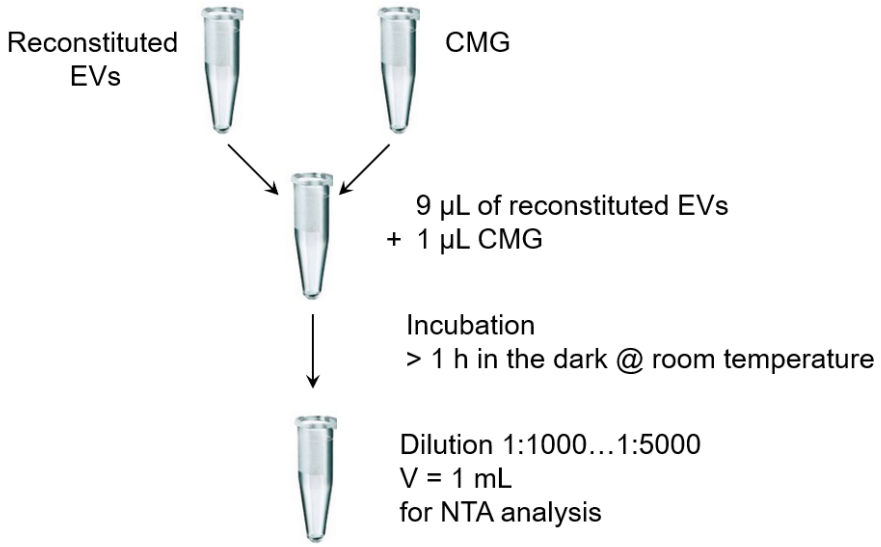
Protocols here are exemplary performed in PBS. When using other buffer, check for background and perform all dilutions in same buffer. The protocols should serve as a starting point for staining of customer samples.

12.8 Protocol for staining of EVs with Cell Mask Green (CMG) for 488 nm laser

1. Needed material
   - 0.5 – 10 µL and 100 – 1000 µL pipette including corresponding pipette tips
   - Lyophilized or fresh EVs
   - 1 µL aliquot of CMG
   - Practically particle free PBS or another customized buffer
   - Centrifuge and Vortex
   - Gloves
   - Microcentrifuge caps

2. Scatter measurement for control
   - For reconstituted EVs, expect dilution factor 1:1000 …. 1:5000, e.g. start with dilution of 1 µl reconstituted EV solution in 999 µl of PBS
   - Measure with the ZetaView® in scatter mode
     sensitivity: 80 - 85; shutter: 100 - 150, Min Brightness: 30; Min Area: 10; Max Area: 1000
     Or use predefined SOP

3. Staining of EVs with CMG



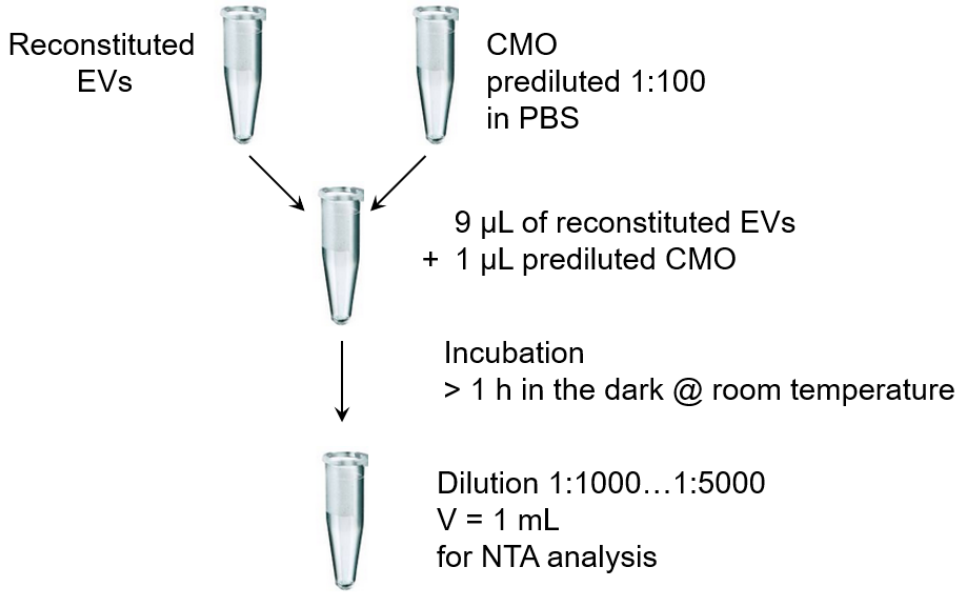
4. Fluorescence measurement of stained EVs
   - Measure with the ZetaView® in fluorescence mode
     sensitivity: 85 - 95; shutter: 100 - 150, Min Brightness: 30; Min Area: 10; Max Area: 1000 or use predefined SOP

12.9 Protocol for staining of EVs with Cell Mask Orange (CMO) for 520 nm laser

1. Needed material
   - 0.5 – 10 µL and 100 – 1000 µL pipette including corresponding pipette tips
   - Lyophilized or fresh EVs
   - 1 µL aliquot of CMO
   - Practically particle free PBS or another customized buffer
   - Centrifuge and Vortex
   - Gloves
   - Microcentrifuge caps

2. Scatter measurement for control
   - For reconstituted EVs, expect dilution factor 1:1000 …. 1:5000, e.g. start with dilution of 1 µl reconstituted EV solution in 999 µl of PBS
   - Measure with the ZetaView® in scatter mode
     sensitivity: 80 - 85; shutter: 100 - 150, Min Brightness: 30; Min Area: 10; Max Area: 1000
     Or use predefined SOP

3. Staining of EVs with CMO
   - Predilute CMO 1:100: add 1 µL of CMO to 99 µL of PBS



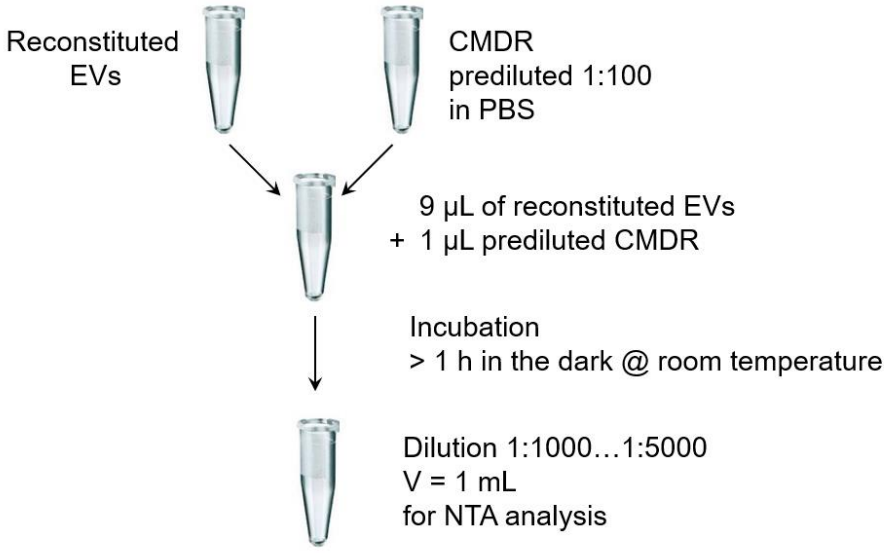
4. Fluorescence measurement of stained EVs
   - Measure with the ZetaView® in fluorescence mode
     sensitivity: 85 - 95; shutter: 100 - 150, Min Brightness: 30; Min Area: 10; Max Area: 1000 or use predefined SOP

12.10 Protocol for staining of EVs with Cell Mask Deep Red (CMDR) for 640 nm laser

1. Needed material
   - 0.5 – 10 µL and 100 – 1000 µL pipette including corresponding pipette tips
   - Lyophilized or fresh EVs
   - 1 µL aliquot of CMDR
   - Practically particle free PBS or another customized buffer
   - Centrifuge and Vortex
   - Gloves
   - Microcentrifuge caps

2. Scatter measurement for control
   - For reconstituted EVs, expect dilution factor 1:1000 …. 1:5000, e.g. start with dilution of 1 µl reconstituted EV solution in 999 µl of PBS
   - Measure with the ZetaView® in scatter mode using 488 nm laser
     sensitivity: 80 - 85; shutter: 100 - 150, Min Brightness: 30; Min Area: 10; Max Area: 1000
     Or use predefined SOP

3. Staining of EVs with CMDR
   - predilute CMDR 1:100: add 1 µL of CMDR to 99 µL of PBS



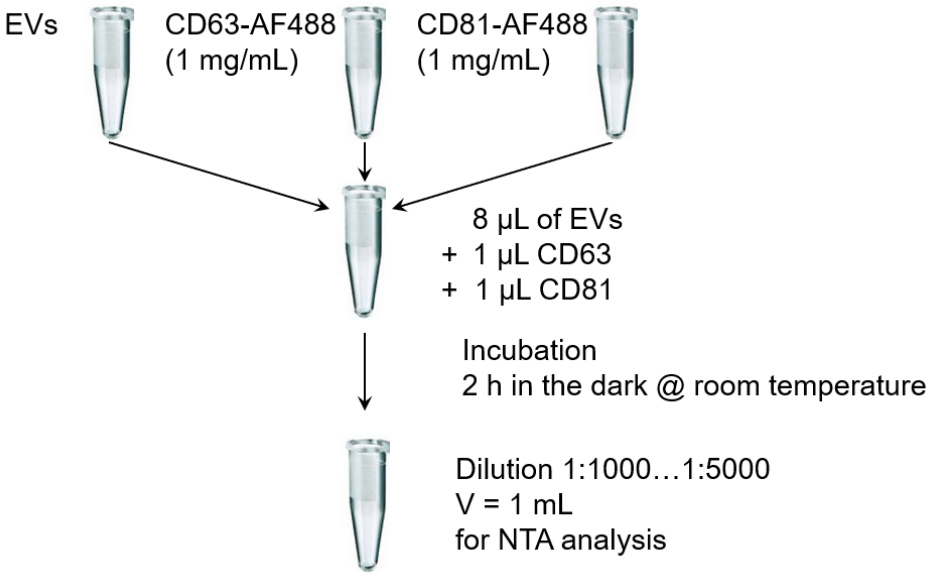
4. Fluorescence measurement of stained EVs
   - Measure with the ZetaView® in fluorescence mode
     sensitivity: 85 - 95; shutter: 100 - 150, Min Brightness: 30; Min Area: 10; Max Area: 1000
     Or use predefined SOP

12.11 Protocol for staining of EVs with Antibody-Mix

1. Needed material for staining:
   - 0.5 – 10 µL and 100 – 1000 µL pipette including corresponding pipette tips
   - Lyophilized or fresh EVs
   - 1 µL aliquot of CD63-AF488 antibody (clone MEM-259 at 1 µg/µl)
   - 1 µL aliquot of CD81-AF488 antibody
   - Practically particle free ddH_2O, PBS or another customized buffer
   - Centrifuge and Vortex
   - Gloves and Microcentrifuge caps

2. Scatter measurement for control
   - For reconstituted EVs, expect dilution factor 1:1000 …. 1:5000, e.g. start with dilution of 1 µl reconstituted EV solution in 999 µl of PBS
   - Measure with the ZetaView® in scatter mode using 488 nm laser sensitivity: 80 - 85; shutter: 100 - 150, Min Brightness: 30; Min Area: 10; Max Area: 1000
     Or use predefined SOP

3. Staining of EVs with antibody mix



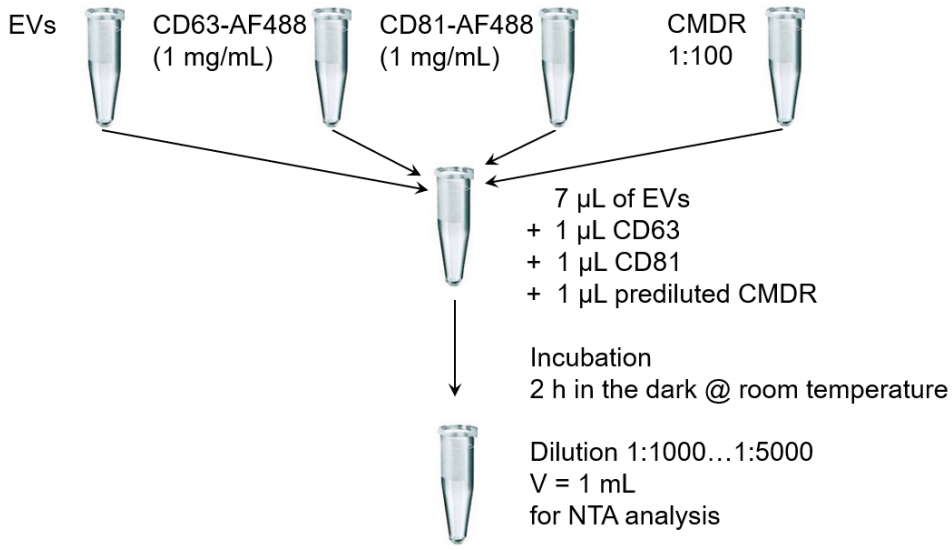
4. Fluorescence measurement of stained EVs
   - Measure with the ZetaView® fluorescence mode sensitivity: 85 - 95; shutter: 50 - 150, Min Brightness: 20; Min Area: 10; Max Area: 1000

12.12 Protocol for double staining of EVs (488/640 TWIN instrument)

1. Needed material for staining:
   - 0.5 – 10 µL and 100 – 1000 µL pipette including corresponding pipette tips
   - Lyophilized or fresh EVs
   - 1 µL aliquot of CMDR
   - 1 µL aliquot of CD63-Alexa488 antibody (clone MEM-259 at 1 µg/µl)
   - 1 µL aliquot of CD81-AF488 antibody
   - Practically particle free ddH_2O, PBS or another customized buffer
   - Centrifuge and Vortex
   - Gloves and Microcentrifuge caps

2. Scatter measurement for control
   - For reconstituted EVs, expect dilution factor 1:1000 …. 1:5000, e.g. start with dilution of 1 µl reconstituted EV solution in 999 µl of PBS
   - Measure with the ZetaView® in scatter mode
     sensitivity: 80 - 85; shutter: 100 - 150, Min Brightness: 30; Min Area: 10; Max Area: 1000
     Or use predefined SOP

3. Staining of EVs with Ab-mix and CMDR



4. Fluorescence measurement of stained EVs

   - Set 488 nm laser
   - Set fluorescence mode (sensitivity: 85 - 95; shutter: 50 - 150, Min Brightness: 20; Min Area: 10; Max Area: 1000) to measure the signal of the antibodies
   - Change laser to 640 nm, set fluorescence mode to measure signal of membrane dye

13 Zeta potential measurement

The zeta potential (ZP) of a sample is determined in the ZetaView® instrument by measuring the electrophoretic mobility (Mb) of particle migration in an electric field. The electrical conductivity of the buffer in which the particles are suspended plays a decisive role as well. The electrophoretic mobility μ_e of each individual particle is calculated via the particle migration v in the applied electric field *E*. The zeta potential, that is considered to be the surface charge of each individual particle, is then calculated using the Helmholtz-Smoluchowski equation.

$$\mu_e = \frac{v}{E} \qquad \zeta = \frac{4\pi\eta}{\varepsilon} f(\kappa a) \cdot \mu_e$$

μ_e = Electrophoretic mobility; v = Velocity of particle in *E*-field; E = Electrical field
ζ = Zeta potential; η = Viscosity of medium; ε = Dielectric constant; $f(\kappa a)$ = Debye function

Figure 13-1: Electrophoretic mobility (left) and Helmholtz-Smoluchowski equation (right).

Zeta potential of a sample can be measured in all 11 measuring positions as profile (prof) or in two positions (stationary layers; SL). A profile measurement is recommended when the sample is unknown and/or heterogeneous. However, stationary layer (SL) measurements result in a higher resolution of the zeta potential distribution and is therefore highly recommended.

In general, before samples are measured for the zeta potential, a zeta potential symmetry correction of the measuring cell should be performed. The symmetry correction is integrated in a fully automated way for the convenience of the user either by performing "AutoAlignment" using polystyrene 100 nm beads (see section 6.2.2) or after one single profile (11-positions) zeta potential measurement. Since the "AutoAlignment" has been already described before, the 11-position zeta potential measurement is described below:

13.1 Procedure for performing a zeta potential profile measurement

1. Power on the instrument
2. Perform daily start up procedure (see chapter 5)
3. Set temperature control if necessary
4. Fill in polystyrene 100 nm beads
5. Switch to “Measurement” and adjust camera parameters or select the SOP “ZP_PS100 nm”
6. Start acquisition with “OK”
7. Wait until acquisition and analysis is done
8. Switch to “Analysis” to obtain the results

Please note that no “Overlay” or “Multiple Acquisitions” functions are activated.

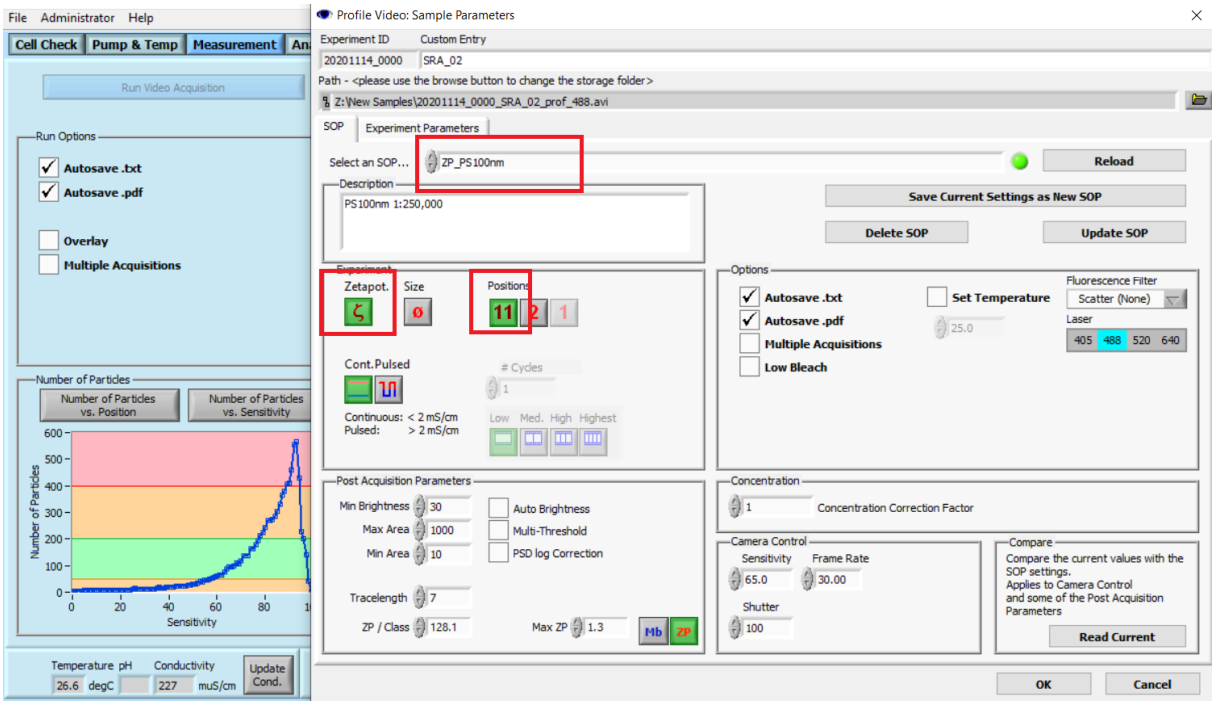


Figure 13-2: Zeta potential profile measurement (11-positions) for symmetry correction. The SOP “ZP_PS100 nm” has been selected for this purpose.

The symmetry correction can also be executed by clicking on the button “Symmetry Correction” in the Analysis tab after a profile measurement (parabola) has been done.

In some cases, an error message may occur after clicking “Symmetry correction”. In this case, a symmetry correction has already been performed by the instrument. Therefore, there is no need to execute an additional one.

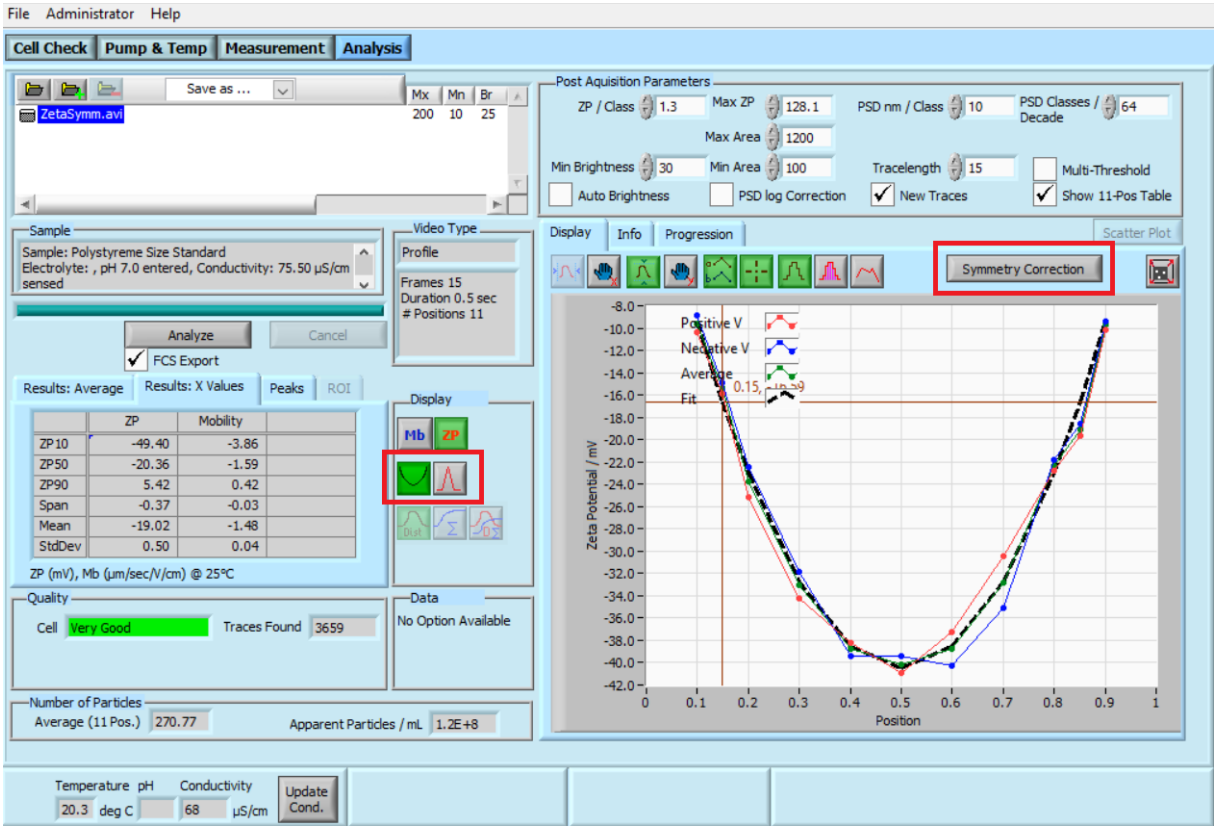


Figure 13-3: Symmetry correction can be performed by clicking the “Symmetry Correction”-button after having completed a zeta potential profile measurement. The result can be presented as a histogram by changing from the parabola button to the distribution button.

13.2 SL-Measurement

As already mentioned above, a zeta potential measurement in the stationary layers (SL) results in a higher resolution compared to a profile measurement. Furthermore, the measurement parameters, such as the number of cycles and the number of video frames (Low, Med., High, Highest) can be more flexibly adjusted in an SL-measurement, compared to a profile measurement.

An SL-measurement is basically carried out in the same way as a profile measurement but at 2 specially designated positions, namely SL1 (that corresponds to position 0.1495) and SL2 (position 0.8505). Depending on the conductivity of the liquid in which the particles are suspended, an SL measurement can be carried out in two different modes.

1. The **continuous** mode should be used if the conductivity of the buffer does not exceed 2mS/cm (milli siemens per centimetre).
2. The **pulsed** mode is an extended mode for ensuring zeta potential measurements at high conductivities. If the conductivity is higher than 2mS/cm. Measurements in continuous mode with high conductivity may lead to incorrect results.

   An example of the settings for an SL measurement is shown in the figure below.

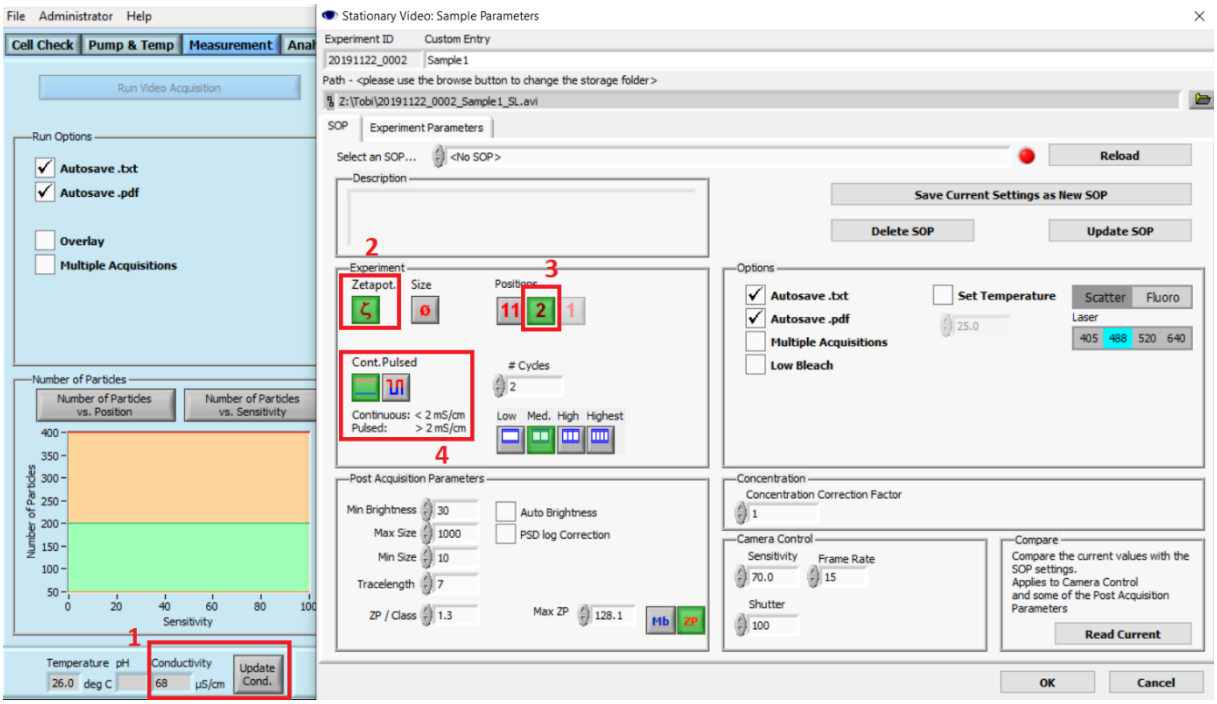


Figure 13-4: SL-zeta potential measurement. The conductivity of the sample buffer can be checked (1) with the button "Update Cond.". Based on that result, either the continuous or the pulsed mode (4) must be adjusted accordingly for the subsequent measurement.

Depending on the sensed conductivity, the selection of continuous or pulsed mode is made automatically, so a manual selection is not necessary.

After an SL-zeta potential measurement, the ZetaView® software can display the result of the measurement as a histogram of the electrophoretic mobility (Mb) or zeta potential (ZP). Both histograms are very similar and differ only by the zeta potential factor (ZP Factor). An example is shown in the figure below.

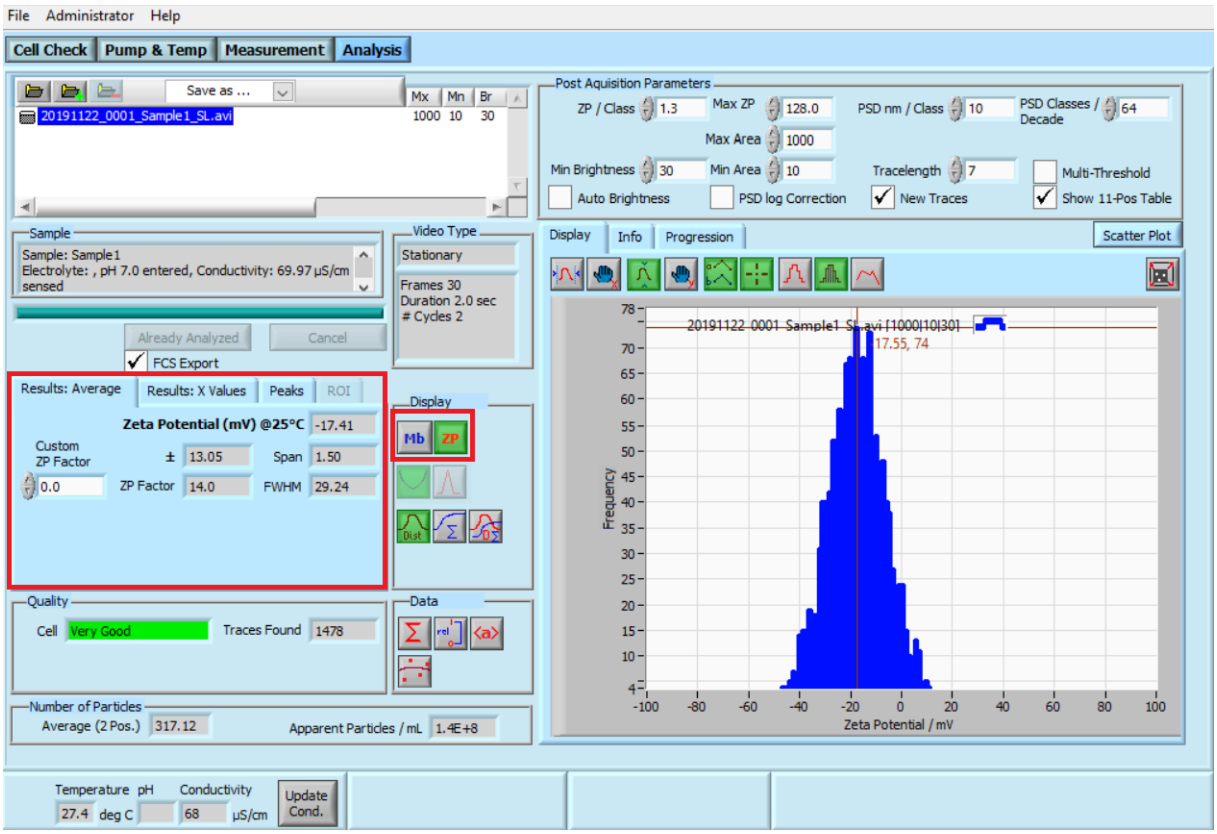


Figure 13-5: Example of a zeta potential distribution after an SL-measurement. On the left side the Average values, the X Values and peaks (big red frame on the left) are shown. The histogram of the zeta potential measurement is shown on the right. The small red frame indicates where to switch between mobility (Mb) presentation and zeta potential (ZP) presentation. The corresponding buttons are then marked in the ZetaView® software.

After an SL-measurement has been performed, the zeta potential, reported in millivolts [mV] and averaged over all histogram values, is reported in the tab "Results: Average" The zeta potential is based on a temperature of 25°C. The “ZP factor” (zeta potential factor) is a multiplicator used to convert the electrophoretic mobility (Mb) into the zeta potential. The “ZP Factor” depends on the temperature and viscosity of the liquid and usually varies between 12 and 14. The standard deviation (+/-), the span and FWHM is reported in the “Results: Average” tab as well.

The X-values (percentiles) and the detected peaks of the histogram can be displayed in the tabs “Results: X Values” and “Peaks”.

Figure 13-6: Left: Percentiles (X-Values) of an SL measurement. Right: Peaks of the histogram of an SL-measurement.

As with size measurements, zeta potential measurements can be plotted in a distributive and cumulative manner. Histograms of zeta potential measurements can also be summed, normalized, averaged and smoothed. The procedure is identical to that for size measurements. This is also true for the presentation of the zeta potential histograms in a step plot, bar plot and line plot as well as for scaling of X- and Y-axis. For more information, the reader is referred to the chapter 10.

13.3 Re-analysis of an existing zeta potential measurement

The ZetaView® software can re-analyze an existing zeta potential measurement with altered post-acquisition parameters. The precondition for this is that the corresponding video file of this measurement is still available, since it contains all raw data for a new analysis. If there is no video file, a re-analysis is not possible.

13.3.1 Load a video file for re-analysis

For a re-analysis, the video file of any desired measurement can be (re-) loaded in the Analysis tab via the button "Load Files / Analyses (Clear List)". The procedure is the same to that described for re-analysis of a size measurement (ref. section 10.7).

Depending on if an 11-position measurement (profile) or a stationary layer measurement (SL) is to be re-analysed, it is important to pay attention to the extension "prof" or “SL” in the file name when re-analyzing a zeta potential measurement. Here, the procedure for an SL-measurement is shown in the figure below, but it also applies for a profile measurement.

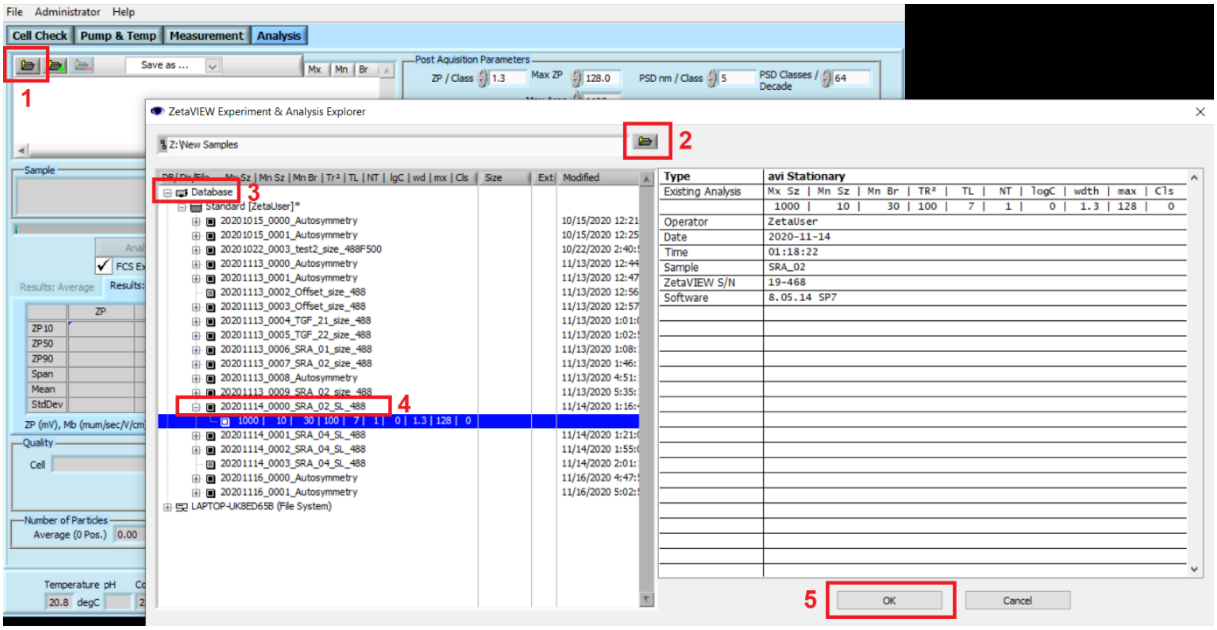


Figure 13-7: Loading the video of an existing zeta potential measurement.

1. Click on "Load Files / Analyzes (Clear List)"
2. Browse to the desired storage folder
3. Open the database
4. Select the desired measurement for (re-) analysis. The sub-item highlighted in blue in this sample already contains parameters from the first analysis
5. Load the histogram by clicking “OK”

As with a size measurement (ref. section 10.7.1), you can either load an already existing analysis that was previously analyzed with the post-acquisition parameters set at the time, or you can start a new re-analysis with the currently set post-acquisition parameters.

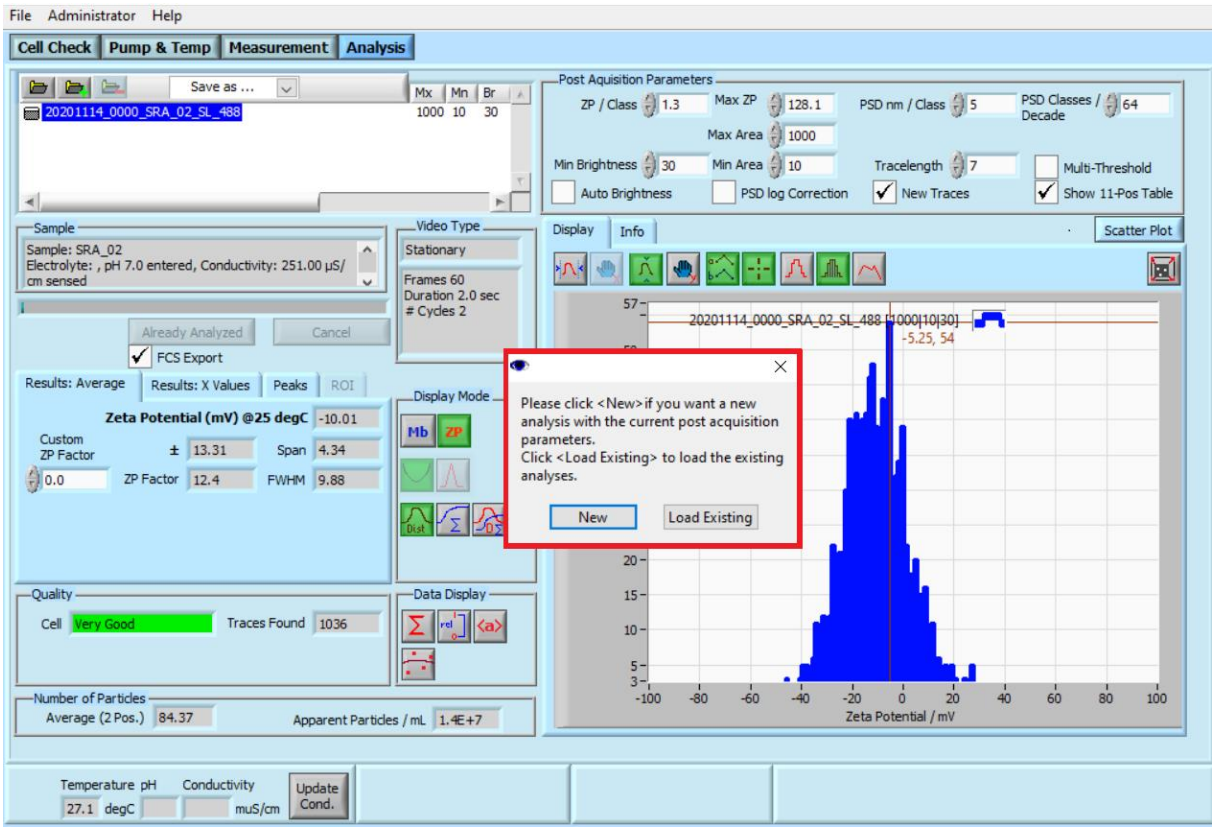


Figure 13-8: You can select whether an existing analysis is to be loaded or a new analysis should be carried out with the current post-acquisition parameters.

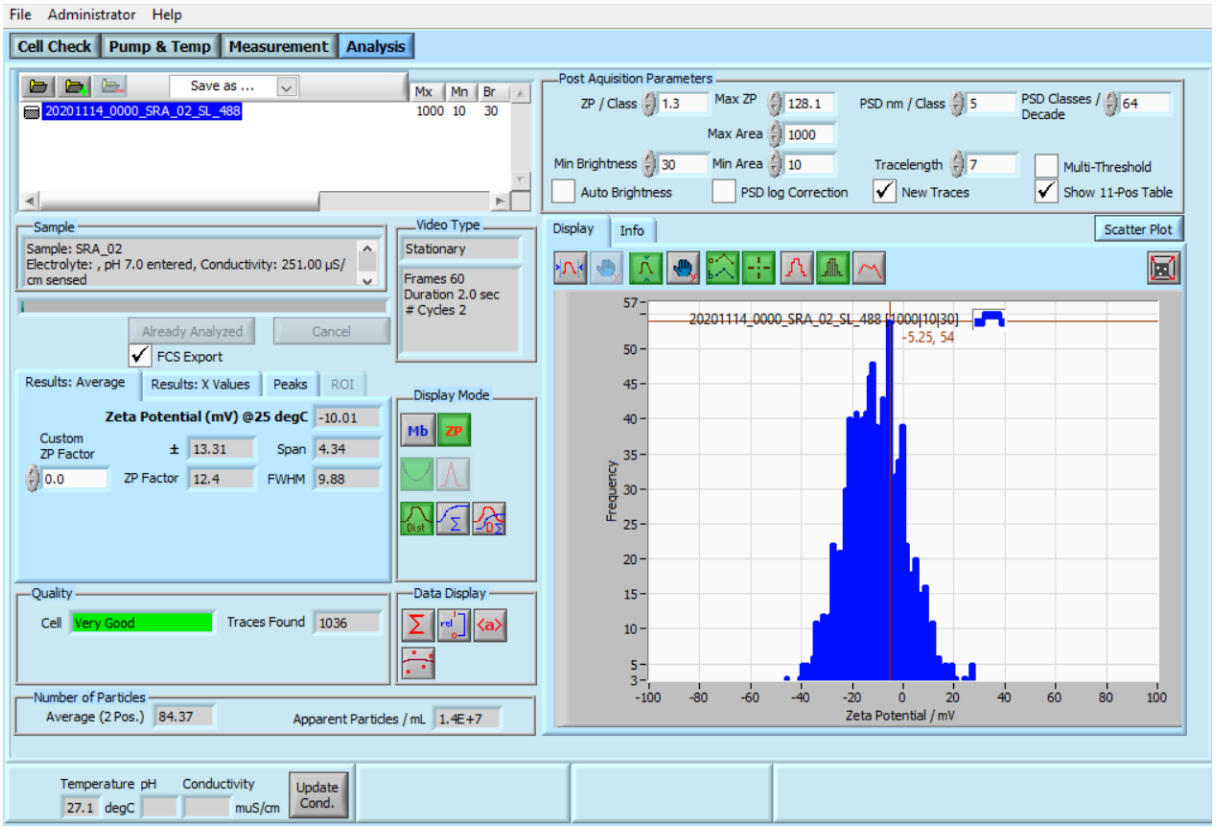


Figure 13-9: An already existing analysis has been loaded.

All newly performed analyzes are saved and displayed as sub-items under the corresponding sample / measurement name. If a measurement has been re-analyzed several times with different post-acquisition parameters, all analyzes performed appear as a compilation of the post-acquisition parameters used. An example of a measurement that was re-analyzed three times with different parameters is shown below.

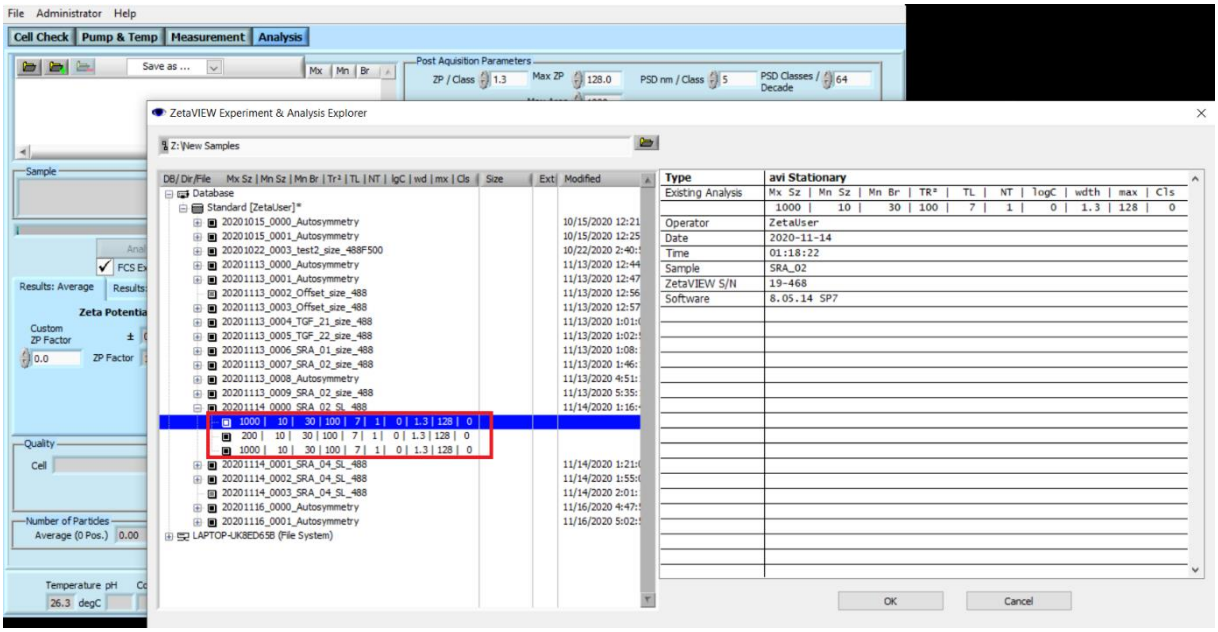


Figure 13-10: 3 analyzes of the measurement "20201114_0000_SRA_02_SL_488" are highlighted in red. The post-acquisition parameters used are listed in each of the three lines. The currently selected analysis is highlighted in blue.

Please note that it is not possible to display an SL-measurement and a profile measurement at the same time in the same diagram. Likewise, it is just as impossible to display any zeta potential measurement (SL or profile) simultaneously with a size measurement in the diagram due to different scaling of the x-axis.

13.3.2 Re-analysis of a zeta potential measurement that has just been carried out

Starting from an already existing or (re-) loaded histogram (video file), the post-acquisition parameters can now be changed according to the requirements of the operator. However, “PSD nm/Class” and “PSD Classes/Decade” cannot be changed as they are related to size measurements. In the example below, the “Max Area” was decreased from 1000 to 200 in order exclude large and high scattering particles in the new analysis. However, additional post-acquisition parameters such as “ZP / Class”, Max ZP (see sections 13.7 and 13.8) or Tracelength (ref. section 8.3) can be changed simultaneously and re-analysed afterwards as well.

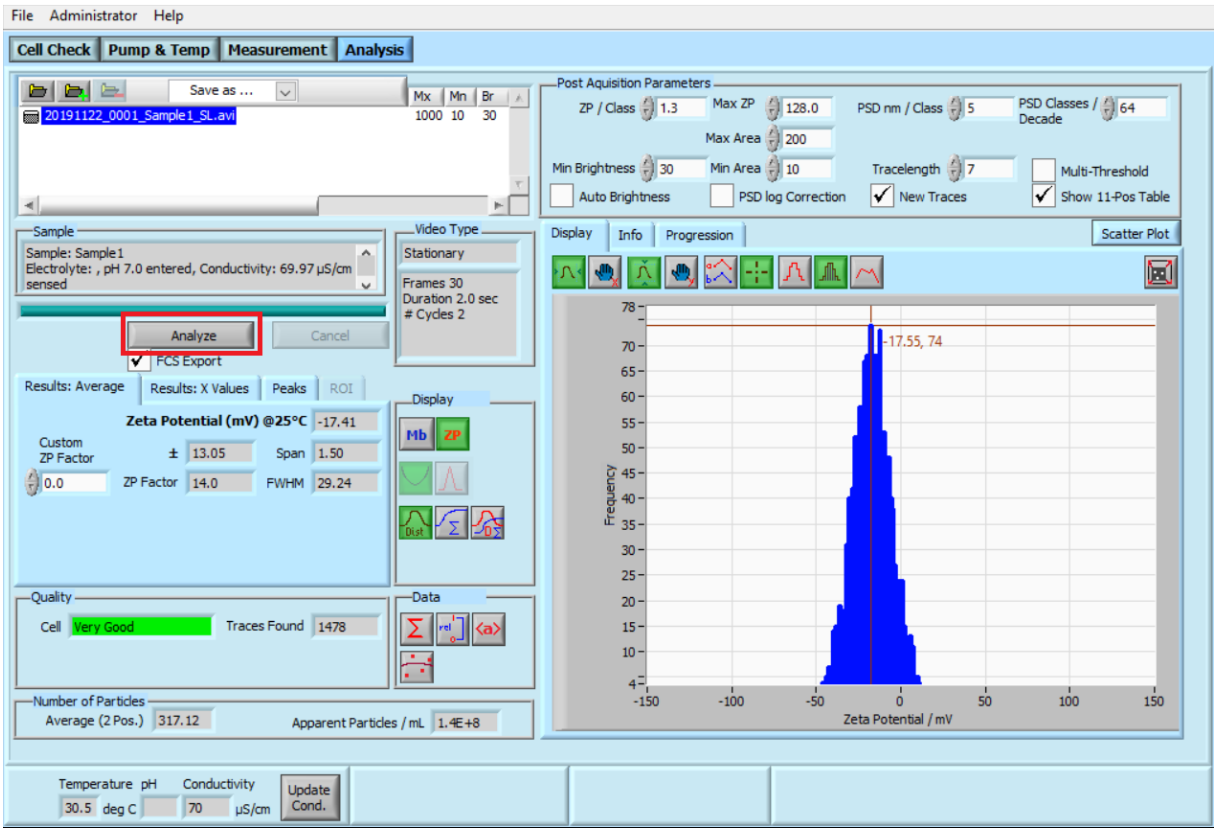


Figure 13-11: In this histogram, the “Max Area” has been decreased from 1000 to 200. After changing at least one post-acquisition parameter, the new analysis can be started by clicking the "Analyze" button highlighted in red. However, it is also possible to change more than one post-acquisition parameter simultaneously for re-analysis.

The duration of the re-analysis depends on which post-acquisition parameters have been changed and how much they have been changed.

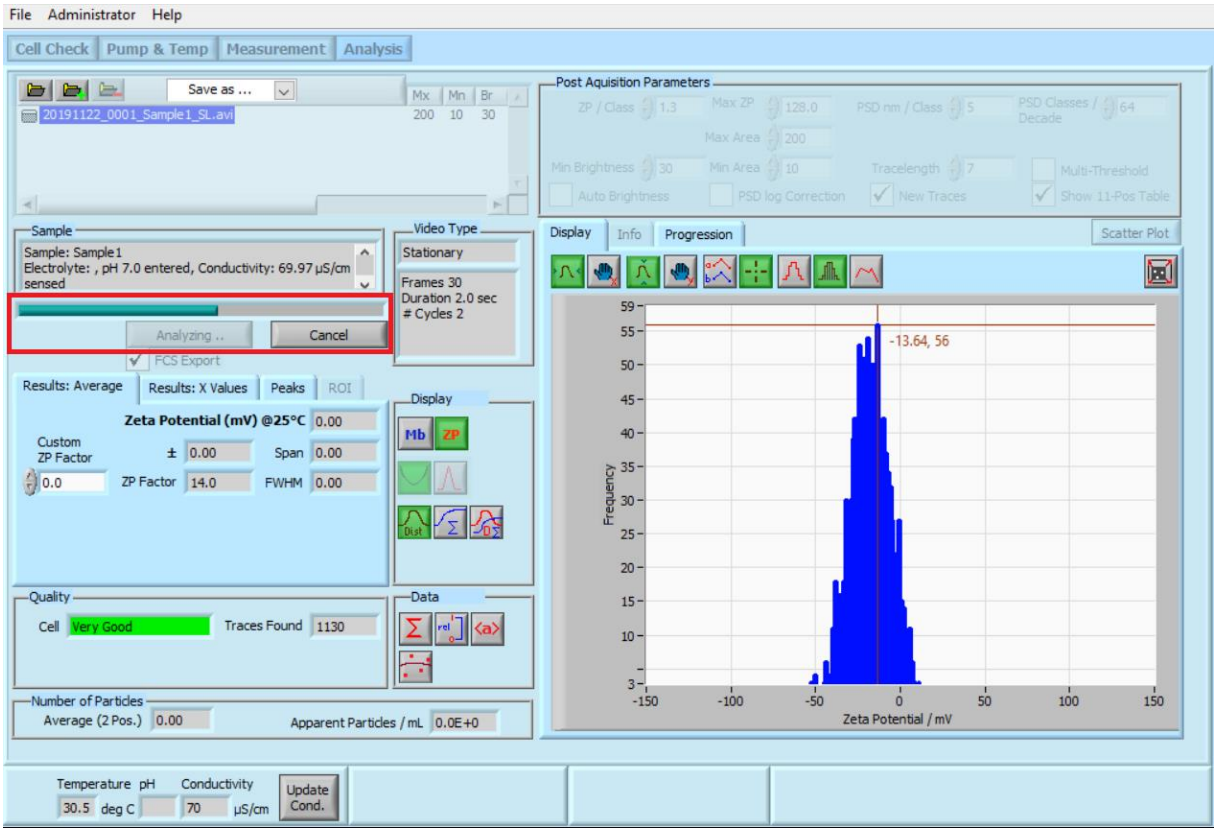


Figure 13-12: The re-analysis is indicated by the progress bar, it can also be stopped at any time.

Since a completed re-analysis is not automatically saved as a pdf report or as a text file, it is strongly recommended to save it manually. This is also important because the X values, peaks and all other values in the Analysis Menu have changed after a re-analysis.

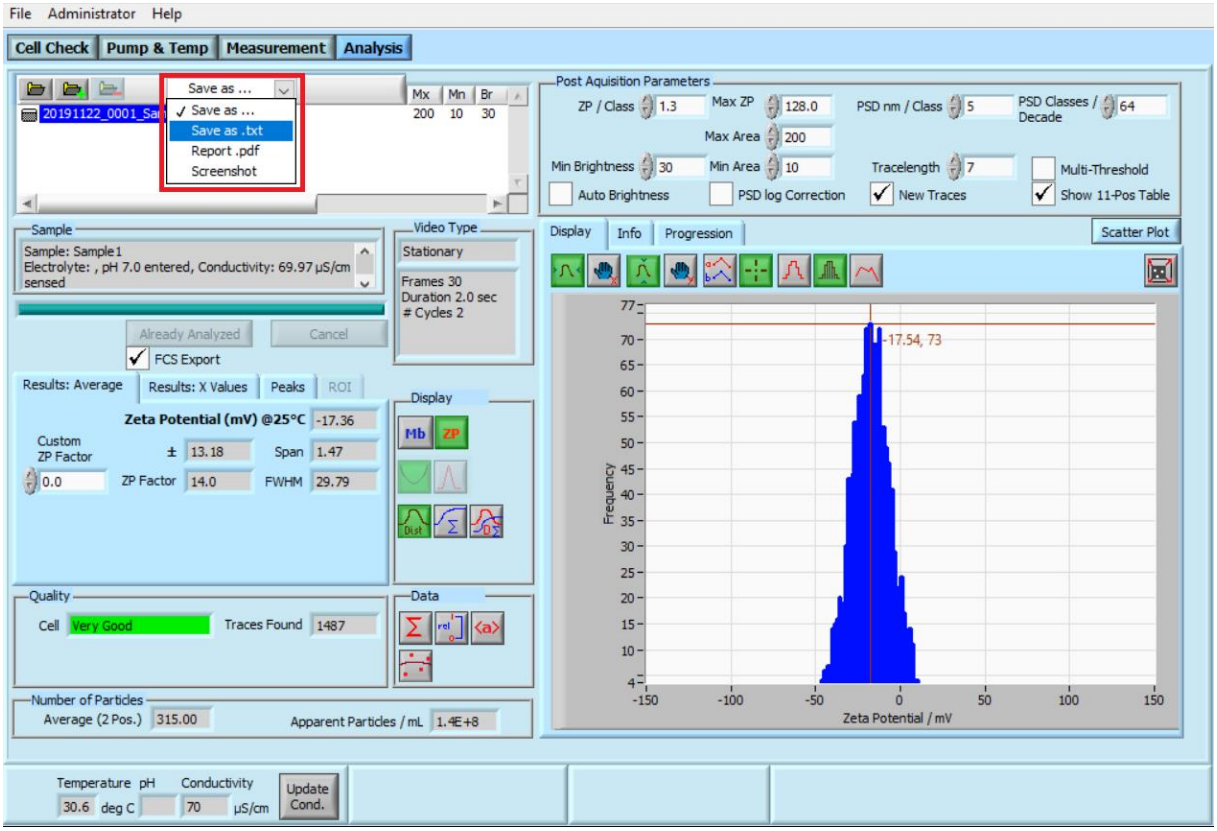


Figure 13-13: Manual saving of the re-analysis of a zeta potential measurement.

13.4 Scatter Plot of an SL zeta potential measurement

By clicking on "scatter plot" at the top right of the histogram, the zeta potential measurement can also be displayed as a scatter plot. For this, an FCS file is required. The precondition for displaying the scatter plot immediately is that "FCS-Export" was switched on in the Analysis Menu before the corresponding measurement has started.

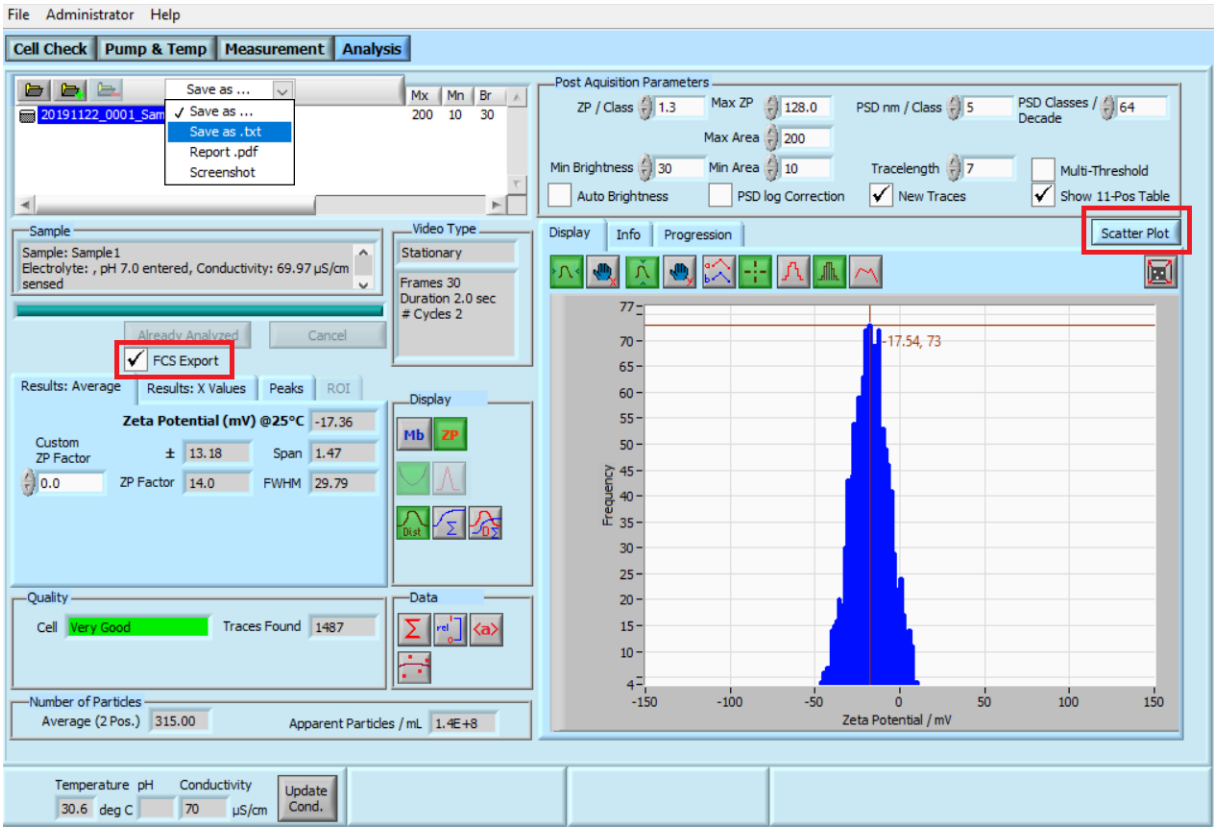


Figure 13-14: The scatter plot can be accessed by clicking the corresponding button right above the histogram. It is important that the "FCS Export" was switched on before the corresponding measurement has started.

An example image of a scatter plot after an SL-measurement is shown below.

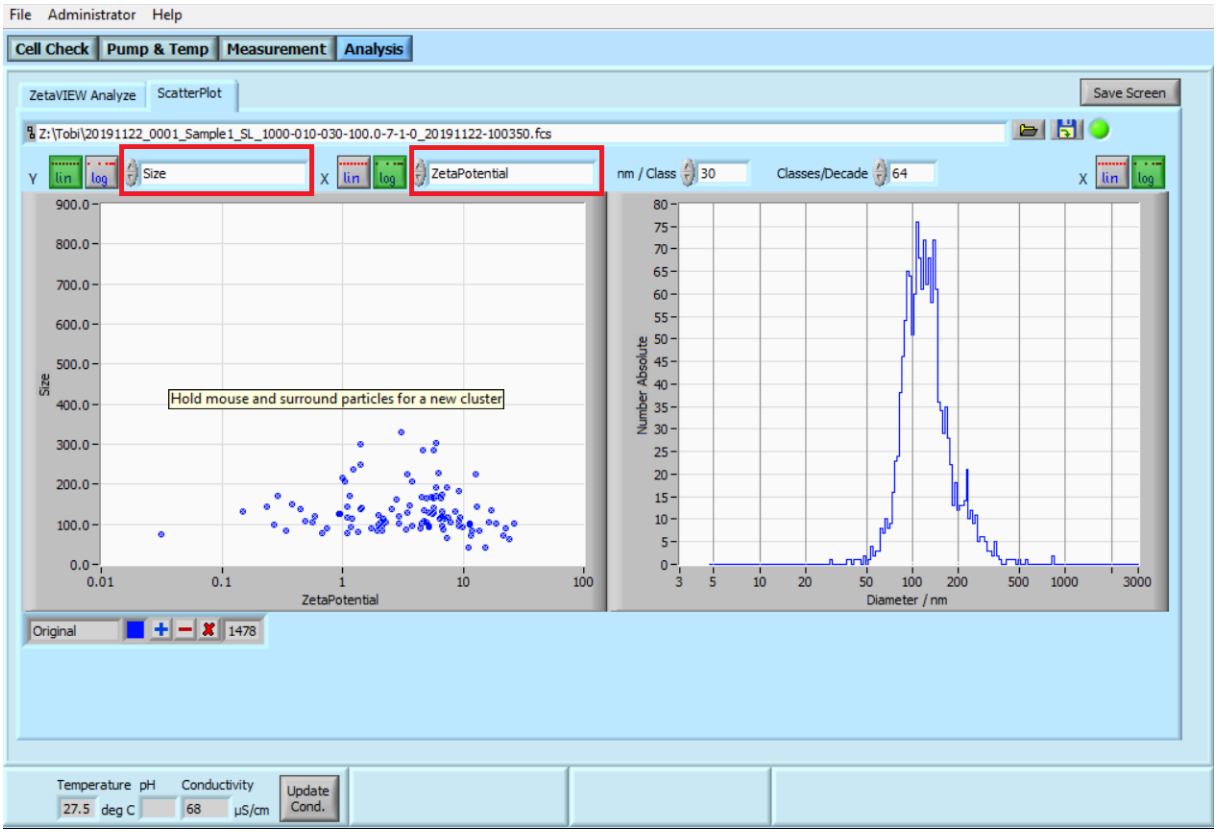


Figure 13-15: For a meaningful representation of the scatter plot after an SL- zeta potential measurement, the Y-axis is plotted with the size and the X-axis with the zeta potential. Particles in the scatter plot can be surrounded by the mouse to create a new cluster which is displayed under the scatter plot. The number of particles of every cluster is displayed as well.

If “Scatter Plot” is clicked and no FCS file is available after the measurement, the software gives a message that no FCS file is available and asks for creating an FCS file for subsequent re-analysis.

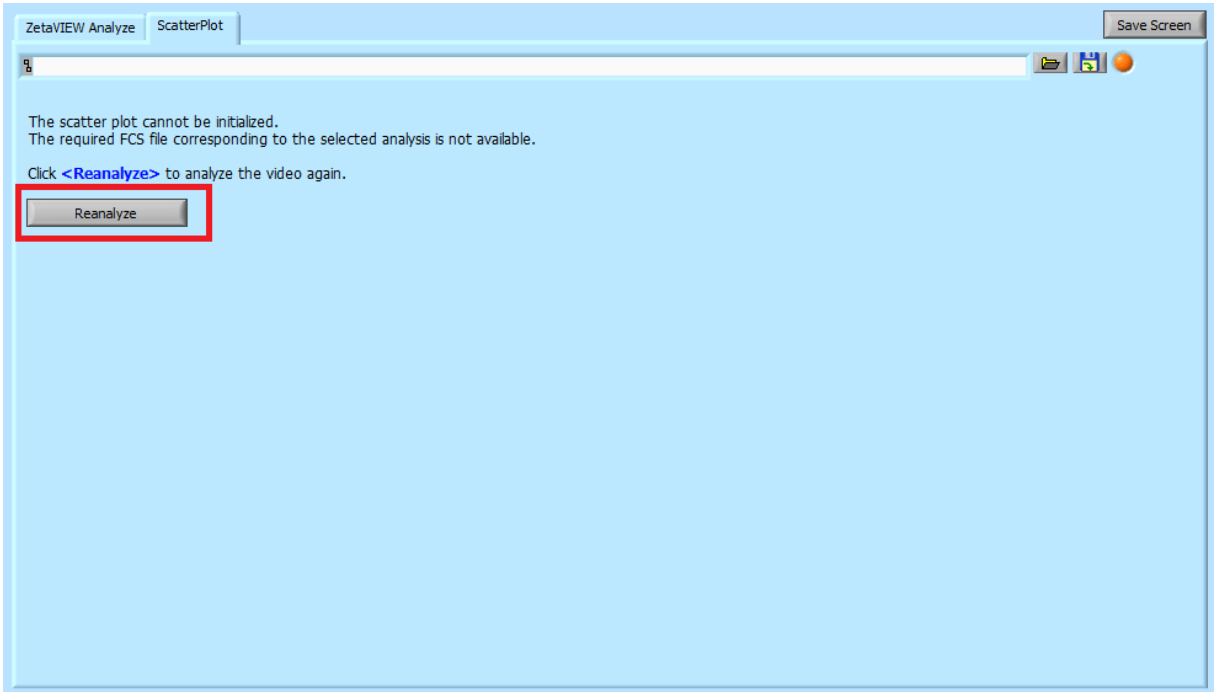
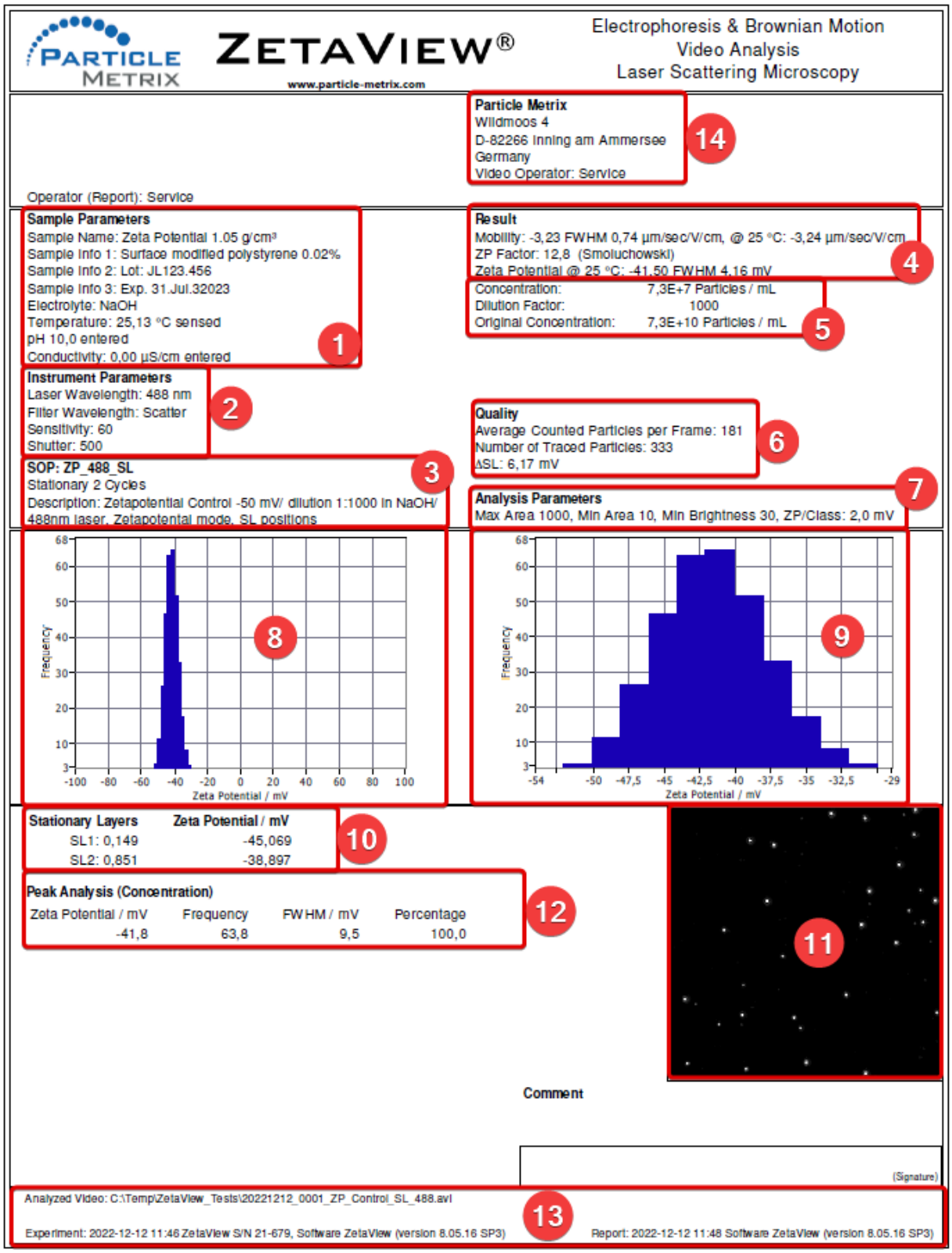


Figure 13-16: If an FCS file is not available for re-analysis, it can be created by clicking “Reanalyse” in the Scatter Plot tab.

13.5 Description of the PDF report of a zeta potential measurement



1. Sample Parameters

Here, the sample information that was entered on the measurement menu are documented. All those information (comment, sample info 1, sample info 2, sample info 3, Electrolyte, Temperature, and pH) can be found and entered in the „Experiment Parameters“ tab (see below).

Note: The *Sample Name* has to be entered each time the measurement menu is opened, there is no auto-fill of that field or any linkage to the *Custom Entry File Name*.

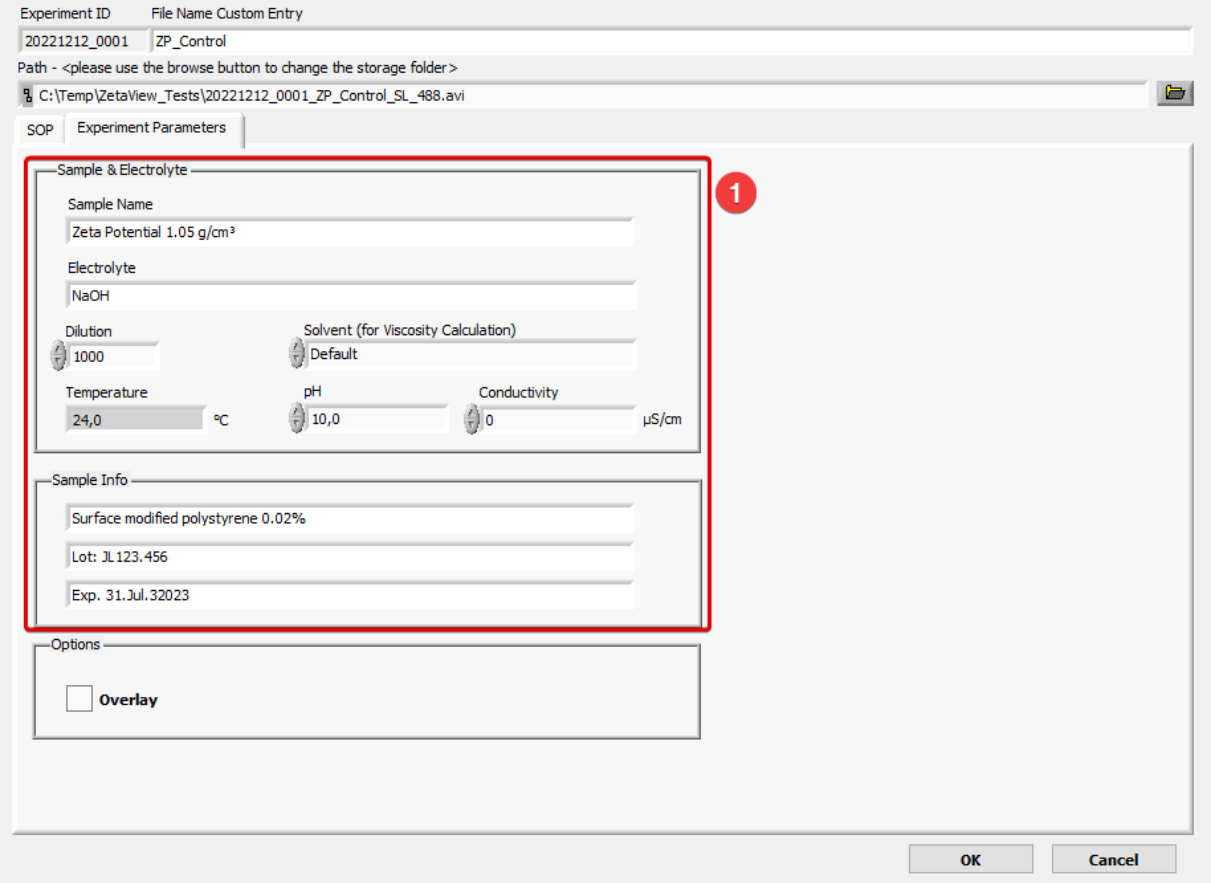


Figure 13-17: The settings for the Experiment Parameters are highlighted in red.

2. Instrument Parameters

This section lists which laser and camera settings have been used during the measurement (e.g. laser wavelength, filter wavelength, shutter and sensitivity settings). If no fluorescence filter was used, “Scatter” is stated on the report.

3. SOP

This section lists the name and description of the used SOP and what kind of measurement has been performed (size distribution or zeta potential measurement). In addition, it lists the number of the cycles. In case an SOP was not saved before starting it, (e.g. status of the SOP shows red), the reports states < No SOP >.

4. **Result** informs about:
   - Electrophoretic mobility
   - FWHM for electrophoretic mobility
   - FWHM calculated for 25°C

   } $\frac{\mu m/sec}{V/cm}$
   - Zeta potential factor according to Smoluchowski
   - Zeta potential calculated for 25°C
   - FWHM of zeta potential distribution calculated for 25°C

5. **Measured concentration** of the particles, dilution factor and original calculated concentration is documented. The original concentration is calculated by the dilution factor multiplied by the measured concentration.

6. **Quality**
   - Average counted particles per frame is a calculated value and describes the number of particles that are detected on average within one video frame. This number is very similar to the „No. of Detected Particles“ that you can see in the lower right bar in the software interface.

   - Number of traced particles is the total number of particles that are analysed in the statistics.

   - ΔSL: Difference of SL1 and SL2. The difference should not exceed 10mV. When the difference is larger than 10mV, symmetry correction should be carried out.

7. **Analysis Parameters** (post-acquisition parameters) that were used for the measurement:
   - Max Area (maximum number of pixels that a particle contains to be analysed in the statistics)
   - Min Area (minimum number of pixels that a particle contains to be analysed in the statistics)
   - Min Brightness (grey level of the particles) describes the minimum grey level that a particle has for analysis in the statistics

8. **Histogram** of the zeta potential distribution

9. **Histogram** of the zeta potential distribution plotted in a wider way

10. Stationary Layers

Description of the stationary layers SL1 and SL2 and their relative position is indicated.

11. Picture of the first image taken during the measurement

12. Peak Analysis

Peaks that were found in the zeta potential distribution.

13. Experiment and Report Details

This section lists the details about the analyzed video file (e.g. Windows path and file name) and when the experiment was conducted (e.g. time stamp, ZetaView serial number and software version used). It lists also details when the report was created (e.g. time stamp and ZetaView software version).

14. Company Information

Starting the ZetaView software in Administratior mode (see section 15.1), the Measurement tab offers a “PDF Report Settings” button to customize the report header. See also section 7.3.14.

13.6 Text file of zeta potential measurement

After a zeta potential measurement has been carried out and evaluated, a text file is stored in addition to a video file (which contains the actual raw data of the measurement) and the pdf report, regardless of whether the sample was measured in scatter mode or in fluorescence mode.

Basically, the text file consists of 2 parts:

The top part represents the header of the text file, and contains the name of the file, operator, software version, and the numerous parameters used to measure the sample. This header is similar to that of a text file derived from a size measurement (see chapter 11).

In the second part of the text file, the electrophoretic mobility (Mb), the corresponding zeta potential (ZP) and the number of particles that have been assigned to a zeta potential bin class are listed. This is also similar to the result table of a size measurement. The figure below shows a section of the text file.

Results:
Mean Velocity (Pos 0.149498) / um/s: -4.299652
Mobility / um/s/V/cm: -1.246194
Mobility @ 25°C / um/s/V/cm: -1.359009
Mobility FWHM / um/s/V/cm: 2.093194
Zeta Potential / mV: -17.409069
Zeta Potential @ 25°C / mV: -17.409069
ZP FWHM / mV: 29.241477
Histogram:

Electroph Mobility @ 25°C / um/s/V/cm	ZP / mV	Frequency
-9.895E+0	-1.267E+2	0.000E+0
-9.793E+0	-1.254E+2	0.000E+0
-9.692E+0	-1.241E+2	0.000E+0
-9.590E+0	-1.228E+2	0.000E+0
-9.489E+0	-1.215E+2	0.000E+0
-9.387E+0	-1.202E+2	0.000E+0
-9.286E+0	-1.189E+2	0.000E+0
-9.184E+0	-1.176E+2	0.000E+0
-9.083E+0	-1.163E+2	0.000E+0
-8.981E+0	-1.150E+2	0.000E+0
-8.880E+0	-1.137E+2	0.000E+0
-8.778E+0	-1.124E+2	0.000E+0
-8.677E+0	-1.111E+2	0.000E+0
-8.575E+0	-1.098E+2	0.000E+0
-8.474E+0	-1.085E+2	0.000E+0
-8.372E+0	-1.072E+2	0.000E+0
-8.271E+0	-1.059E+2	0.000E+0
-8.169E+0	-1.046E+2	0.000E+0
-8.068E+0	-1.033E+2	0.000E+0
-7.966E+0	-1.020E+2	0.000E+0

Figure 13-18: Detail of a text file generated after an SL-measurement. The bin classes of the electrophoretic mobility and the zeta potential are listed in the first and second column highlighted in red and blue.

The first column highlighted in red describes the electrophoretic mobility at 25°C, which is divided into bin classes, similarly to the bin classes in the text file of a size measurement (ref. section 11). The unit for the electrophoretic mobility is µm/second/Volt/centimeter.

The second column highlighted in blue represents the zeta potential classes in millivolts.

The last column highlighted in green shows the number of particles that have been assigned to a particular mobility or zeta potential class.

13.7 Adjusting the zeta potential per class

Similar to a size measurement the bin classes of the zeta potential can be adjusted and changed with the parameter “ZP / Class". Since this parameter is a post-acquisition parameter, the video must be re-analyzed as described previously in section 13.3. This function allows the user to modify and adjust the resolution of the displayed zeta potential histogram according to match the desired presentation. The smaller the bin class, the higher the resolution of the histogram, and the more classes that are considered when displaying the histogram.

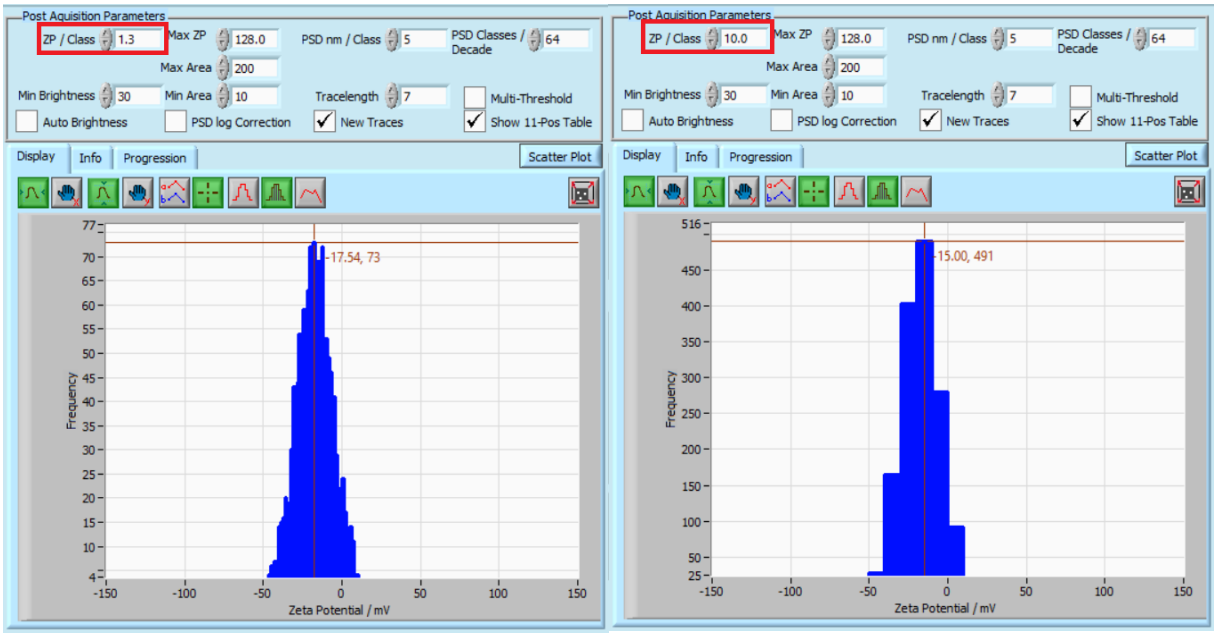


Figure 13-19: In this example, on the left side “ZP/Class” is adjusted to 1.3 mV. The histogram has a higher resolution than the histogram on the right where the bin class is set to 10 mV. The peak of the histograms may differ a bit depending on how the bin classes are adjusted.

Each individual value in the column for zeta potential (second column) represents the center of the adjusted bin class. This is shown in the figures below where the bin class is adjusted to 1.3 and 10, respectively.

The class width for a specific “ZP/Class” can be calculated by adding or subtracting half of the “ZP/Class” values to the value of the corresponding bin class. The resulting values represent the upper and lower limits of the respective class. For better understanding this is shown figuratively below.

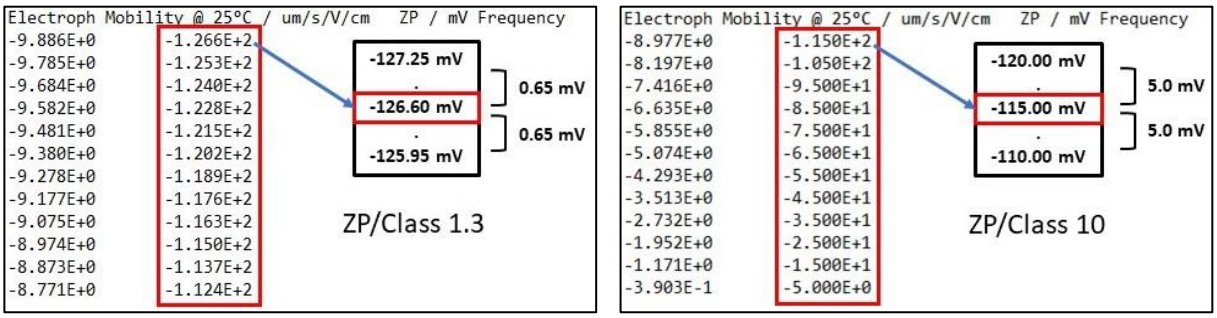


Figure 13-20: The distance of the values right above and below the centers is always exactly half of the adjusted “ZP/Class”. This is shown for “ZP/Class” adjusted to 1.3 (left) and 10 (right).

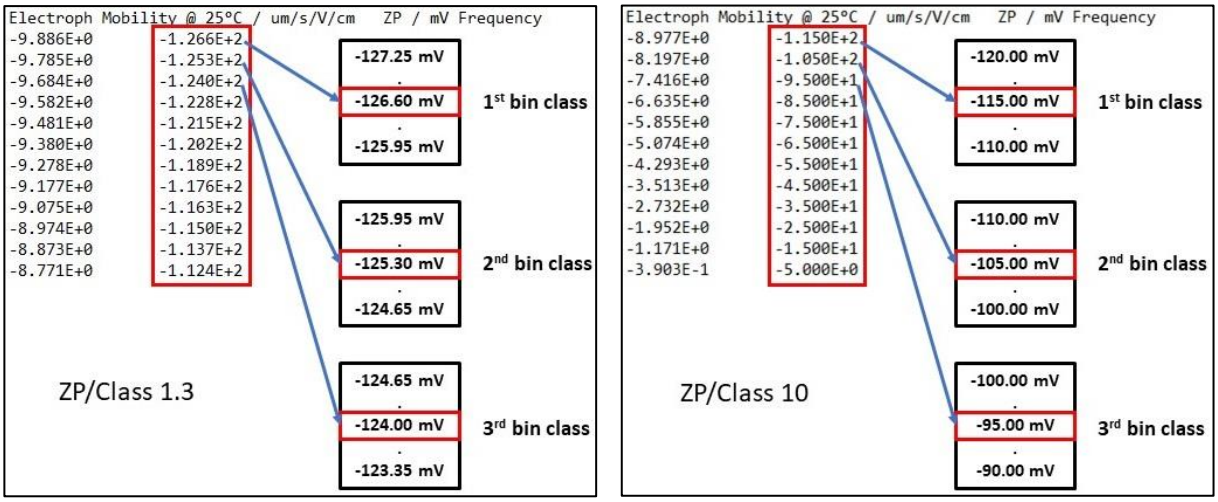


Figure 13-21: Comparison between ZP/Class adjusted to 1.3 and 10

The left side shows an extract from the text file. “ZP/Class” was set to 1.3 mV. The first bin class -126.60 mV ranges from -127.25 mV to -125.95 mV. -125.30 mV corresponds to the second bin class, ranging from -125.95 mV to -124.65 mV, etc. On the right side a bin class of 10 is adjusted. Here, -115.00 mV corresponds to the first bin class. It ranges from -120.00 mV to -110.00 mV. In the second one it ranges from -110.00 mV to -100.00 mV, etc.

In this example, when “ZP/Class” is set to 1.3, all particles with a zeta potential between -127.25 mV and -125.95 mV are assigned to the first bin class, all particles with a zeta potential between -125.95 mV and -124.65 mV are assigned to the second bin class etc. When “ZP/Class” is adjusted to 10 mV in this example, however, these particles are not taken into account in the analysis because the adjusted range here is too small. It is therefore advisable to keep the bin class small in order to be able to analyze all particles and to keep the resolution high.

The best possible histogram resolution is achieved when “ZP/Class” is adjusted to 1-1.3. A smaller class width is available but not recommended because the accuracy of the ZetaView® at the range mentioned above is maximized.

13.8 Adjusting the maximum zeta potential

With the "Max ZP" setting, the maximum zeta potential value on the X-axis can be shown and limited in the diagram in positive and negative directions. “Max ZP” is also a post-acquisition parameter. If this parameter is to be changed for a measurement that has already been completed, the corresponding video must be re-analyzed (see section 13.3). The following figure shows an example of how the histogram changes after changing the "Max ZP" setting.

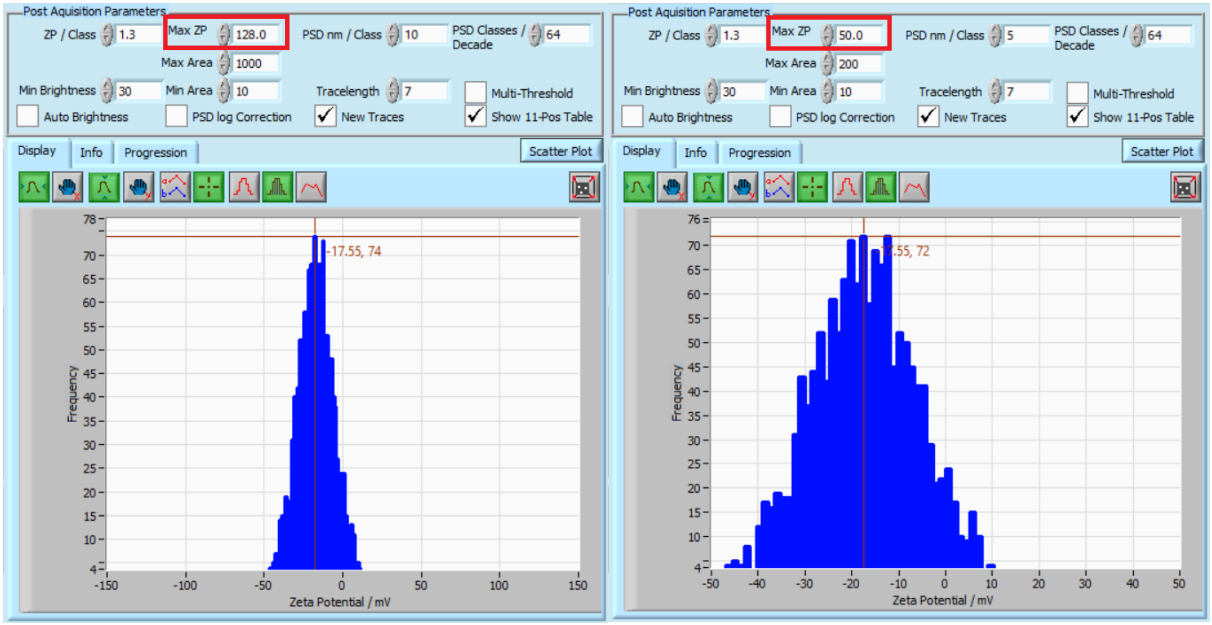


Figure 13-22: “Max ZP” is adjusted to 128 (left) and 50 (right). The X-axis is scaled accordingly.

The "Max ZP" value is a factor that determines the scaling of the X-axis. Depending on the scaling, the peak and the number of particles in the peak of the zeta potential distribution may vary slightly.

13.9 Adjusting the mobility per class and maximum mobility

Similar to the zeta potential, the bin classes of the (electrophoretic) mobility can be adjusted and changed with the parameter “Mobility / Class" and Max Mobility. The precondition for this is that the histogram must be switched to “Mb”. This function allows the user to modify and adjust the resolution of the displayed mobility histogram according to requirements.

Analog to the zeta potential, the smaller the bin class, the higher the resolution of the histogram, and the more classes are considered when displaying the histogram.

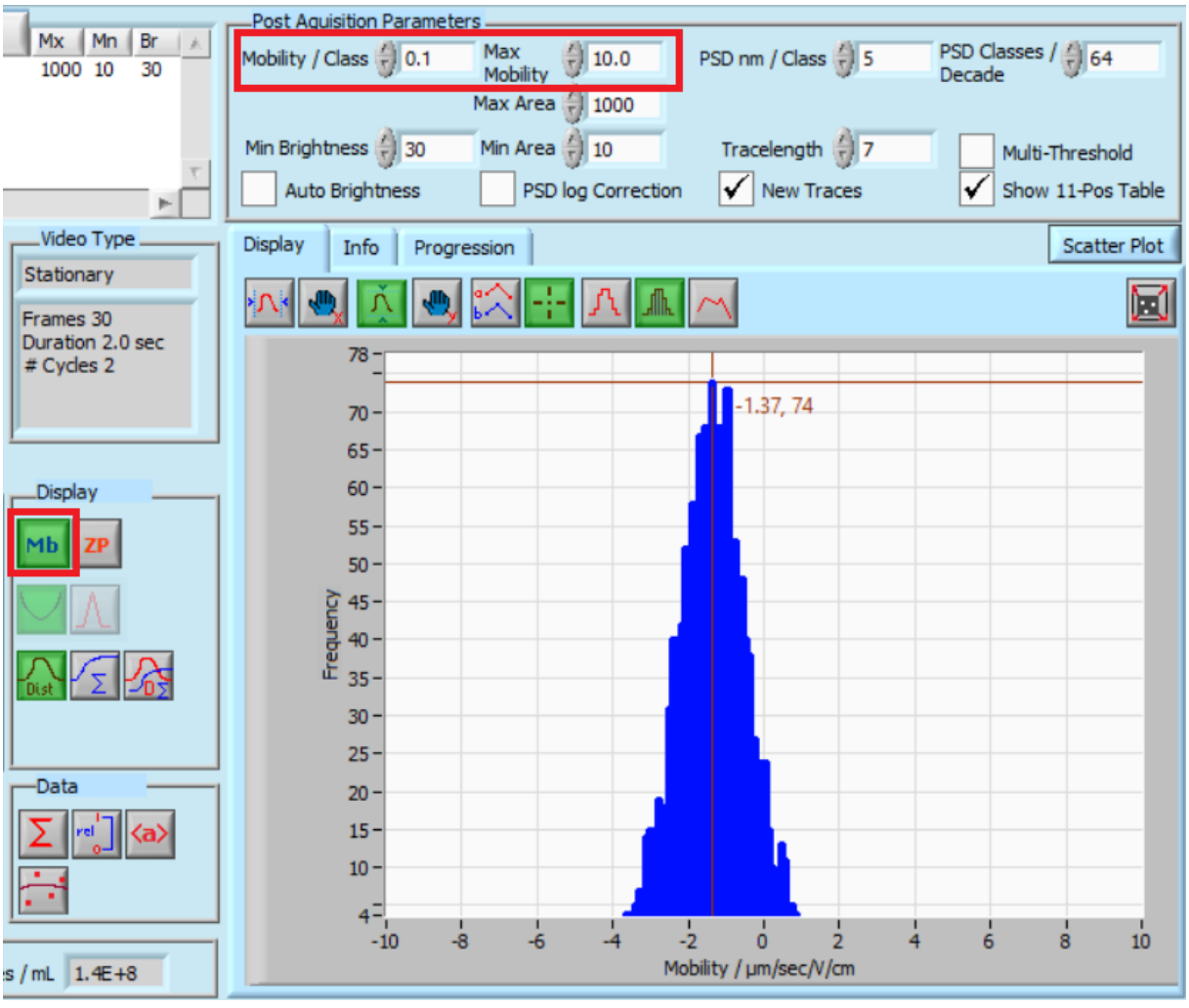


Figure 13-23: Mobility histogram of a zeta potential measurement.

All explanations and procedures that have been described about the zeta potential in the sections above also apply to mobility without restriction, since the zeta potential is calculated from the electrophoretic mobility. Therefore, the reader is referred to the corresponding sections on zeta potential described previously.

14 Cleaning and flushing the measuring cells

In order to achieve reproducible and accurate results it is necessary to clean the cell from time to time. The cleaning intervals are dependent on how often the ZetaView® instrument is used and what type of samples are measured. It is generally advisable to clean the measuring cell at least once a week. When measuring biological samples e.g. from the cell culture or if samples tend to leave possibly sticky residues (some cell culture media), or if the samples tend to generate background due to non-ionic detergents, more frequent cleaning is recommended.

At any time when background scattering becomes annoying, cleaning is useful. The procedure is explained in detail below. It is strongly recommended to rinse the cell with DI water or with the buffer used for suspending the particles in the sample after each measurement. In addition, it is advisable to leave DI water or air after a measurement session. Many experiments can be carried out by following this rinsing advise.

Cationic samples may coat the negatively charged cell walls leading to an increased background scattering. If this coating cannot be removed by flushing, mechanical or chemical cleaning may be necessary.

Never try to clean the cell channel with dry material! Always rinse wet, with water if the cell is still in inside the Cell assembly or by any other liquid when the cell is separate from the Cell assembly. The liquid, into which the sample is dispersed is often good for cleaning. If the sample was coated with a polyelectrolyte, a surfactant, an emulgator, and this coating was dome in the cell, this can be removed with the appropriate solvent for these coating materials.

Take care not to drop any O-ring or silicon washer into any organic cleaning liquid!

14.1 Cleaning the Z-NTA cell

To remove the Z-NTA Cell assembly from the instrument, click on “Remove Cell Assembly”.

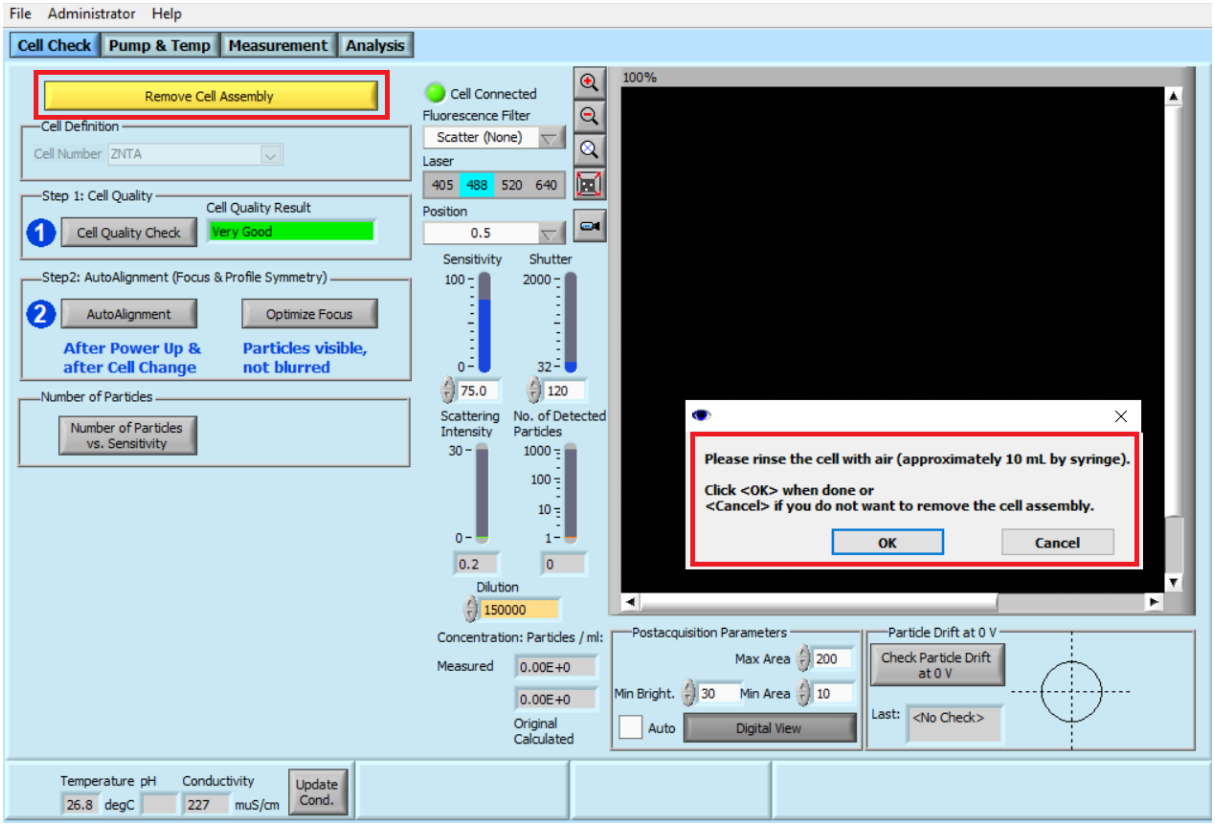
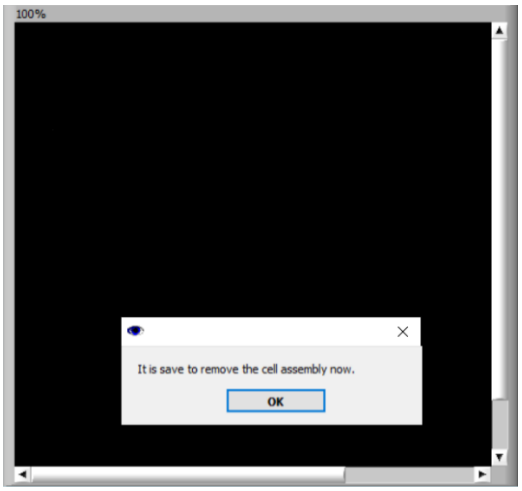


Figure 14-1: Follow the message and rinse the cell with 10-20 ml air from a syringe until only air bubbles rise from the tube in the waste bottle.

Click “OK” when done. The software shows a message that it is safe to remove the Cell assembly.



For unlocking the Cell assembly, pull the knob downwards, turn it into the red position as indicated below and let the knob release. The red position is the “unlock” position.

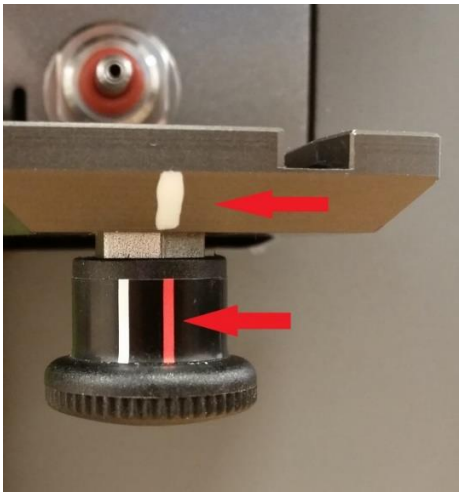


Figure 14-2:Unlock position.

The cell is usually mounted in a black or blue colored cell carrier. The cell carrier features two holes that are located on the left and right side for fixing it by two screws at the Cell assembly. The following description shows how to remove the cell carrier from the Cell assembly for cleaning purposes.

1. Remove both screws that fix the cell carrier on the Cell assembly by using the wrench provided in the toolkit.

Figure 14-3: Remove both screws on top of the cell carrier.

2. After removing the screws hold the protruding parts of the cell carrier with your thumb and fore finger and pull the cell carrier slowly and evenly upwards (green arrows) out of the Cell assembly by applying some counter pressure with your middle fingers (red arrows pointing down).

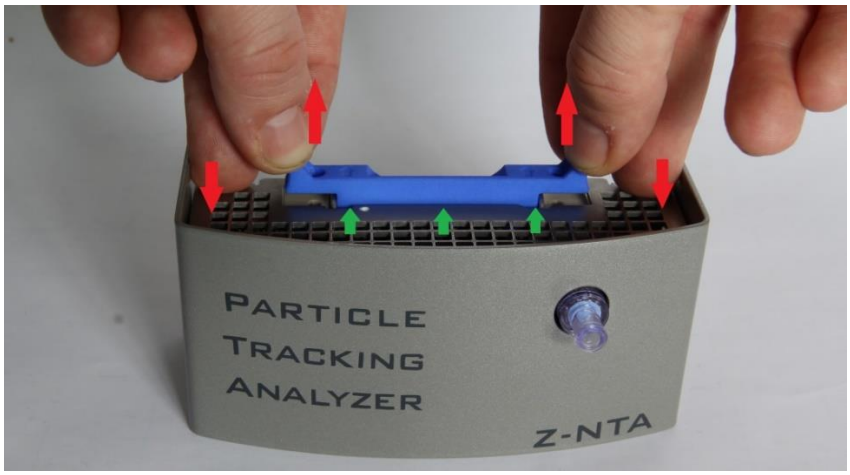


Figure 14-4: Pull the cell carrier carefully out evenly.

3. Prepare the cleaning solution by dripping 8-10 drops of the blue (dries a bit faster) or green (stronger detergent) cleaning detergent in 50ml DI water. For zeta potential measurements, the green solution is recommended. Mix the solution thoroughly. For more intensive cleaning, a particle-free mixture of 90 % distilled water and 10 % acetone can be used.

4. Wet a soft brush with the cleaning solution, insert the wet brush into the measuring cell and move it back and forth 5-10 times. Repeat this procedure on the opposite site of the cell.

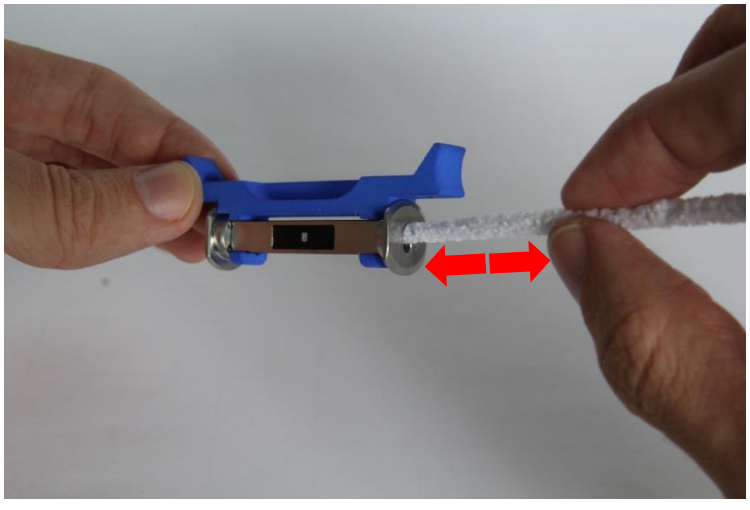
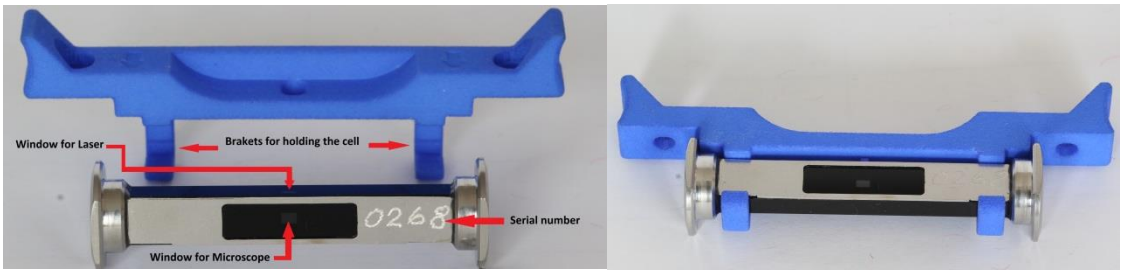


Figure 14-5: Insert the wet brush into the cell and move it back and forth.

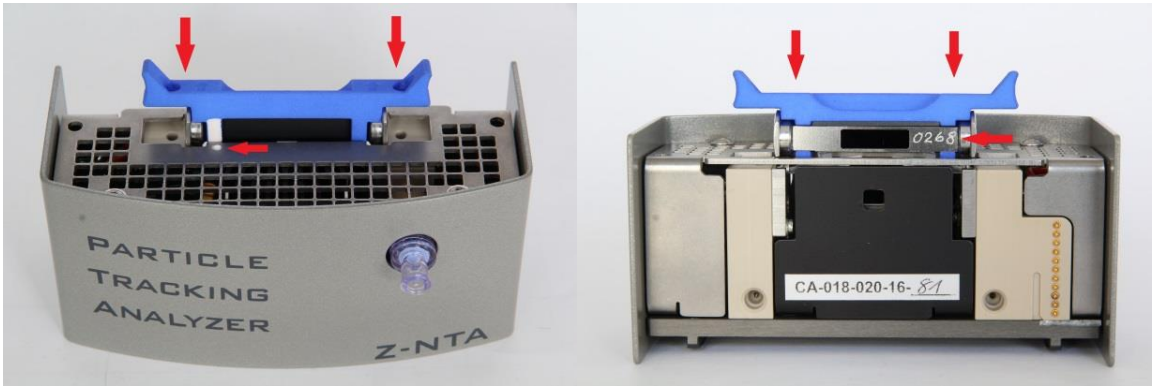
5. Rinse the cell with distilled water afterwards, dry it completely on the outer surface and place it back into the Cell assembly.

6. Care must be taken not to touch the two optical windows of the cell for the laser and the microscope with your bare fingers. Fingerprints on the windows of the cell can interfere with the measurements. If fingerprints are visible on the windows, they can be wiped off with a 50% acetone-water mixture using a Q-tip cotton swab. You should also make sure that the acetone evaporates without streaks after removing fingerprints.

7. While re-attaching the cell to the cell carrier, both must be mounted to each other in one specific orientation. When the cell is mounted on the cell carrier, the serial number must be visible at the open part of the cell carriers' right bracket (from this point of view as shown below)



8. Re-inserting the cell carrier (with the cell) must be done in one specific orientation too. Please make sure that the white painting on the cell carrier faces the white dot located on top of the Cell assembly. Please also note that the serial number of the cell faces towards the back side of the Cell assembly (see below).



9. Fix the cell carrier with the cell by using the screws on the Cell assembly.

Figure 14-6: Fix the screws tightly. Do not overtight them.

Place the Cell assembly back on the ZetaView® instrument and start the start-up routine as described in chapter 5.

14.2 Cleaning the NTA cell

The procedure of removing the NTA Cell assembly is the same as for the Z-NTA Cell assembly (refer to section 14.1).

Remove the purple check valves at the inlet and outlet of the cell by unscrewing them at both sides.

Figure 14-7: The left and right check valves must be unscrewed.

Wet the provided brush with the freshly prepared cleaning solution, insert it into the right side of the measurement cell and move it back and forth 5-10 times. Please note that the brush will not go completely through the entire measurement cell due to constructive reasons of the cell.

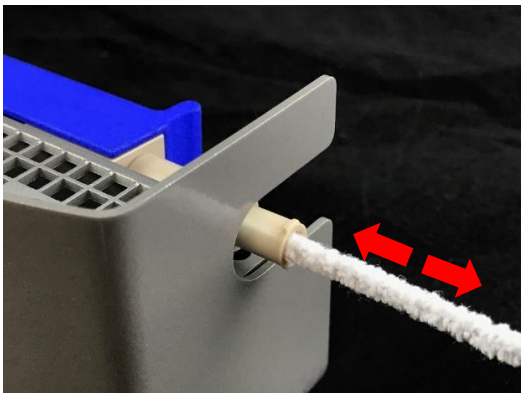


Figure 14-8: For cleaning, insert the wet brush into the right opening of the cell.

Rinse the cell with distilled water afterwards, dry the cell completely on the outer surface and place the Cell assembly back into the instrument. Start the start-up routine as described in chapter 5.

15 Troubleshooting

15.1 Administrator mode

The software interface of the ZetaView® instrument includes an administrator mode in which quality, measurement and calibration parameters can be checked, adjusted and, if necessary, corrected. The administrator mode is only accessible with a password which can be made known to the user during installation or training by Particle Metrix personnel or a trained distributor upon request.

Untrained personnel should only work in the administrator mode under the supervision or at the direction of Particle Metrix personnel or trained distributors, as changes to the parameters may result in incorrect calibration, misalignment or malfunction of the ZetaView® device.

The administrator mode extends the ZetaView® software interface with the tabs “**Alignment”**, “**Manual Acquisition”** and “**Calibration”**, whereby only “Alignment” and “Calibration” are described here.

15.1.1 Alignment tab

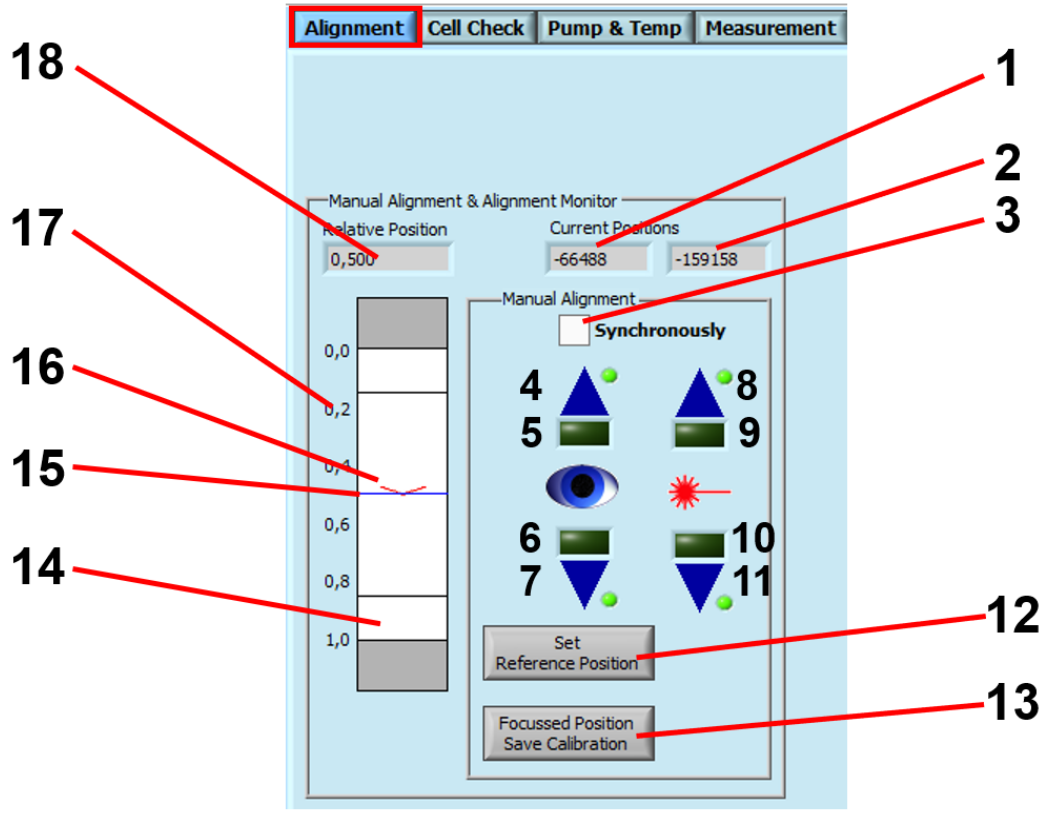


Table 15.1: Parameters and functions in the Alignment tab

Number	Function	Remarks
1	Current motor position of the microscope	
2	Current motor position of the laser	
3	Synchronous and asynchronous movement of microscope und laser	Depending on the status, microscope and laser can be moved synchronously or independently of each other
4	Stepwise movement of the microscope towards position 0	The microscope is moved step by step with each mouse click
5	Continuous movement of the microscope towards position 0	The microscope is moved continuously by holding down the mouse button
6	Continuous movement of the microscope towards position 1	The microscope is moved continuously by holding down the mouse button
7	Stepwise movement of the microscope towards position 1	The microscope is moved step by step with each mouse click

Number	Function	Remarks
8	Stepwise movement of the laser towards position 0	The laser is moved step by step with each mouse click
9	Continuous movement of the laser towards position 0	The laser is moved continuously by holding down the mouse button
10	Continuous movement of the laser towards position 1	The laser is moved continuously by holding down the mouse button
11	Stepwise movement of the laser towards position 1	The laser is moved step by step with each mouse click
12	For setting the reference position within the measuring cell	The reference position is the centre position within the measuring cell
13	Saves the relative positions of the microscope and laser	May only be clicked after successful manual focusing
14	Sketch of the inside of the measuring cell over all measuring positions	
15	The blue line shows the position of the microscope within the measuring cell	
16	The red arrow shows the position of the laser within the measuring cell	
17	Scaling of the measuring positions within the cell	
18	Relative Position	Always refers to the currently set position

15.1.2 Calibration tab

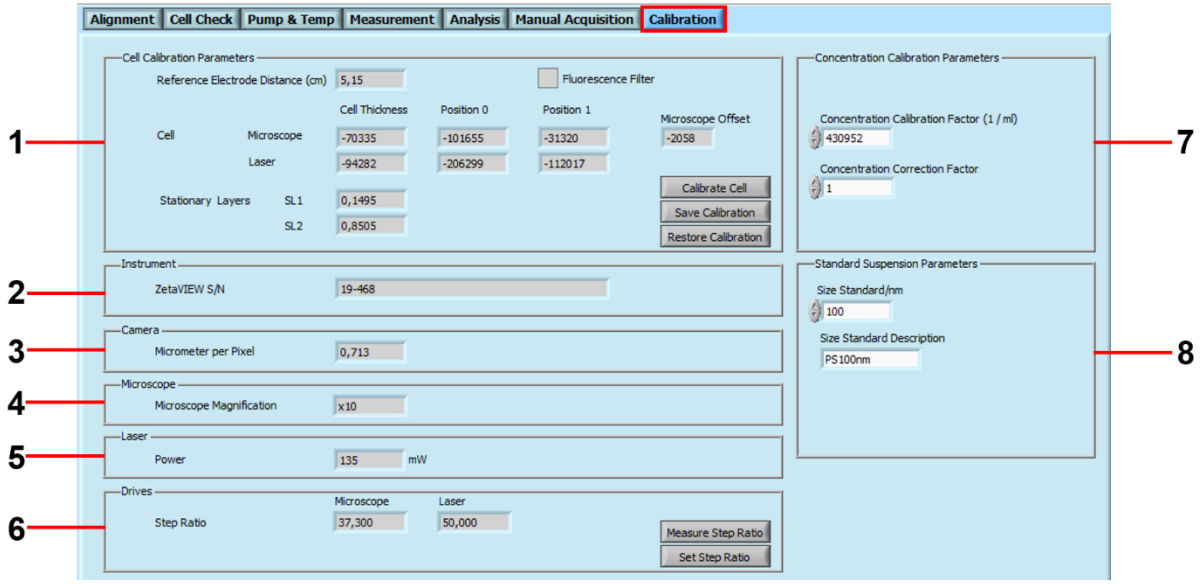


Table 15.2: Parameters and functions in the Calibration tab

Number	Function	Remarks
1	Essential calibration values for the measuring cell and the detection optics	Under no circumstances should these values be changed without consulting or guidance from Particle Metrix
2	Serial number of the instrument	
3	Pixel size	
4	Optical magnification of the microscope	
5	Power of the laser used	
6	Step ratio of microscope and laser	
7	Concentration calibration factor	Under no circumstances should these values be changed without consulting or guidance from Particle Metrix
8	Parameters for the Standard Suspension	Actual size of the used size reference standards. Used to calculate the *Trueness* (Accuracy) during the Daily Performance script.

15.2 Manual focusing of the particles

If the particles are out of focus despite running the AutoAlignment or focus optimization function, manual focusing of the particles is required.

Manual focusing may only be carried out under the supervision or guidance of Particle Metrix personnel, trained distributors or a trained ZetaView® user.

Manual focusing is performed in the Alignment tab and is described in the following figure.

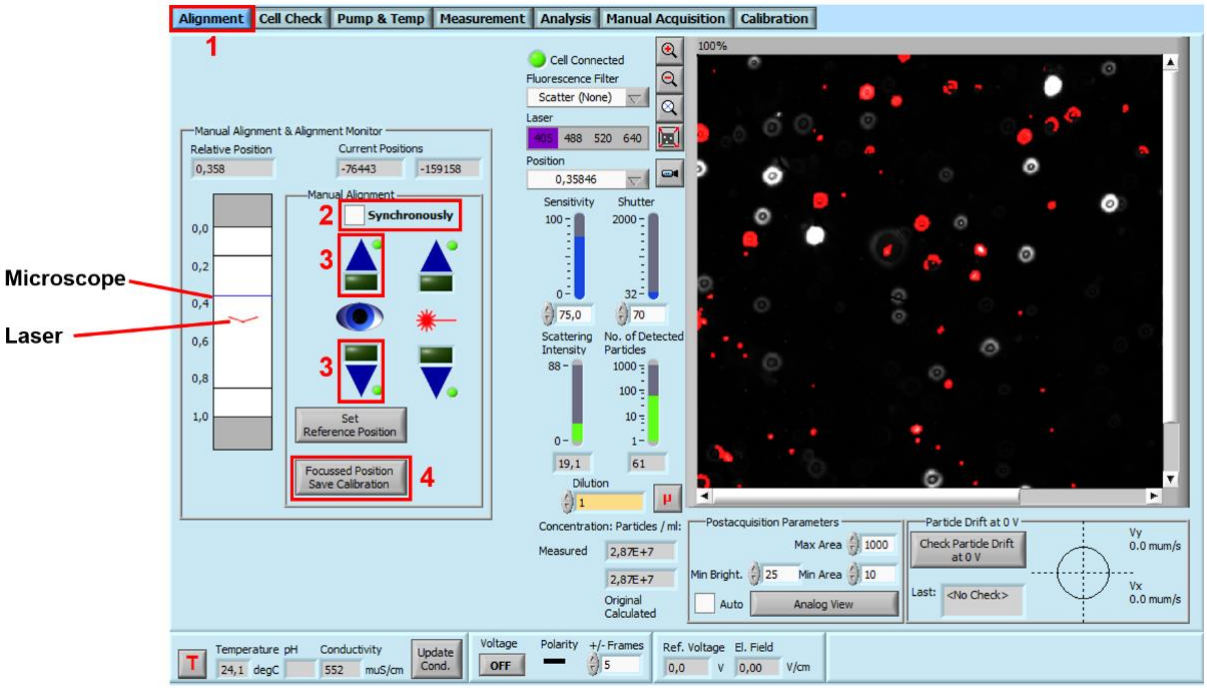


Figure 15-1: Procedure for manual focusing. The movement of the microscope during manual focusing can be recognized by the blue line representing the microscope on the left edge of the image.

1. Click on the Alignment tab
2. Deactivate the "Synchronously" function
3. Move the microscope up or down. Press and hold the green buttons to move the microscope continuously. Click the blue triangles to step the microscope with each mouse click. Observe whether the focus of the particles improves or deteriorates. The focus is optimal when the particles are displayed as small, and the number of detected particles is at maximum.
4. After the focus is found, press "Focussed Position Save Calibration". The focus is now saved.

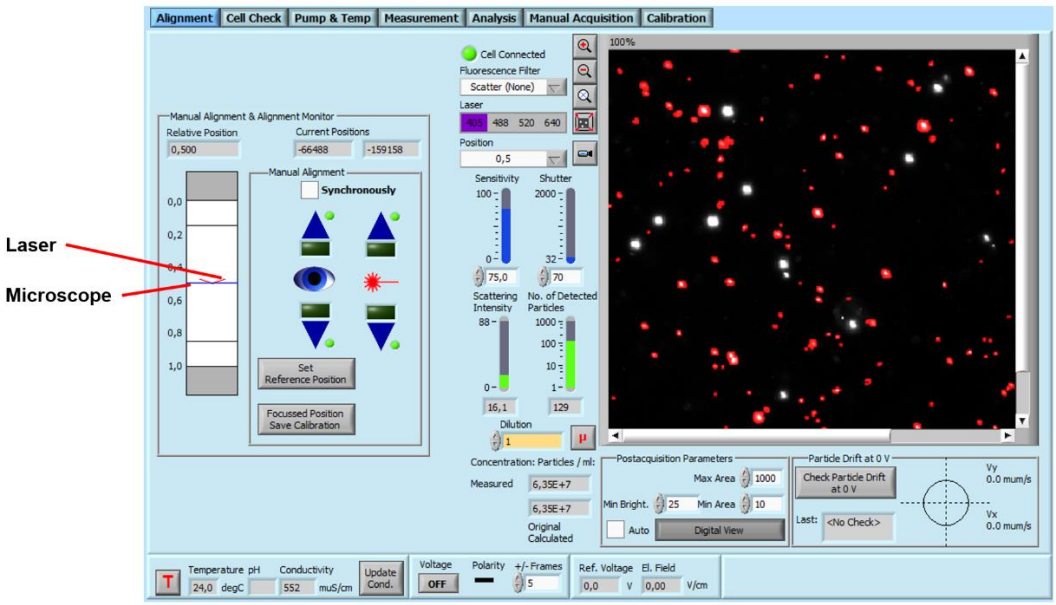


Figure 15-2: Manual focusing is complete.

15.3 Restore Calibration

The cell calibration is stored in the Calibration tab. If no particles can be observed even though they have been correctly injected into the ZetaView® instrument, this may be due to incorrect cell calibration. If the cell calibration is no longer correct, it can easily be restored.

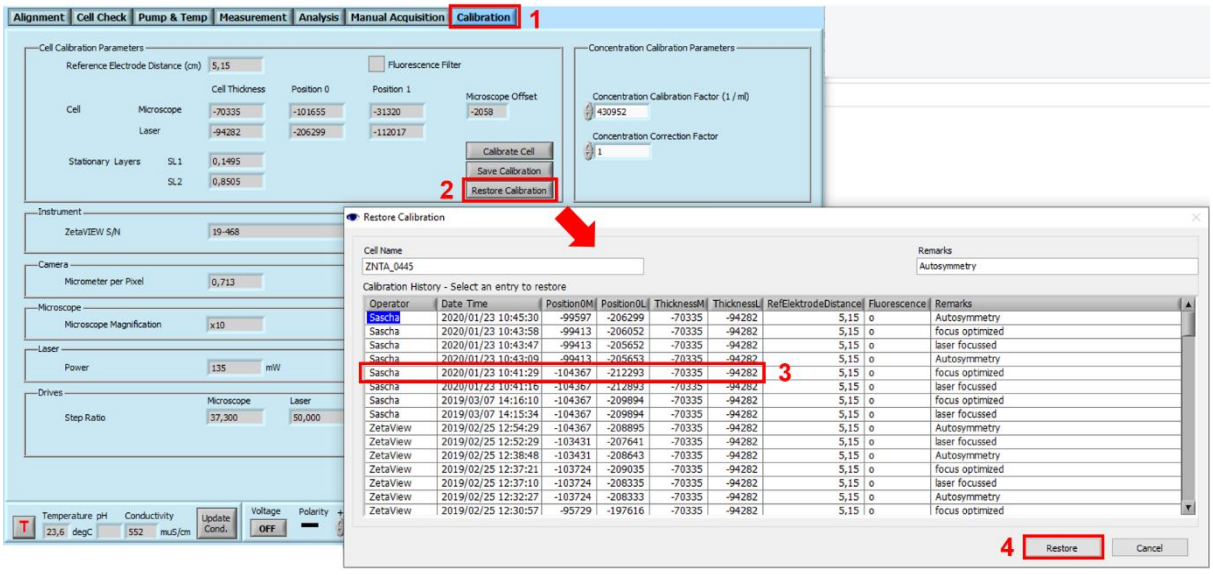


Figure 15-3: Procedure how to restore the calibration.

1. Click on the Calibration tab
2. Click on "Restore Calibration"
3. A list of calibration parameters, sorted by date, opens in a new window. Click on an older date or time that shows different calibration parameters compared to the current date or time and of which you know that particles were visible at that time a measurement could be carried out.
4. Click “Restore” to restore the older calibration parameters.

15.4 Setting up new calibration parameters

Usually, calibration parameters must only be reset or adapted when a new measuring cell is installed in the cell assembly. The calibration parameters are provided by Particle Metrix personnel or a trained distributor. The following figure shows an example of how to proceed in order to enter new calibration parameters.

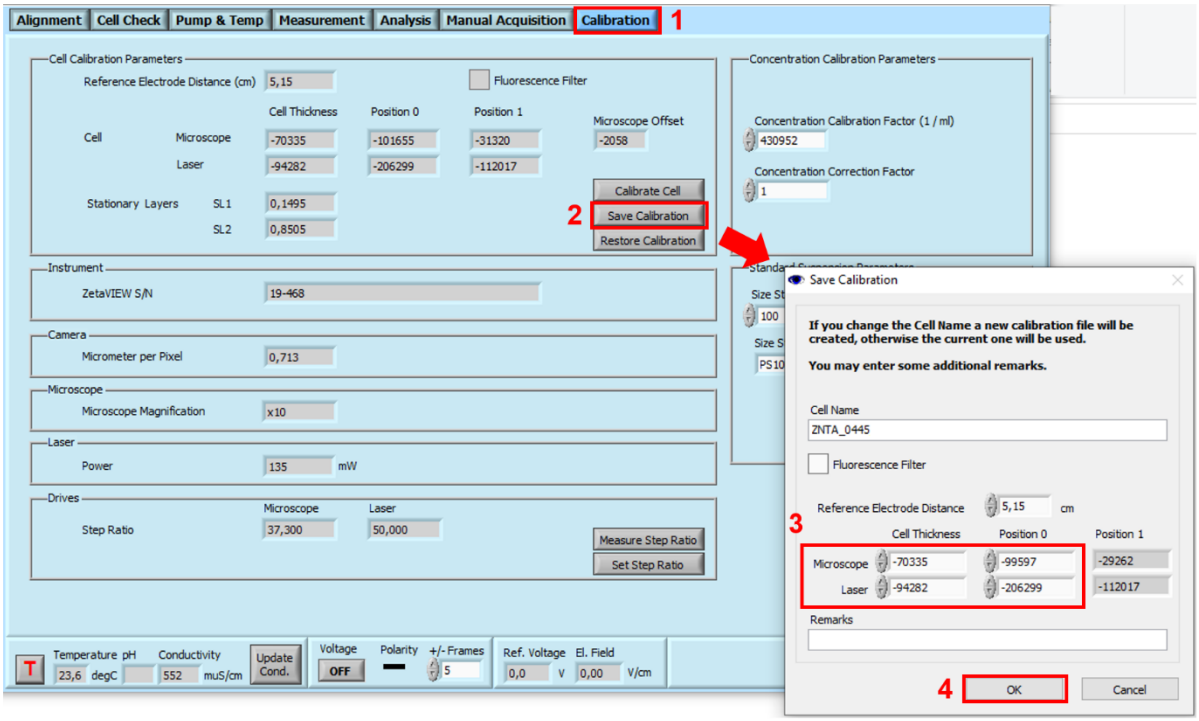
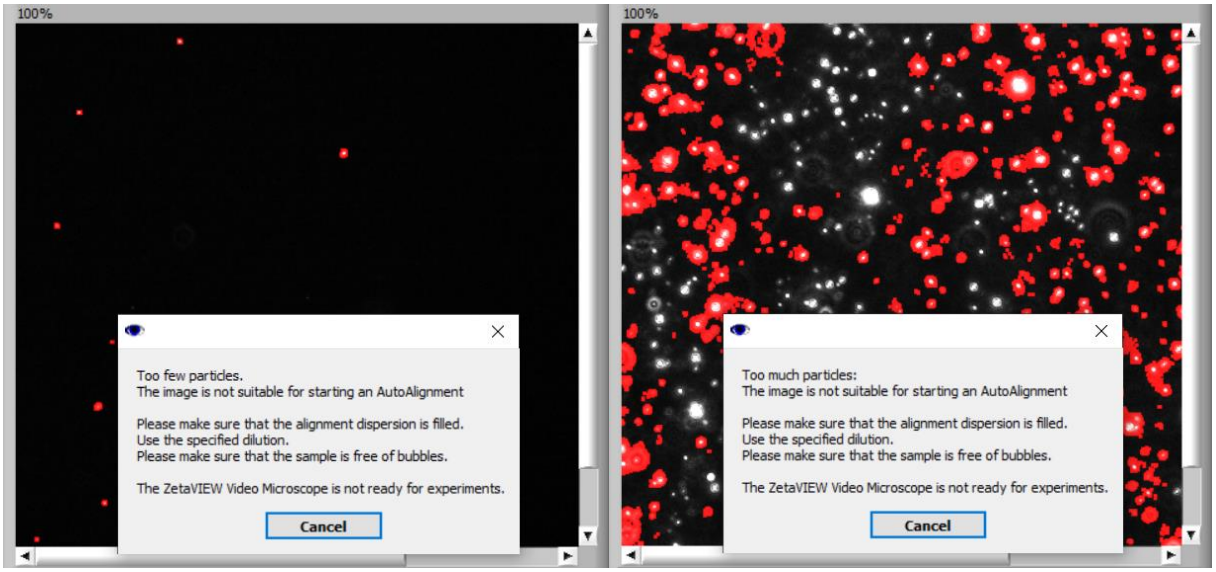


Figure 15-4: Procedure for setting up calibration parameters.

1. Click on the Calibration tab
2. Click on "Save Calibration"
3. You can enter new calibration parameters in a new window. The parameters must be provided by Particle Metrix personnel or the distributor.
4. Click OK to save the calibration parameters.

15.5 Issues during or after AutoAlignment

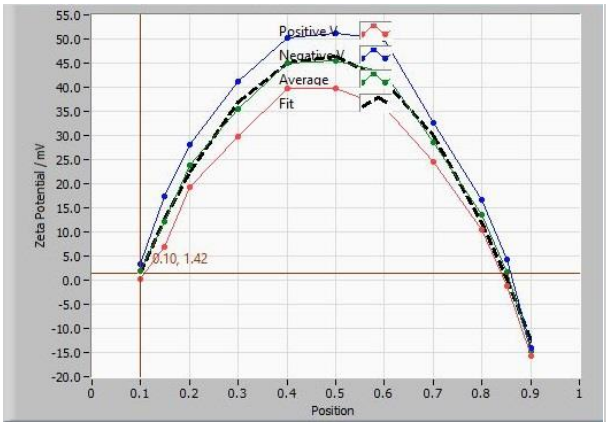
15.5.1 Too few or too many particles

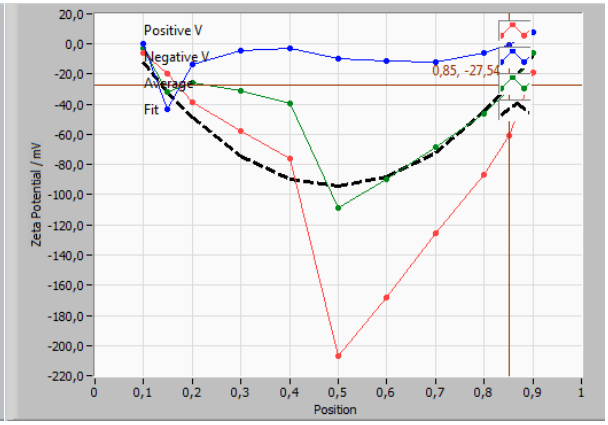


Cause	Remedy
Incorrect dilution of the PS100 nm alignment suspension.	• Verify the dilution of the alignment suspension and prepare a new dilution if necessary.
Particles are not in focus. As a result, fewer particles are detected than are actually present in the measuring cell.	• Perform a focus optimization. • Try to adjust the focus manually.
Too much background in the field of view. As a result, more particles are displayed than are actually present in the measuring cell.	• Clean the cell.
Air bubbles in the measuring cell.	• Inject some PS100 nm alignment suspension with a little more pressure to move the air bubble out of the measuring cell. • Inject 5-10ml of water and fill the measuring cell again with PS100 nm alignment suspension. • Empty the measuring cell by injecting air, then inject 5-10 ml of water, followed by PS100 nm alignment suspension. • Make sure to avoid air bubbles during injection.

15.5.2 Inverted or zigzag parabola

This message can only be displayed if the ZetaView® instrument is equipped with a Z-NTA cell assembly and if there are issues with the symmetry correction after the AutoAlignment or after an 11-position zeta potential measurement with PS100 nm beads has been performed.

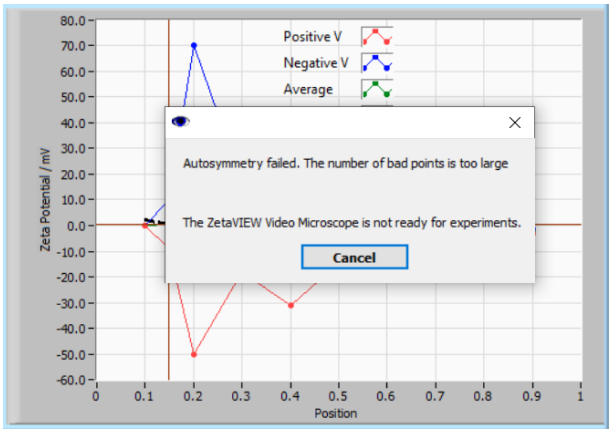




Cause	Remedy
Air bubbles in the fluidic system.	• Inject some PS100 nm alignment suspension with a little more pressure to move the air bubble out of the measuring cell. • Inject 5-10ml of water and fill the measuring cell again with PS100 nm alignment suspension. • Empty the measuring cell by injecting air, then inject 5-10 ml of water, followed by PS100 nm alignment suspension. • Make sure to avoid air bubbles during injection.
Dirty measuring cell.	• Clean the measuring cell.
Cationic impurities on the walls of the measuring cell.	• Clean the measuring cell.

15.5.3 Autosymmetry failed

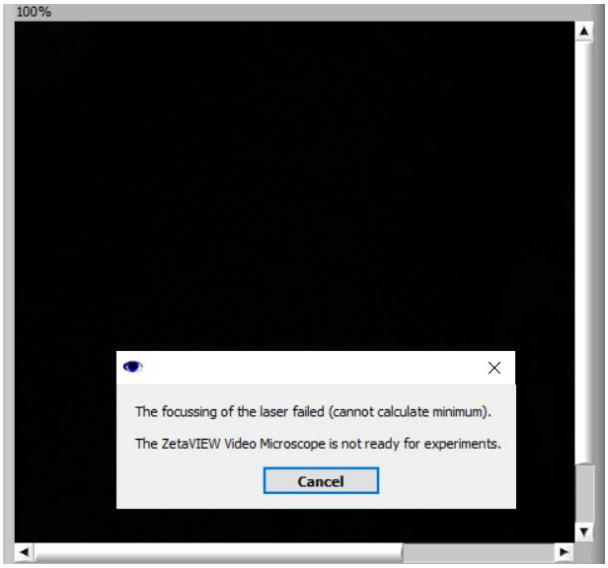
The following message is displayed if the ZetaView® instrument cannot perform an autosymmetry / symmetry correction after the autoalignment has ended.



Cause	Remedy
Air bubbles in the fluidic system.	• Inject a little more PS100 nm alignment suspension with more pressure to move the air bubble out of the system. • Inject 5-10ml water and fill the measuring cell again with alignment suspension. • Empty the measuring cell by injecting air, then inject 5-10ml of water, followed by alignment suspension. • Make sure that the injection is free of air bubbles.
Measuring cell is not clean.	• Clean the measuring cell
Cationic impurities on the walls of the measuring cell.	• Clean the measuring cell.
The beads in the alignment suspension are too old.	• Prepare new alignment suspension with fresh beads.

15.5.4 Focussing of the laser failed

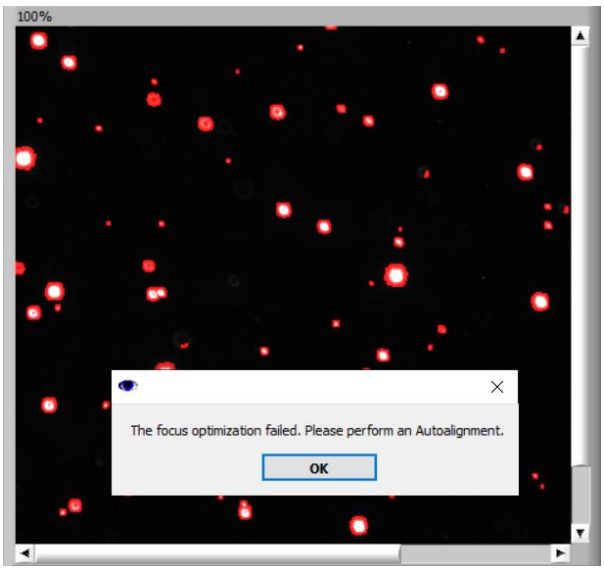
The following message is displayed if the ZetaView® instrument cannot focus the laser during autoalignment.



Cause	Remedy
Air bubbles in the fluidic system.	• Inject a little more PS100 nm alignment suspension with more pressure to move the air bubble out of the system. • Inject 5-10ml water and fill the measuring cell again with alignment suspension. • Empty the measuring cell by injecting air, then inject 5-10ml of water, followed by alignment suspension. Make sure that the injection is free of air bubbles.
Measuring cell is not clean.	• Clean the measuring cell.
There are too many particles in the cell.	• Verify the dilution of the alignment suspension 1:250,000.
Particle drift is too high.	• Reduce the particle drift (see section 15.8).
Laser and microscope are misaligned.	• Contact the Particle Metrix Support
The fluorescent filter was accidentally moved into the optical path.	• Switch the instrument into the scatter mode.

15.6 Issues with the focus optimization

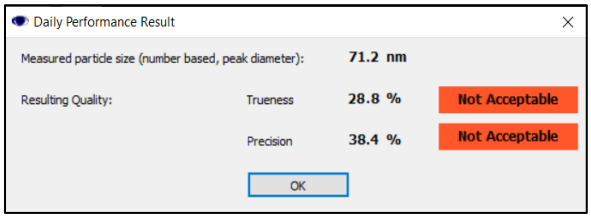
The following message is displayed if the ZetaView® instrument could not optimize the focus after starting "opimize focus" manually.

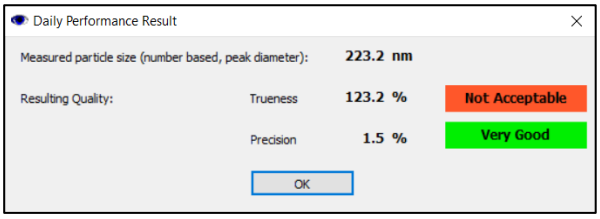


Cause	Remedy
Air bubbles in the fluidic system.	• Inject a little more sample with more pressure to move the air bubble out of the system. • Inject 5-10ml buffer and fill the measuring cell again with sample. • Empty the measuring cell by injecting air, then inject 5-10ml of buffer, followed by sample. Make sure that the injection is free of air bubbles.
Alignment suspension is too old.	• Prepare some fresh alignment suspension.
Focussing on small and very large particles in one sample is too difficult.	• Perform manual focusing. Observe the number of detected particles. The focus is best when the number of detected particles is maximum.

15.7 Daily Performance is "Not Acceptable"

When this message appears, in most cases the particle size is usually too large. Trueness (and precision) are not acceptable.





Cause (trueness too large)	Remedy
Quality of the water in which the particles are dissolved is not good.	• Make sure that you use particle free water. • You may filter the water by using an 0.1µm syringe filter before preparing a new alignment suspension.
The dilution of the alignment suspension is too old.	• The 1:250,000 dilution of the alignment suspension should always be prepared freshly. The shelf life of the 1:250,000 dilution is about 30-60 minutes after preparation.
The original (concentrated) PS100 nm beads have expired.	• Order new PS100 nm standard beads.
Some containers release nano particles which disturb the daily performance measurement since they are larger than the PS100 nm beads.	• Prepare the alignments suspension in different vials.
Contaminations in the original (concentrated) PS100 nm beads.	• Order new PS100 nm standard beads.
Background in the field of view of the measuring cell. The background could be considered as particles and makes the daily performance incorrect.	• Clean the measuring cell.

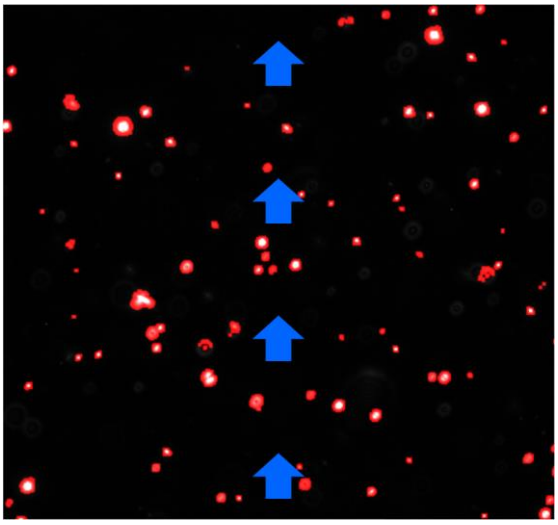
Cause (trueness too small)	Remedy
Vibrations in the vicinity of the ZetaView® instrument.	• Make sure there aren´t any devices that cause vibrations nearby (vortexer, centrifuge, devices with a fan, etc.).

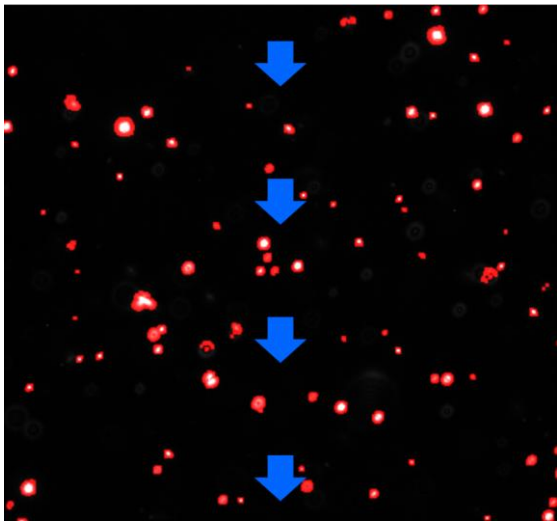
15.8 Excessive particle drift

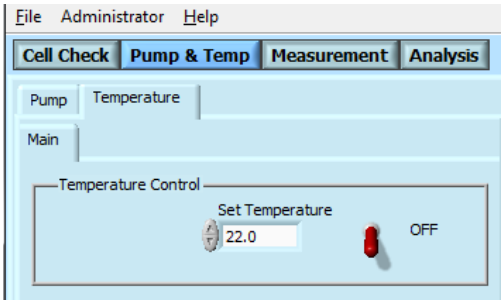
Drift is considered to be the vertical, horizontal or turbulent movement of the particles in the field of view, which is triggered due to other influencing factors than Brownian motion.

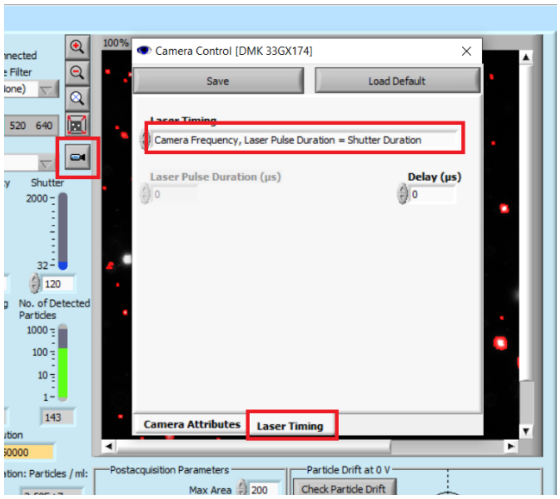
15.8.1 Drift in vertical direction

Vertical drift is the result of thermal influences.



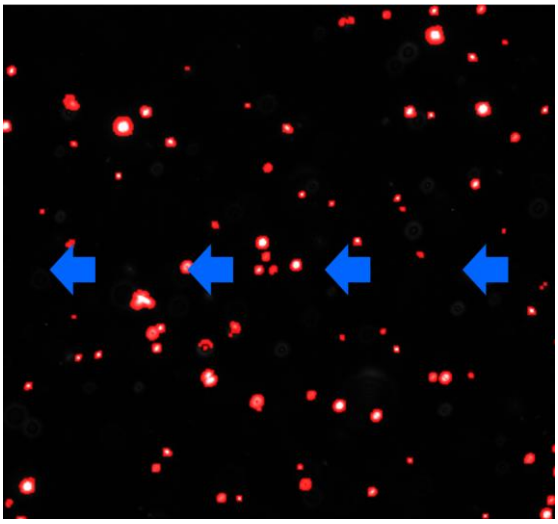


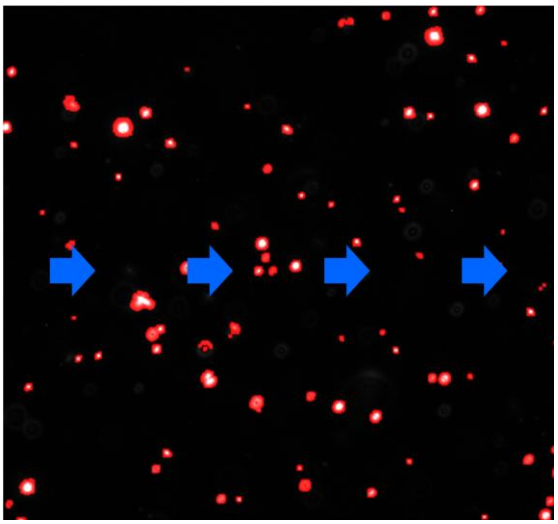
Cause	Remedy
Overheating of the measuring cell by the laser. Since the laser is synchronized with the shutter of the camera, the laser gives pulses when the shutter is open. Low shutter values result in longer exposure times and longer laser pulses. If the shutter value is <50, the laser pulses are long enough to heat up the cell and thus the sample inside. The particles migrate upwards. Slight downward drift can occur near wall position 1 and is the result of better heat dissipation near the heat sink. The liquid is cooler on this side and convection occurs along the long cell axis.	• Increase the shutter value >50 or 60. The particle size and composition must be taken into account when setting the shutter in order to avoid thermal drift. • Some multilaser instruments have more powerful lasers. If necessary, change to a different laser wavelength. • Switch on the temperature control and cool the cell. 

Cause	Remedy
Ambient temperature is too high.	• Switch on the temperature control. • It is also possible to set the cell temperature (not the ambient temperature) just above the measured cell temperature. The system is more agreeable when going slightly higher as opposed to lower than the cell temperature. It stabilizes more quickly, and the system doesn't have to work as hard.
High absorption capacity of laser light by components in the sample (e.g. chemical indicator, etc.).	• Dilute the sample if possible. • Reduce the concentration of suspected absorbing components in the sample, if possible.
The laser timing is set to continuous.	• Make sure that the laser timing is set to "Camera Frequency, Laser Pulse Duration = Shutter Duration". 

15.8.2 Drift in horizontal direction

Horizontal drift is the result of mechanical influences.

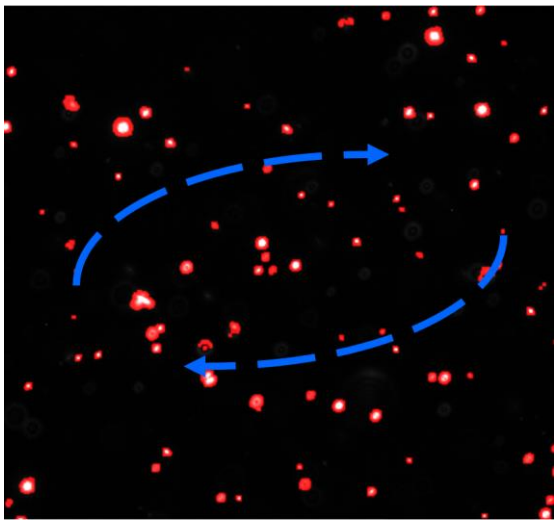


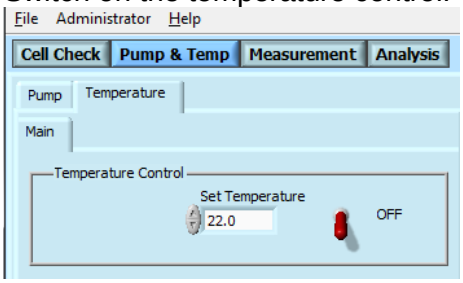


Cause	Remedy
A defective check valve on the silicone tube of the waste bottle or on the cell assembly. A defective check valve no longer closes tightly.	• Replace the check valve.
A damaged or broken measuring cell in the cell assembly.	• The measuring cell must be replaced.
Leaking tubes inside or outside the ZetaView® instrument.	• Check and, if necessary, replace the tubes on the water, buffer or waste bottle. • Have the tubes inside your ZetaView® device checked and, if necessary, replaced.
Air bubbles in the fluidic system.	• Inject a little more sample with more pressure to move the air bubble out of the system. • Inject 5-10ml buffer and fill the measuring cell again with sample. • Empty the measuring cell by injecting air, then inject 5-10ml of buffer, followed by sample. • Make sure that the injection is free of air bubbles.

15.8.3 Turbulent drift

Turbulent drift is mainly caused by mixing two different liquids.



Cause	Remedy
Mixing liquids with different viscosities or ionic strengths inside the measuring cell.	• Prime the fluidic system of your ZetaView® instrument with the liquid / buffer that you use to dilute your sample. • Make sure that the liquid you use to dissolve the particles in your sample is identical or very similar to the liquid you use or have used to rinse the cell. • Wait until the turbulent drift has settled.
Mixing liquids at different temperatures.	• Use liquids of the same temperature. • Switch on the temperature control.  • Wait until the turbulent drift has settled.

15.9 Disturbing stray light patterns or background

Background or different stray light patterns can appear in the field of view of the particles, which may or may not have a disruptive effect. The interference patterns can occur in an empty measuring cell as well as in a measuring cell filled with sample.

15.9.1 Stray light patterns in an empty measuring cell

Stray light patterns can occur if the measuring cell is filled with air at the end of the measuring day and the device is shut down. These patterns may look differently and are usually not a sign of damage. The patterns are created by the fact that after the liquid has been removed by injection with air, liquid droplets remaining within the measuring cell scatter the laser light. The patterns can vary, depending on the measuring position in which the detection optics of the device are located.

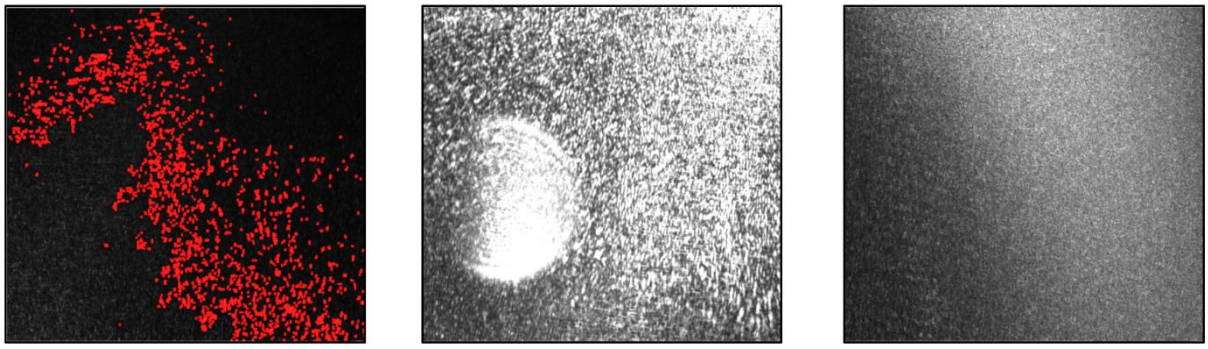


Figure 15-5: Examples of scattered light patterns in an empty measuring cell. Left: in the digital view, middle and right: in the analog view.

15.9.2 Stray light patterns or background in a measuring cell filled with sample

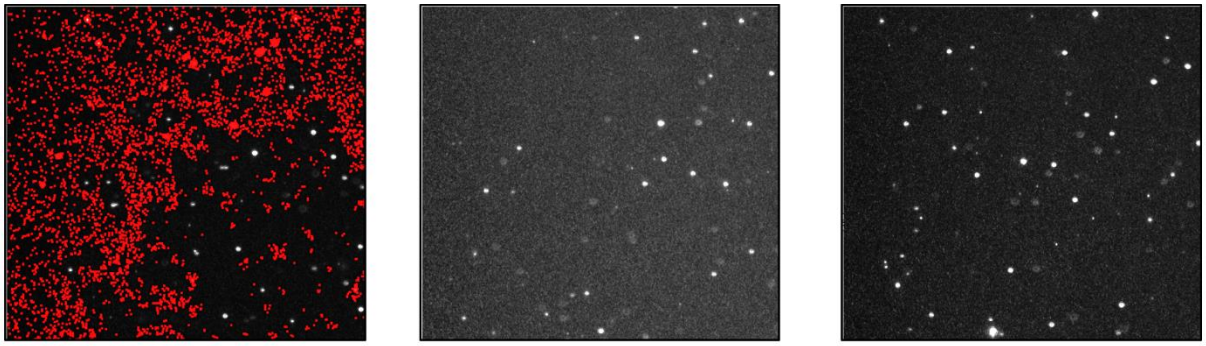
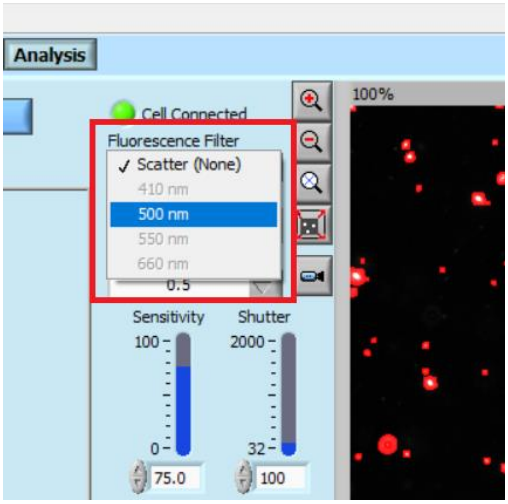
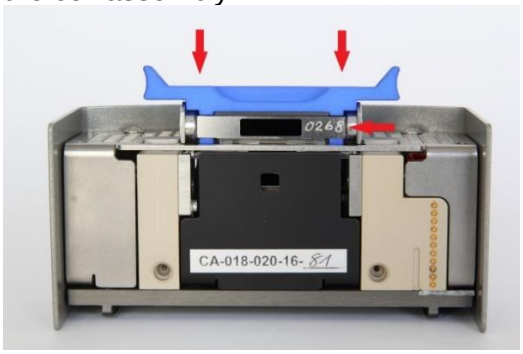


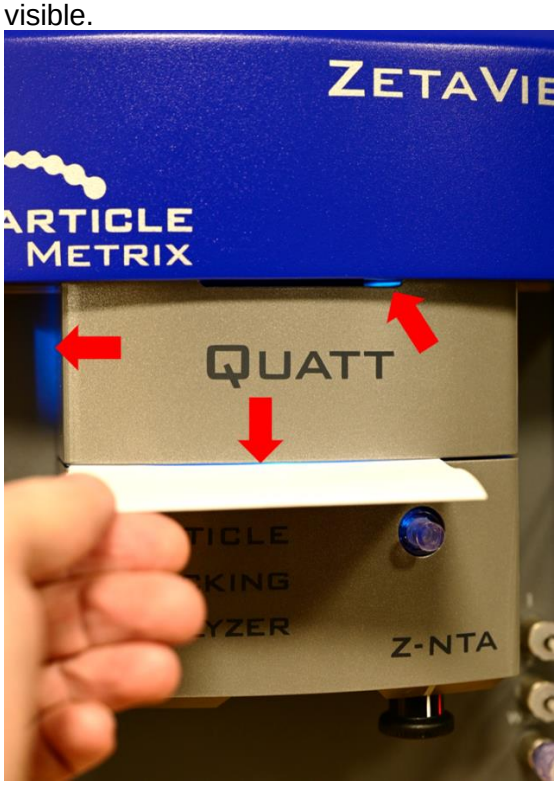
Figure 15-6: Examples of stray light patterns in a measuring cell filled with sample. Left: in the digital view. Middle and right: in the analog view with background of different intensity.

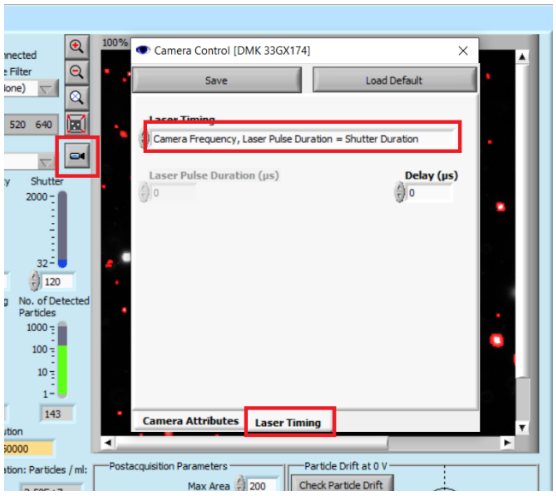
Cause	Remedy
Introduced air bubbles.	• Inject a bit more sample with the syringe with a quick, forceful push to force the air bubble away.
Dirty measuring cell	• Clean the measuring cell.
Too much unbound fluorescent dye or too high concentration of unbound antibodies.	• Reduce the concentration of fluorescent dye if possible. • Reduce the concentration of the antibodies. • If possible, use clean-up columns to remove excess dye or antibodies from your sample.
Too high concentration of detergents (e.g. Tween, Triton, NP40, etc.).	• Reduce the concentration of detergents or omit them if possible.
Too high concentration of indicator (e.g. phenol red from cell culture).	• If possible, dilute the sample. • Use indicator-free cell culture media.
Too high concentration of serum (FCS, FBS from cell culture).	• Dilute the sample or, if possible, reduce the concentration of the serum.
Remaining residues of cleaning solution.	• Rinse the measuring cell again with particle-free water.

15.10 No particles in the field of view

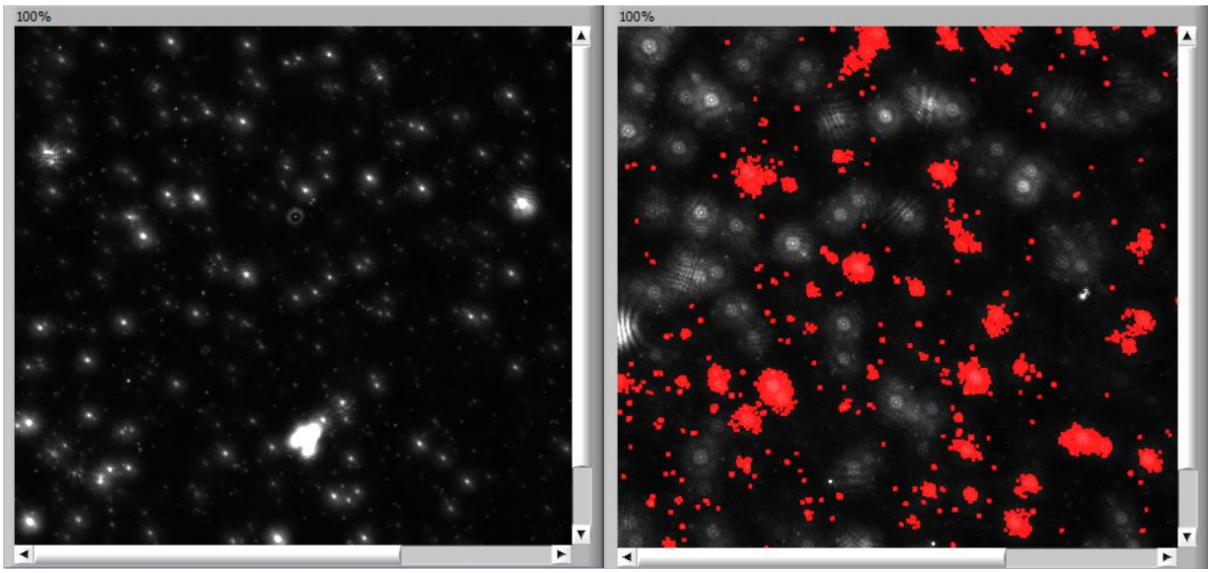
The field of view appears dark.

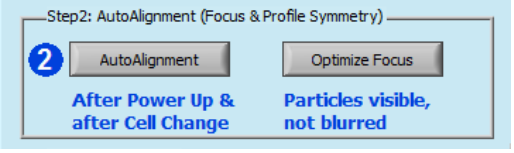
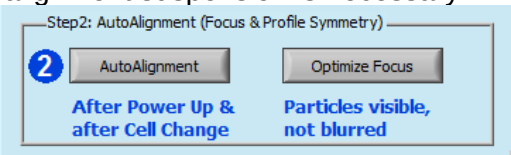
Cause	Remedy
The fluorescence filter is pushed up, although you want to measure non-fluorescent particles in the scatter mode (only for ZetaView® devices with **manual** fluorescence filter).	• Slide the fluorescence filter down. 
The automatic fluorescence filter was activated although you want to measure non-fluorescent particles in the scatter mode (only for ZetaView® devices with **automatic** fluorescence filter) 	• Switch your ZetaView® device to the scatter mode. 
Laser and camera are misaligned. As a result, the particles are not visible or may appear blurred.	• Perform manual focusing. • Calibration may need to be restored. • It may be necessary to reset to the factory calibration. Contact the Particle Metrix Support.
The measuring cell is incorrectly inserted in the cell assembly.	• Insert the measuring cell correctly into the cell assembly.  As shown above, the serial number of the cell must be visible upright on the right-hand side.

Cause	Remedy
The laser does not work.	• Check the function of the laser by placing a white piece of paper in the gap between the cell assembly and the front plate. The reflection of the laser light should be visible.  • If the laser does not work, contact the Particle Metrix Support.
The camera does not work.	• Check the function of the camera by pointing a flash light into the microscope objective with the ZetaView® software open and the cell assembly removed. The instrument should be switched to the scatter mode. The light from the flash light should be visible in the live image. 

Cause	Remedy
Incorrect Laser-Timing.	• Make sure that the laser timing is set to “Camera Frequency, Laser Pulse Duration = Shutter Duration”. 

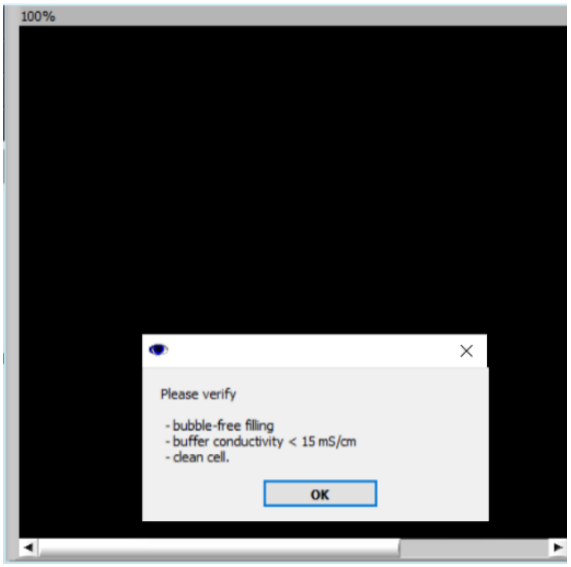
15.11 Particles not in focus

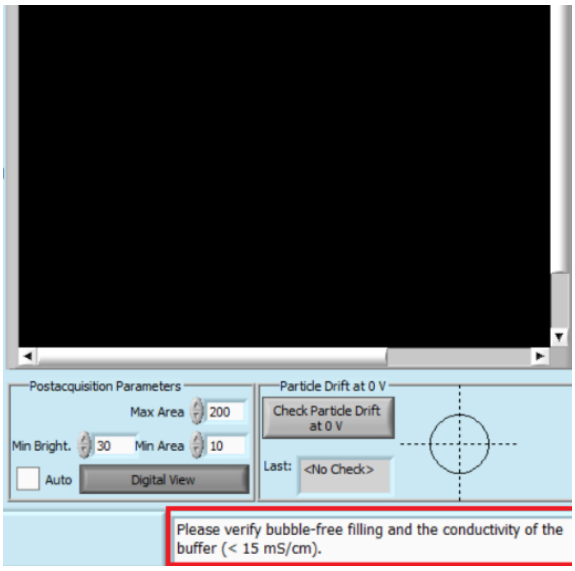


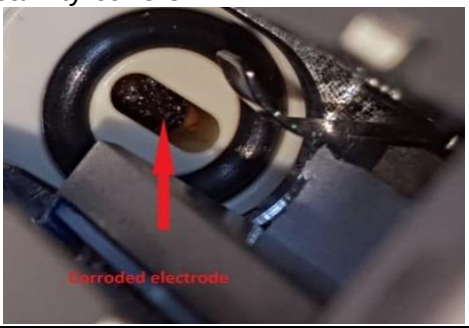
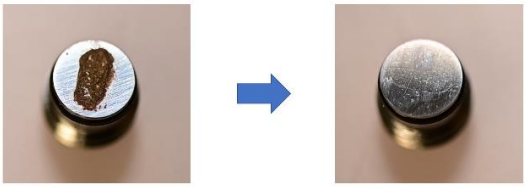
Cause	Remedy
Incorrect focusing. If small particles are in focus, large particles may appear out of focus in a subsequent sample.	• Perform a focus optimization with the particles in the sample. • Under certain circumstances a new AutoAlignment with PS 100 nm alignment suspension is necessary. 
Significant temperature differences between successive samples or other thermal reasons such as constant temperature rise within the measurement day at a warm location of the device.	• Perform a focus optimization with the particles in the sample. • Under certain circumstances a new AutoAlignment with PS 100 nm alignment suspension is necessary.  • Switch on the temperature control and cool the cell down.
Misalignment of laser and camera.	• Perform manual focusing. • The calibration may need to be restored.
Incorrect step ratio between microscope and laser.	• Contact the Particle Metrix Support to check and readjust the step ratio.

15.12 Voltage issues (applies only for the Z-NTA cell assembly)

The message below is displayed if the ZetaView® instrument cannot establish an electrical field during the “AutoAlignment” function or during a zeta potential measurement.



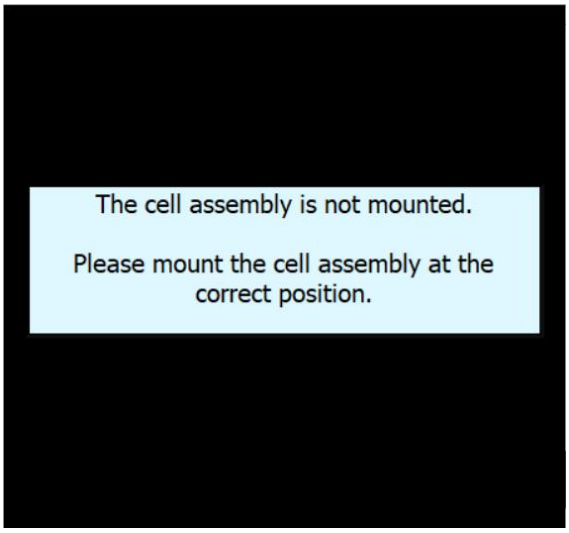


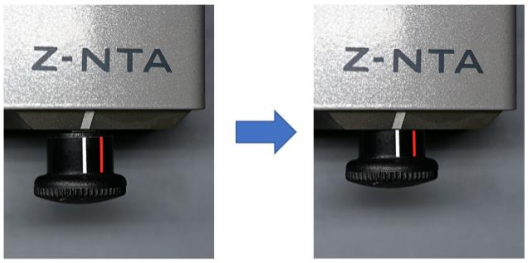
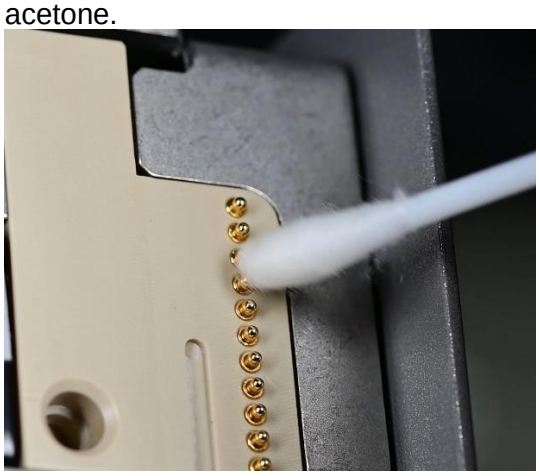
Cause	Remedy
Air bubbles inside the measuring cell that prevent the electrical current flow from one cell flange to the other. 	• Rinse the ZetaView® instrument with air and then again with water and PS 100 nm alignment suspension. Start the autoalignment again. • Clean the measuring cell.
A layer of water on the outside of the measuring cell that connects both cell flanges. This causes an electrical short circuit.	• Dry the outer surface of the cell and the flanges well.
Driver electrodes are corroded. Corrosion is a normal occurrence and is caused by frequent zeta potential measurements in high salinity buffers. 	• The electrodes must be refurbished or replaced by Particle Metrix. 

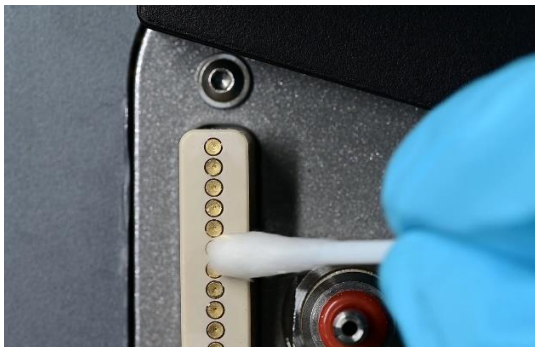
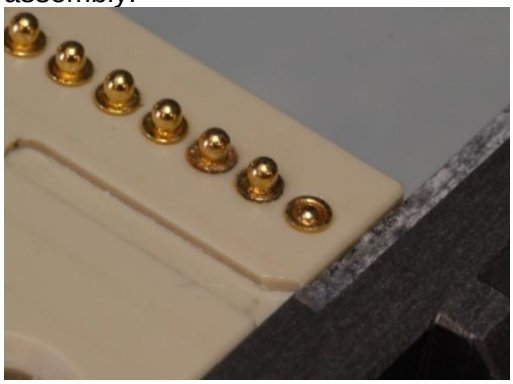
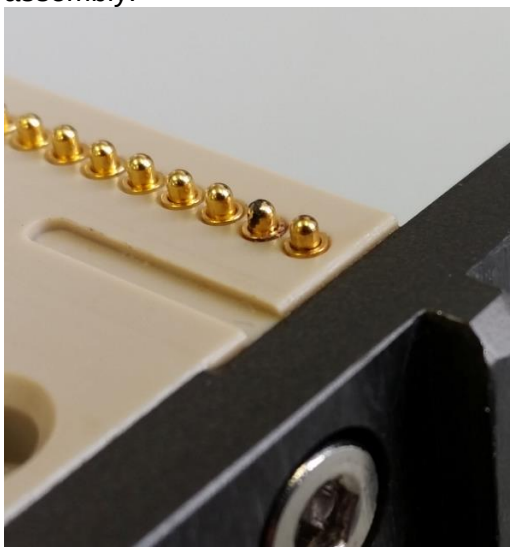
Cause	Remedy
Insufficient electrical connection between cell and cell assembly.	• Tighten both screws that secure the cell carrier to the cell assembly a little more firmly. 

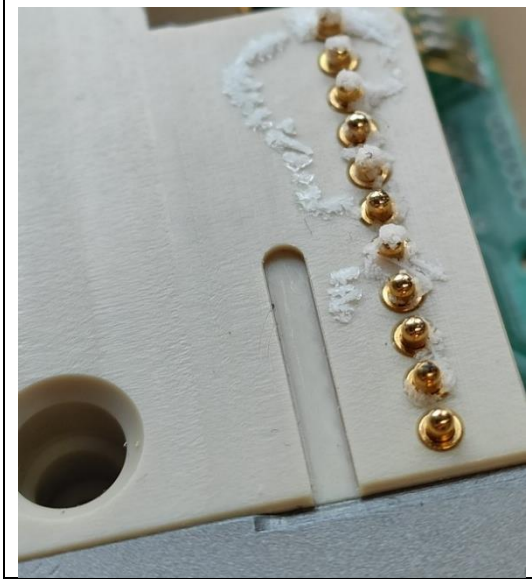
15.13 Connection issues with the cell assembly

The message below is displayed if your ZetaView® device does not recognize the cell assembly although it is installed on the ZetaView® device.



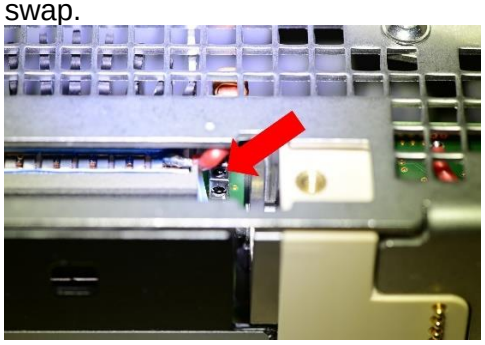
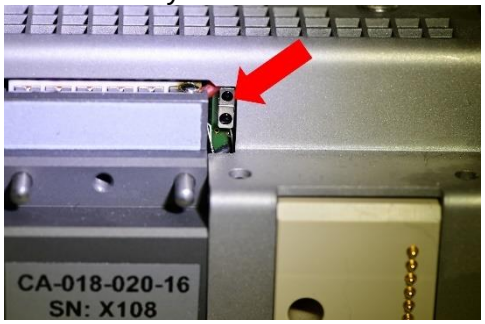
Cause	Remedy
The cell assembly is not locked into position.	• Make sure that the black locking button is fully engaged in the cell assembly. 
The electrical contacts on the cell assembly are not clean.	• Carefully clean the contacts with 70% ethanol or isopropanol or with 50% acetone. 

Cause	Remedy
The electrical contacts on the ZetaView® device are not clean.	• Carefully clean the contacts with 70% ethanol or isopropanol or with 50% acetone. 
Stuck gold pin on the back of the cell assembly. 	• Contact the Particle Metrix Support.
Corroded gold pin on the back of the cell assembly. 	• Contact the Particle Metrix Support.

Cause	Remedy
Crystallized deposits of salty buffers.  	• Carefully remove the deposits with a soft paper tissue (Kleenex or similar) and/or or cotton swap until no more deposits can be seen.

15.14 Connection issues with the measuring cell

The message below is displayed if your ZetaView® device does not recognize the measuring cell.

Cause	Remedy
The measuring cell is not installed.	• Install the measuring cell (ref. chapter 4.3 and 4.4).
The photo sensor is mechanically blocked or dirty.	• Verify that the photo sensor has a clear view on the cell. If necessary, carefully clean the photo sensor with a damp cotton swap.  Location of the photo sensor in the Z-NTA cell assembly.  Location of the photo sensor in the NTA cell assembly.

16 Standard Technical Data

16.1 PMX-120 ZetaView® Monolaser Instrument

General Features

Measurement Principle

- Precision-engineered motorized scanning Nanoparticle Tracking Analysis (NTA) instrument for tracking the movement of individual visualized nanoparticles in suspension
- Real-time visualization of Brownian Motion and electrophoretic mobility, for measuring size, concentration and zeta potential in scattering and fluorescence modes
- One software-controlled laser for enhanced fluorescence measurements
- Manual controlled emission filter for quick changes between scatter and fluorescence measurements
- Fast scanning to acquire and analyze typically 1000 particles in ~ 1 minute
- Software-controlled pumps for flushing and sample sub-dosing

Samples

- Nanoparticles suspended in polar liquids (e.g. water, alcohols) for size, concentration, fluorescence and zeta potential* studies

Hardware

Equipment

- ZetaView® PMX-120 main unit is equipped with cell assembly, laser (see section Lasers) and bottles for buffer rinse
- Power of statistics by automated unique scan and dose control for measurement of 1 - 100 independent sub-volumes
- Optional: Zeta potential option (Z-NTA)*
- Fluorescence option features short acquisition time to avoid negative effect of photo bleaching

Optical Layout

- 90° laser scattering video microscope with x10 magnification
- Automated alignment and focusing of laser and microscope

Lasers

- Available laser wavelengths: 405 nm, 488 nm, 520 nm, 640 nm, and 660 nm at typical laser power of >30 mW
- Pulse duration 0.1 ms up to continuous

Camera

- High sensitive CMOS camera 1920 x 1080 pixels
- Variable frame rate from 1 to 60 Hz for optimum resolution and fast acquisition

Fluorescence Filters

- Long wave-pass (LWP) cut-off filters:
  @405 nm: 430 nm
  @488 nm: 500 nm
  @520 nm: 550 nm
  @640 nm: 680 nm
  @660 nm: 680 nm
- Bandpass filter available on request

Cell Assemblies

- NTA – slide-in assembly for size and concentration measurements in aqueous and organic solvents
- X-NTA – as NTA, for isolating samples from contact with others
- Z-NTA – slide-in assembly for size, concentration and fluorescence measurements plus zeta-potential experiments in aqueous and organic solvents with pumps for 2 different liquids/buffers – for rinsing and sub-dosing experiments, electrical field sensing

Cleaning

- Cell cleaning recommended weekly – cell resistant to >1000 brush cleanings
- Cleaning of driver electrodes required after 1000 zeta potential runs*
- Cleaning kit and basic replacement parts included in delivery

Temperature Range/Control

- Working external temperature range: 5˚C to 45˚C
- Sample temperature control: Peltier temperature control from RTP-5˚C to 55˚C with dew-point sensing

Software

Communication

- Software provided on pre-configured PC, communication via Ethernet

Quality Control

- Cell quality check, daily performance check, outlier control with automatic Grubbs statistical analysis of measurement data

Live Monitoring

- Number of detected particles in scatter and fluorescence mode, scattering intensity, conductivity*, temperature, particle drift

Standard Operating Procedures (SOP)

- Fully customizable SOPs for different samples/applications

Analysis and Reports

- Data Analysis: particle size distribution profiles, concentration, overlays and averaging, scatter plots, zeta-potential distribution profiles, sub-population analysis (using additional ‘Particle Explorer’ software)
- Data export format: AVI, TXT, CSV, FCS
- PDF reports containing key results

Measurement Specifications

Size/Concentration

- Concentration range: 10^5 – 10^9 particles/ml
- Reproducibility: ±5% (for 100 nm polystyrene latex)
- Particle size: 10 nm – 1000 nm (dependent on sample and laser selection)
- Accuracy: ±5 nm (for 100 nm polystyrene latex)
- Reproducibility: ±2 nm (for 100 nm polystyrene latex)

Fluorescence

- Concentration range: 10^5 – 10^9 particles/ml
- Reproducibility: ±5% (for 100 nm polystyrene latex)
- Particle size: 20 nm – 1000 nm (dependent on fluorescent dye and laser selection)
- Accuracy: ±5 nm (for 100 nm polystyrene latex)
- Reproducibility: ±2 nm (for 100 nm polystyrene latex)

Zeta-Potential*

- Working range: -500 to +500 mV
- Concentration range: 10^6 – 10^{10} particles/ml
- Particle size: 20 nm – 5000 nm (dependent on sample and laser selection)
- Conductivity range: 3 uS/cm – 15 mS/cm
- Accuracy: ±4 mV (for alumina zeta-potential standard)
- Reproducibility: ±2 mV (for alumina zeta-potential standard)

General

- Minimum sample quantity: 500 µl of sample at 10^5 particles/ml
- pH range: 1 – 13
- Temperature: 5˚C to 45˚C (external temperature)
- Sample volume visualized and tracked by the camera for a single measurement: 11 x 3.3 nL

Reference Materials

- Nominal 100 nm reference suspension for size
- Nominal 100 nm reference suspension for fluorescence
- Nominal -50mV reference suspension for zeta potential*

Dimensions

Physical

- Footprint (W x D x H): 20 x 30 x 25cm
- Weight: 8.5kg (main unit, PC extra)
- Shipping box with standard content: 48 x 62 x 63cm; 22kg

Electrical

- 90-240V, 47-63Hz, 50VA

Warranty & Support

Warranty

- 1 year (glass excluded)

Service & Support

- Reaction time: 48 hours
- Maintenance, service and IQ/OQ contracts available on demand
- Support via telephone, email and via remote session for trained users free of charge during warranty period
- Training courses for new users available on demand
- Special arrangements and specifications available on demand – quotation required

16.2 PMX-220 ZetaView® TWIN Laser Instrument

General Features

Measurement Principle

- Precision-engineered motorized scanning Nanoparticle Tracking Analysis (NTA) instrument for tracking the movement of individual visualized nanoparticles in suspension
- Real-time visualization of Brownian Motion and electrophoretic mobility, for measuring size, concentration and zeta potential in scattering and fluorescence modes.
- Two simultaneous aligned and software-controlled lasers for enhanced fluorescence measurements
- Software controlled emission filter changer for quick changes between the fluorescence measurements
- Fast scanning to acquire and analyze typically 1000 particles in ~ 1 minute
- Software-controlled pumps for flushing and sample sub-dosing

Samples

- Nanoparticles suspended in polar liquids (e.g. water, alcohols) for size, concentration, fluorescence and zeta potential studies

Hardware

Equipment

- ZetaView® PMX-220 TWIN Laser main unit is equipped with cell assembly, two simultaneous aligned lasers (see section Lasers) and bottles for buffer rinse
- Power of statistics by automated unique scan and dose control for measurement of 1 - 100 independent sub-volumes
- Zeta potential option
- Software controlled double Fluorescence option features short acquisition times to avoid negative effect of photo bleaching

Optical Layout

- 90° laser scattering video microscope with x10 magnification
- Automated alignment and focusing of laser and microscope

Laser sets

- Special TWIN Laser design
- Available laser wavelengths combinations:

  405 nm / 488 nm
  405 nm / 520 nm
  405 nm / 640 nm
  488 nm / 520 nm
  488 nm / 640 nm
  520 nm / 640 nm

  On request, the 640 nm laser can be exchanged by a 660 nm excitation laser free of charge

  at typical laser power of >30 mW per laser
- Pulse duration each laser 0.1 ms up to continuous

Camera

- High sensitive CMOS camera 640 x 480 pixels
- Variable frame rate from 1 to 60 Hz for optimum resolution and fast acquisition

Fluorescence Filter sets

- Software controlled, automated filter changer
- Available long wave-pass (LWP) filter combinations:

  430 nm / 500 nm for 405 / 488 laser combination
  430 nm / 550 nm for 405 / 520 laser combination
  430 nm / 680 nm for 405 / 6X0 laser combination
  500 nm / 550 nm for 488 / 520 laser combination
  500 nm / 680 nm for 488 / 6X0 laser combination
  550 nm / 680 nm for 520 / 6X0 combination

- Bandpass filter available on request

Cell Assembly

- Z-NTA – slide-in assembly for size, concentration and dual fluorescence measurements plus zeta-potential experiments in aqueous and organic solvents with pumps for 2 different liquids/buffers – for rinsing and sub-dosing experiments, electrical field sensing

Cleaning

- Cell cleaning recommended weekly – cell resistant to >1000 brush cleanings
- Cleaning of driver electrodes required after more than 1000 zeta potential runs
- Cleaning kit and basic replacement parts included in delivery

Temperature Range/Control

- Working external temperature range: 5˚C to 45˚C
- Sample temperature control: Peltier temperature control from RTP-5˚C to 55˚C with dew-point sensing

Software

Communication

- Software provided on pre-configured PC, communication via Ethernet

Quality Control

- Cell quality check, daily performance check, outlier control with automatic Grubbs statistical analysis of measurement data

Live Monitoring

- Number of detected particles in scatter and fluorescence mode, scattering intensity, conductivity*, temperature, particle drift

Standard Operating Procedures (SOP)

- Fully customizable SOPs for different samples/applications

Analysis and Reports

- Data Analysis in scatter and / or fluorescence mode: particle size distribution profiles, concentration, overlays and averaging, scatter plots, zeta-potential distribution profiles, sub-population analysis
- Data export format: AVI, TXT, CSV, FCS
- PDF reports containing key results

Measurement Specifications

Size/Concentration

- Concentration range: 10^5 – 10^9 particles/ml
- Reproducibility: ±5% (for 100 nm polystyrene latex)
- Particle size: 10 nm – 1000 nm (dependent on sample and laser selection)
- Accuracy: ±5 nm (for 100 nm polystyrene latex)
- Reproducibility: ±2 nm (for 100 nm polystyrene latex)

Fluorescence

- Concentration range: 10^5 – 10^9 particles/ml
- Reproducibility: ±5% (for 100 nm polystyrene latex)
- Particle size: 20 nm – 1000 nm (dependent on fluorescent dye and laser selection)
- Accuracy: ±5 nm (for 100 nm polystyrene latex)
- Reproducibility: ±2 nm (for 100 nm polystyrene latex)

Zeta-Potential*

- Working range: -500 to +500 mV
- Concentration range: 10^6 – 10^{10} particles/ml
- Particle size: 20 nm – 5000 nm (dependent on sample and laser selection)
- Conductivity range: 3 uS/cm – 15 mS/cm
- Accuracy: ±4 mV (for alumina zeta-potential standard)
- Reproducibility: ±2 mV (for alumina zeta-potential standard)

General

- Minimum sample quantity: 500µl of sample at 10^5 particles/ml
- pH range: 1 – 13
- Temperature: 5˚C to 45˚C (external temperature)
- Sample volume visualized and tracked by the camera for a single measurement: 11 x 3.3 nL

Reference Materials

- Nominal 100 nm reference suspension for size
- Two nominal 100 nm reference suspensions for fluorescence
- Nominal +50 mV reference suspension for zeta potential*

Dimensions

Physical

- Footprint (W x D x H): 20 x 30 x 25cm
- Weight: 8.5kg (main unit, PC extra)
- Shipping box with standard content: 48 x 62 x 63cm; 22kg

Electrical

- 90-240V, 47-63Hz, 50VA

Warranty & Support

Warranty

- 1 year (glass excluded)

Service & Support

- Reaction time: 48 hours
- Maintenance, service and IQ/OQ contracts available on demand
- Support via telephone, email and via remote session for trained users free of charge during warranty period
- Training courses for new users available on demand
- Special arrangements and specifications available on demand – quotation required

16.3 PMX-420 ZetaView® QUATT Laser Instrument

General Features

Measurement Principle

- Precision-engineered motorized scanning Nanoparticle Tracking Analysis (NTA) instrument for tracking the movement of individual visualized nanoparticles in suspension
- Real-time visualization of Brownian Motion and electrophoretic mobility, for measuring size, concentration and zeta potential in scattering and fluorescence modes.
- Four simultaneous aligned and software-controlled lasers for enhanced fluorescence measurements
- Software controlled emission filter changer for quick changes between the fluorescence measurements
- Fast scanning to acquire and analyze typically 1000 particles in ~ 1 minute
- Software-controlled pumps for flushing and sample sub-dosing

Samples

- Nanoparticles suspended in polar liquids (e.g. water, alcohols) for size, concentration, fluorescence and zeta potential studies

Hardware

Equipment

- ZetaView® PMX-420 QUATT Laser main unit is equipped with cell assembly, four simultaneous aligned lasers (see section Lasers) and bottles for buffer rinse
- Power of statistics by automated unique scan and dose control for measurement of 1 - 100 independent sub-volumes
- Zeta potential option
- Software controlled four-fold Fluorescence option features short acquisition times to avoid negative effect of photo bleaching

Optical Layout

- 90° laser scattering video microscope with x10 magnification
- Automated alignment and focusing of laser and microscope

Laser sets

- Special QUATT Laser design with 405 nm / 488 nm / 520 nm / 640 nm excitation lasers at typical laser power of >30 mW per laser
- Pulse duration each laser 0.1 ms up to continuous

Camera

- High sensitive CMOS camera 1920 x 1080 pixels
- Variable frame rate from 1 to 60 Hz for optimum resolution and fast acquisition

Fluorescence Filter sets

- Software controlled, automated filter changer with four long-pass fluorescence emission filters (LWP) with cut-off at 430 nm / 500 nm / 550 nm / 680 nm
- Bandpass filter available on request

Cell Assembly

- Z-NTA – slide-in assembly for size, concentration and dual fluorescence measurements plus zeta-potential experiments in aqueous and organic solvents with pumps for 2 different liquids/buffers – for rinsing and sub-dosing experiments, electrical field sensing

Cleaning

- Cell cleaning recommended weekly – cell resistant to >1000 brush cleanings
- Cleaning of driver electrodes required after more than 1000 zeta potential runs
- Cleaning kit and basic replacement parts included in delivery

Temperature Range/Control:

- Working external temperature range: 5°C to 45°C
- Sample temperature control: Peltier temperature control from RTP-5°C to 55°C with dew-point sensing

Software

Communication

- Software provided on pre-configured PC, communication via Ethernet

Quality Control

- Cell quality check, daily performance check, outlier control with automatic Grubbs statistical analysis of measurement data

Live Monitoring

- Number of detected particles in scatter and fluorescence mode, scattering intensity, conductivity*, temperature, particle drift

Standard Operating Procedures (SOP)

- Fully customizable SOPs for different samples/applications

Analysis and Reports

- Data Analysis in scatter and / or fluorescence mode: particle size distribution profiles, concentration, overlays and averaging, scatter plots, zeta-potential distribution profiles, sub-population analysis
- Data export format: AVI, TXT, CSV, FCS
- PDF reports containing key results

Measurement Specifications

Size/Concentration

- Concentration range: 10^5 – 10^9 particles/ml
- Reproducibility: ±5% (for 100 nm polystyrene latex)
- Particle size: 10 nm – 1000 nm (dependent on sample and laser selection)
- Accuracy: ±5 nm (for 100 nm polystyrene latex)
- Reproducibility: ±2 nm (for 100 nm polystyrene latex)

Fluorescence

- Concentration range: 10^5 – 10^9 particles/ml
- Reproducibility: ±5% (for 100 nm polystyrene latex)
- Particle size: 20 nm – 1000 nm (dependent on fluorescent dye and laser selection)
- Accuracy: ±5 nm (for 100 nm polystyrene latex)
- Reproducibility: ±2 nm (for 100 nm polystyrene latex)

Zeta-Potential*

- Working range: -500 to +500 mV
- Concentration range: 10^6 – 10^{10} particles/ml
- Particle size: 20 nm – 5000 nm (dependent on sample and laser selection)
- Conductivity range: 3 uS/cm – 15 mS/cm
- Accuracy: ±4 mV (for alumina zeta-potential standard)
- Reproducibility: ±2 mV (for alumina zeta-potential standard)

General

- Minimum sample quantity: 500 µl of sample at 10^5 particles/ml
- pH range: 1 – 13
- Temperature: 5°C to 45°C (external temperature)
- Sample volume visualised and tracked by the camera for a single measurement: 11 x 3.3 nL

Reference Materials

- Nominal 100 nm reference suspension for size
- Four nominal 100 nm reference suspensions for fluorescence
- Nominal +50 mV reference suspension for zeta potential*

Dimensions

Physical

- Footprint (W x D x H): 20 x 30 x 25cm
- Weight: 8.5kg (main unit, PC extra)
- Shipping box with standard content: 48 x 62 x 63cm; 22kg

Electrical

- 90-240V, 47-63Hz, 50VA

Warranty & Support

Warranty

- 1 year (glass excluded)

Service & Support

- Reaction time: 48 hours
- Maintenance, service and IQ/OQ contracts available on demand
- Support via telephone, email and via remote session for trained users free of charge during warranty period
- Training courses for new users available on demand
- Special arrangements and specifications available on demand – quotation required

With 'Z-NTA' cell assembly only

Particle Metrix, December 2022

17 Chemical resistance

When measuring nanoparticles of various materials, the solvent needs to be fit for purpose, i.e. it should be ideal to disperse the nanoparticles to prevent agglomeration.

The following table lists the materials of the ZetaView® instrument that come into direct contact with the liquid.

When choosing your buffer, make sure that the chemical resistance is given from all materials to prevent damage of the instrument. When working with acidic or alkaline buffer solutions or aggressive buffers, reduce exposure time (measurement time) as much as possible and rinse thoroughly with water after the measurements. Using aggressive buffers leads to shorter maintenance intervals; change the O-rings, injection ports/check valves and, if necessary, silicon tubes more frequently.

Table 17.1: Chemical resistance of materials in the ZetaView® instrument

Part		**Injection port Bottles Connectors**	**Tubes Connectors O-rings**	**Measuring cell**	**Measuring cell Z-NTA cell assembly Fluidic feedthrough**	**Cell assembly**
Material		Polycarbonate	Silicone	Fused silica	Stainless steel	PEEK
Medium	tested?					
Distilled water	YES	YES	YES	YES	YES	YES
Phosphate buffered saline(PBS)	YES	YES	YES	YES	YES	YES
Acetone	YES	NO	YES	YES*	YES	YES
Isoproanol	YES	YES	YES	YES**	YES	YES
EtOH 98%	YES	YES	YES	YES	YES	YES
NaOH (1M)****	YES	YES	YES	YES	YES	YES
Sodium perchloride (5%)	YES	YES	YES***	YES	YES	YES

* with pipe cleaner or cotton swap / Q-tip
** resistant but not recommended for cleaning
*** No experience. Avoid exposure, may lead to discoloration or corrosion of silicone.
**** pH >13 only briefly for disinfection purposes

This table is subject to continuous development. If you have any questions about the compatibility of other chemicals not mentioned here, please contact the Particle Metrix Support at service@particle-metrix.de.

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