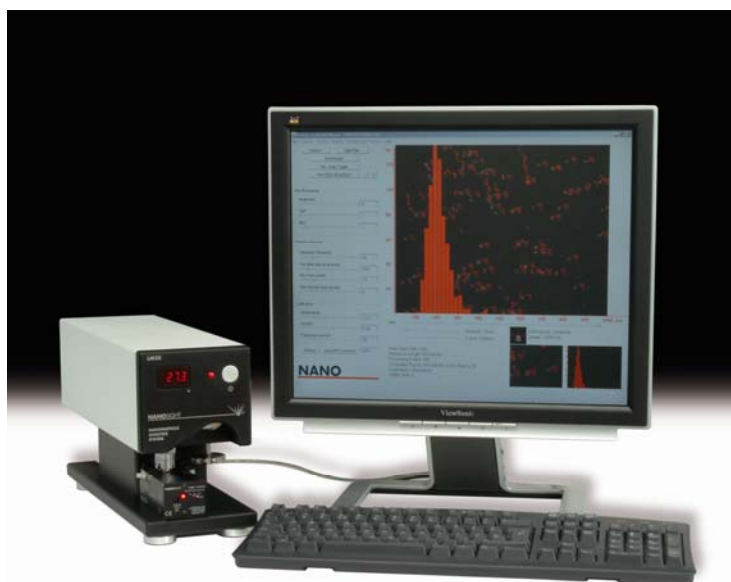




NanoSight LM20 Nanoparticle Analysis System



Operating Manual

Part 1

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The information in this Operating Manual correct Aug 2007. Our instruments are continually being developed. Consequently, we reserve the right to alter the information without prior notice.

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1 Important Notes

1.1 Usage

The Nanosight LM20 has been designed to be used only in conjunction with a LM12 viewing unit.

! CAUTION – USE OF CONTROLS OR ADJUSTMENTS OR PERFORMANCE OF PROCEDURES OTHER THAN THOSE SPECIFIED HEREIN MAY RESULT IN HAZARDOUS RADIATION EXPOSURE

1.2 Safety

While the NanoSight LM12 viewing device is classified (to BS EN 60825-1 (2001)) as a Class1 laser device, it is vital that the following instructions are adhered to.

- The instrument contains a Class 3B laser source which must never be removed from the main instrument housing.
- The unit should only be viewed via a camera fitted within the unit.
- The instrument must not be used in hazardous areas.
- It is the responsibility of the operator to determine the requisite protection for each application.
- Use only the accessories supplied with the instrument.
- Do not use a power adapter other than that supplied with the instrument.
- Laser label located at optical head and on the side of the device.

Do contact support@nanosight.co.uk or NanoSight at +44 1722 349 439 if you have any queries about these safety instructions.

1.3 Servicing

- The LM series of instruments must only be serviced by qualified NanoSight personnel, or approved agents.
- The Nanosight LM20 and LM12 viewing unit contains no user serviceable parts.
- Opening the casing of either the Nanosight LM20 or LM12 viewing unit voids all warranties and could expose hazardous Class 3B laser radiation.

1.4 Handling

The Nanosight LM20 incorporates a rugged housing in a sealed case designed to protect the integrity of the instrument. However, it is a sensitive scientific instrument and should be treated as such. This is particularly important in packing and unpacking the unit from the case. In particular, the **objective lens should not be used as a lever** for moving the system.

The unit contains a static sensitive laser diode and should never be used in circumstances when a static discharge may damage the diode. The Nanosight LM20 should not be dropped or subjected to significant impacts as there are delicate parts contained within.

If transporting the LM20 it should be packed carefully in its original casing. If the system is to be transported by plane and/or motor vehicle, the unit should also be packed in a polystyrene filled cardboard box to protect against vibrations.

1.5 Warranty

NanoSight warrants that the Nanosight LM20 as supplied with its accessories is free from defects in materials and workmanship for a period of one year from shipping to the customer.

During this warranty period, NanoSight will, at its discretion, repair or replace defective products/parts.

Any liability under this warranty extends only to the replacement value of the equipment.

This warranty is void if:

- The Nanosight LM20 or its accessories have been partly or completely disassembled, modified or repaired by persons not authorised by NanoSight Ltd.
- The instrument or instrument system is installed or operated other than in accordance with these operating instructions.
- No other warranty is expressed or implied. NanoSight is not liable for consequential damages except as limited by English law.

2 Introduction

2.1 Contents

The standard instrument is supplied with the following accessories

- LM20 unit and Viewing unit
- Carrying case
- Instruction manual
- NanoSight NTA Training DVD and NanoSight NTA Installation CD
- Fluid injection and removal syringe
- Mains power lead
- Firewire lead
- USB to RS232 lead
- Dongle
- PC
- Calibration latex sample
- Spare 'o' rings

2.2 Unpacking and Initial Inspection

Inspect the shipping container when the Nanosight LM20 is received. If any damage is found, carefully check the contents for completeness and condition. Notify NanoSight Ltd if the contents are incomplete, or if the instrument or accessories appear to be damaged in any way. Keep all damaged packaging, materials and goods for inspection by the carrier.

2.3 LM20 Viewing Unit technical specification

Electrical

Power supply:	110v / 220V 50Hz auto adjusting
Physical Size:	92mm x 66mm x 47mm
Weight:	360g
Operating temperature:	5-40 °C
Humidity:	5-95% non condensing
Casing:	Anodised Aluminium Alloy
Wetted Parts :	303 Stainless Steel, optical glass and acetal
Seal:	Fluorocarbon Elastomer

Laser Classification

Laser Classification	Class 1 to BS EN 60825-1 (2001)
Product measured	638nm CW,
Radiation output	max power <50mW
Embedded laser	638nm CW, max power < 40mW

2.4 LM20 technical specification

Electrical

Power supply:	110v / 220V 50Hz auto adjusting
Physical Size:	360mm x 215mm x 125mm
Weight:	9kg
Operating temperature:	5-40 °C
Humidity:	5-95% non condensing
Wetted Parts :	303 Stainless Steel, optical glass and acetal
Seal:	Fluorocarbon Elastomer

2.5 LM12 Viewing Unit layout

Top plate securing nuts

Caution - Laser aperture located
under top plate

Sample inlet and outlet ports
(Luer fitting)

On-Off switch (not used)

Power socket (not required)

Laser on warning light

Laser Warning label



2.6 LM20 layout

2.6.1 Front Panel

On/Off push button

Power indicator

Y-axis adjuster

X-axis adjuster

Z-axis adjuster

Objective lens

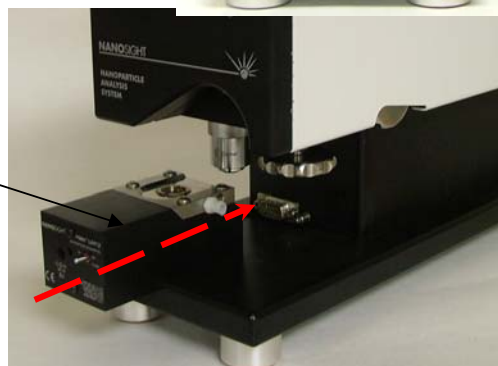
D-connector for LM12

Slide for LM12



2.6.2 Combined view

Viewing unit insertion

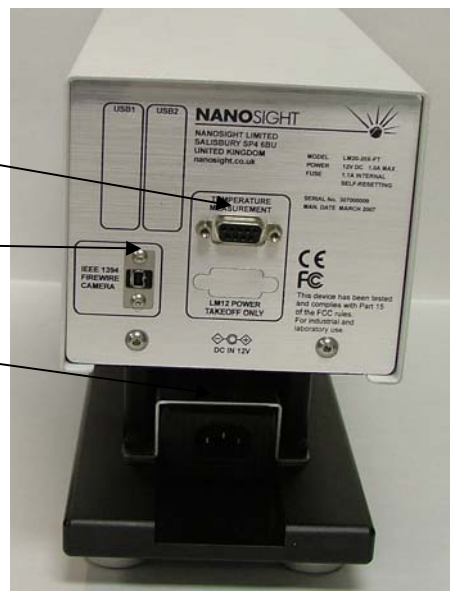


2.6.3 Rear panel

Position for USB-RS232 temperature lead

Position for Firewire lead

Position for power supply



2.7 Maintenance

Although the Nanosight LM20 is easy to use, we recommend that all users read this operating manual prior to use.

Your instrument has been functionally tested prior to shipping. In order to maintain it in perfect working order, we recommend that it is returned to NanoSight Ltd for routine servicing every twelve months.

The instrument should not be left for extended periods of time in strong sunlight as this may damage the components inside.

The Nanosight LM20 contains no user serviceable part.

The casing should be kept clean with the use of a damp cloth in a safe area. To maintain best functionality and to protect against cross contamination during and following use, the flow cell should be cleaned as described in the accompanying manual.

If, for any reason, you experience problems with your instrument, contact NanoSight Ltd (support@nanosight.co.uk).

2.8 Electrical connection and communications

2.8.1 Power

The LM20 unit should be connected to mains power using the suitable power lead supplied or equivalent at the back of the LM20 unit. Unit power can then be checked by pressing and depressing the front panel power switch as shown in section 2.6.

2.8.2 Communications

2.8.2.1 Video

Video communication is through the 4-4 or 4-6 pin Firewire cable that was supplied between the LM20 unit and the supplied PC. The LM20 unit accepts a 4 pin Firewire connection.

2.8.2.2 Temperature

The temperature measurement is relayed to the PC via the USB-RS232 converter cable supplied with the system. The USB end is connected to the PC whilst the RS232 (serial) end of the lead is connected to the back of the LM20 system.

3 Operation

The Nanosight LM20 device is a patented laser-based light scattering system in which particles suspended in a liquid in close proximity to the optical element within the device are illuminated by a specially aligned and focussed laser beam. This allows extremely small particles (down to 10nm) to be seen directly and individually by conventional microscopy.

The system can be used to visualise any particle type in any solvent as long as they scatter light sufficient light.

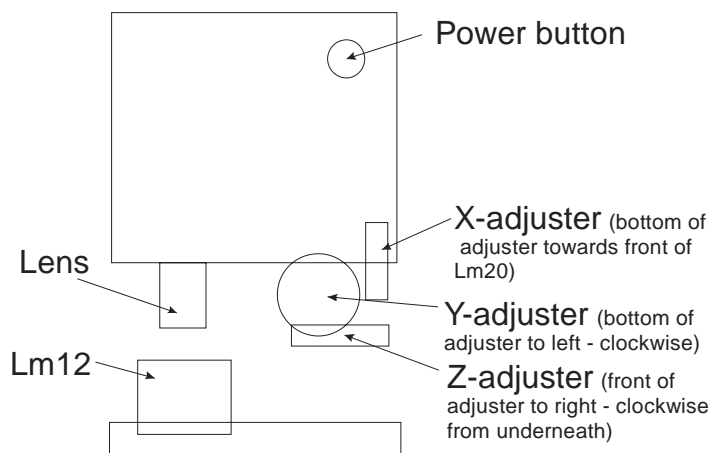
3.1 Adjusting the LM20 to obtain a video

The LM20 is designed to have good beam relocation once set-up. The system is set-up and calibrated at the factory, but it is also necessary to run a quick set-up after shipping. To do this, complete the following steps. If you have problems with this see the troubleshooting section at the end of the manual.

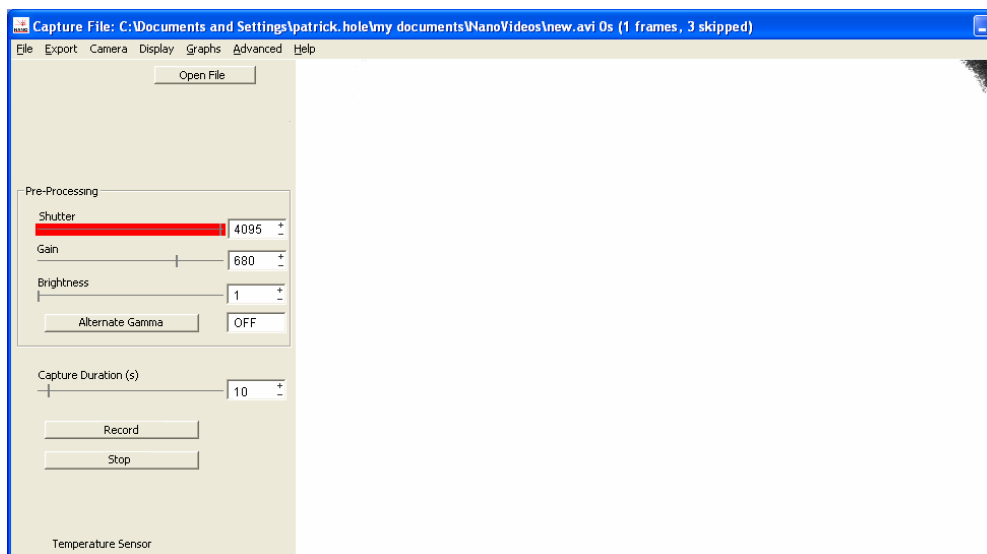
- 1) Load a concentrated sample into the LM12 as described above. This could be, for example, the 100nm calibration particles supplied by NanoSight diluted by a factor of 100.
- 2) Place the Nanosight LM12 viewing unit into the slide and gently push forward until it mates with the D type connector and comes to a hard stop.

! EXTREME CARE MUST BE TAKEN TO ENSURE THE NOSE OF THE OBJECTIVE DOES NOT IMPACT OR TOUCH THE WINDOW OF THE NANOSIGHT LM12 DEVICE WHICH MAY BREAK OR CRACK IF THIS IS ALLOWED TO HAPPEN.

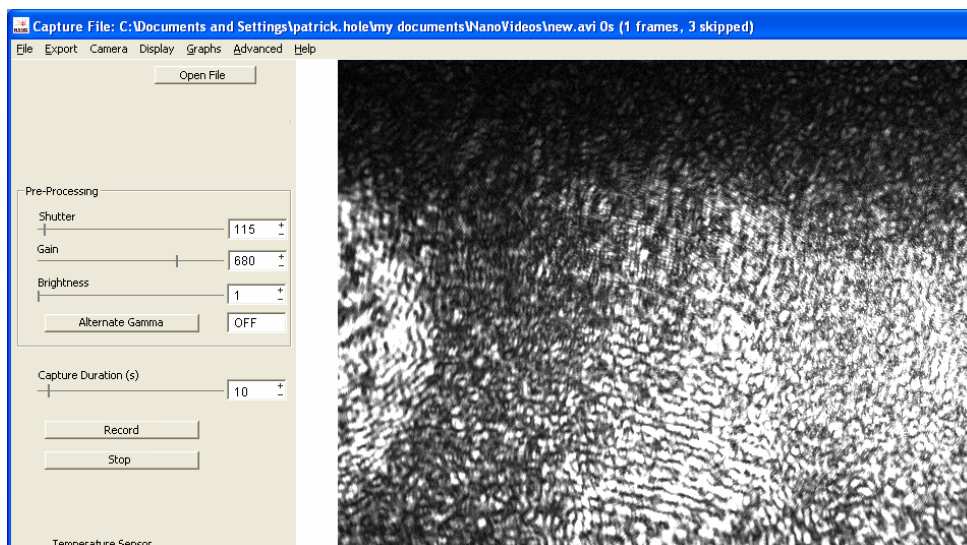
- 3) Ensure all cables are connected between the LM20 and PC and that the LM20 is turned on before loading the NTA software.
- 4) Load the NTA software, click the Capture button and set the capture settings to maximum exposure and gain.
- 5) The LM20 has stops fitted to the slides for greater ease with finding the beam. To reach the origin screw the three (X, Y and Z) adjusters clockwise until there is significantly increased pressure felt (see below). **Note: The stops are not hard stops, so it is possible, with force, to move past the stops. It should not be attempted to move past the point at which an increase in resistance is felt as it is likely to lead to the slide breaking.**



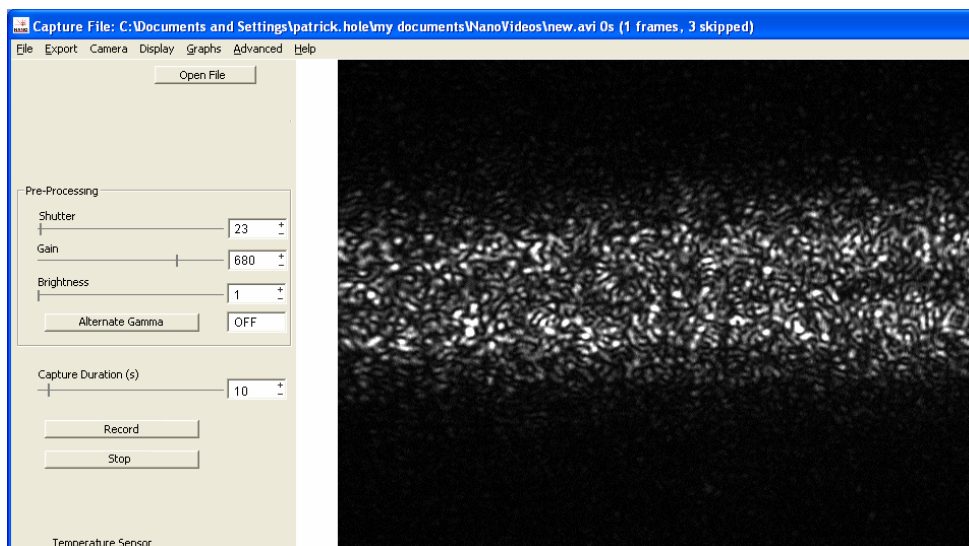
- 6) The approximate area for imaging can be located by unscrewing each of the slides a number of knurls. The numbers are particular to each unit and will have been supplied on a separate sheet supplied.
- a. **X-axis = ? recesses.** The vertical knob on the side of the unit, to move the imaging unit laterally across the beam
 - b. **Y-axis = ? recesses.** The vertical knob at the front of the unit, to move the imaging unit forward or backwards along the beam.
 - c. **Z-axis = ? recesses.** The horizontal knob at the front of the unit to move the imaging unit up or down.
- 7) It is likely that the whole screen will be saturated in this case as shown below.



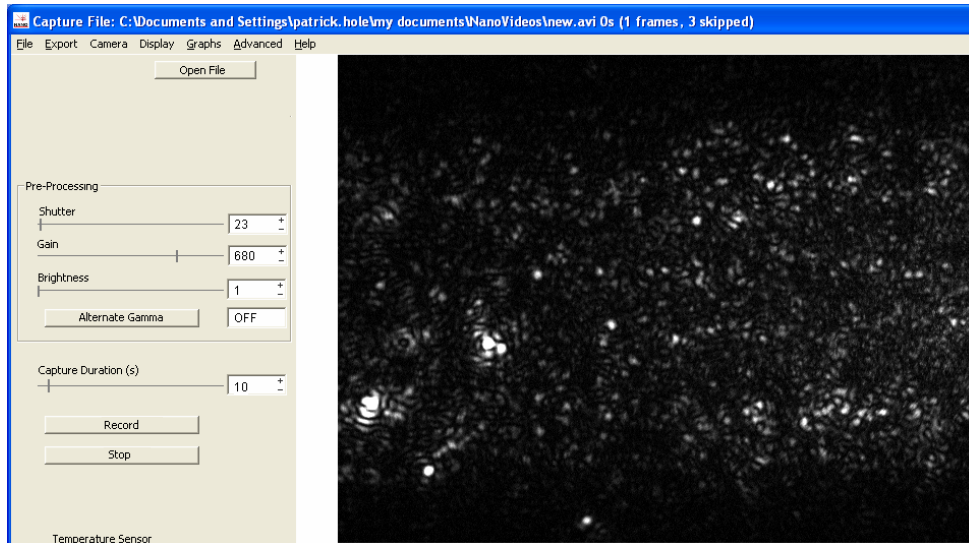
- 8) Reduce the shutter until a small proportion of the frame is saturated – as shown below.



- 9) Refocus and centre the beam as necessary.



- 10) The beam should approximately fill the field of view. If the beam is very narrow (as shown above) turn the y-axis adjuster clockwise, continuously refocusing until a full-frame beam is observed.



- 11) It is likely that the sample is too concentrated (as shown above) to make a good measurement. However now you can remove the LM12, clean the unit and upon reassembly and injection of your sample (at a suitable concentration) when the LM12 is inserted again, the alignment should be very reproducible, with only minor adjustments required for recording.

3.2 Capture a video image

Once a clearly focused image is seen on the NTA software suite screen, an image can be taken using whichever settings are most appropriate. (See Nanoparticle Analysis System – Customer Training Manual).

4 Troubleshooting and Fault finding

4.1 Fault finding chart

Symptom	Cause	Remedial Action
No laser beam visible by eye	LM20 system not turned on (red LED not on).	Check and Connect Switch on.
	LM12 not engaged with D-connector.	Press LM12 firmly into position.
	No sample in cell – beam is reflected into absorbing glass.	Fill cell with sample.
	Sample too dilute – no particles to scatter light.	Increase sample concentration.
	Laser beam misaligned.	Re-alignment required Refer to manufacturer
	Laser not working.	Replacement laser required. Refer to Manufacturer
Beam visible by eye, but not by camera	Looking in wrong area (1)	Follow section 3.3.
	Looking in wrong area (2)	If section 3.3 does not work refer to Appendix A.
Presence or formation of air/gas bubbles	Incorrect sample introduction Dissolved gas in (cold) sample Dirty window or optical element	Introduce sample slowly whilst tilting device De-gas sample before analysis Clean as instructed above
Fluid leaking from unit	Top plate retaining nuts loose	Tighten to finger tight only
	'O' ring seal damaged	Replace – contact NanoSight
Scratched metal surface on optical flat	Optical element damaged	Replace – contact NanoSight
	Incorrect cleaning	Inspect and Replace
Unable to detect or resolve particles	Sample too concentrated – haze of particles seen	Dilute by factor of ten and re-analyse
	Sample too dilute – no particles or beam seen	Use higher concentration sample
	Mis-aligned system	Discuss with NanoSight
	Particles too small	Discuss with NanoSight

Large light scattering centres present	Bubble formation Contaminant particles in sample	De-gas sample Filter or Centrifuge sample
High static (i.e not moving) background	Optical surfaces contaminated	Disassemble and clean as instructed especially optical flat.
Greater range, number of particles than expected or larger particles found	Unclean solvent Carrier fluids causing cross contamination.	Test any solvent before running an analysis to confirm cleanliness of NanoSight unit and solvent. Care should be exercised when using tap water or other unfiltered media, leave tap running for 60 seconds before using. Ensure no carrier liquid left in Luer fittings by blowing through with compressed air.
Beam is not parallel to surface	Solvent refractive index is out of range 1.30 – 1.36	Modify solvent refractive index or, if not possible, contact NanoSight

4.2 Returning Equipment

In the unlikely event you experience a problem with the Nanosight LM20 unit, the device must be returned to NanoSight for repair following contact with us.

Prior to returning any equipment to NanoSight, call to obtain a Material Return number for inclusion on all correspondence.

All equipment should be packed securely in the original packaging, or sufficient to prevent damage.

The following information is to be included with any return.

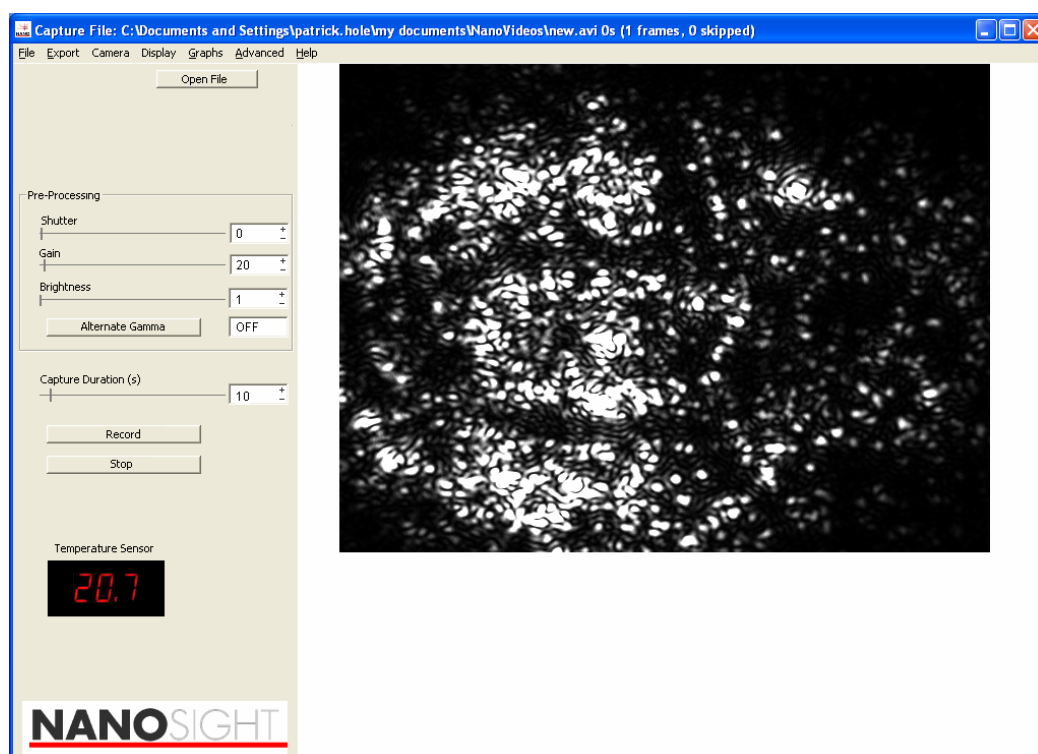
- Sender's name, address, contact telephone number and email address.
- Complete list of equipment being returned including serial numbers
- A detailed description of the problem or reason why the equipment is being returned
- The Material Return number

5 Appendix A – Relocating the beam

If the steps in section 4.3 have not allowed you to find the beam, and you are not encountering problems addressed in the fault finding section, then it is necessary to follow the following set of instructions/tests.

- 1) Check that the slides are operational.
 - a. Locate the origin as outlined in steps 1-5 in section 4.3.
 - b. Observing the screen unscrew each of the adjusters three complete turns.
 - c. Confirm that for all three adjusters the image does change slightly as the adjuster is unscrewed. If not your slides could be stuck and it is necessary to contact NanoSight.
- 2) Relocate the beam manually.
 - a. Locate the origin.
 - b. Unscrew the Y-axis three complete turns.
 - c. Slowly unscrew the X-axis

Use the **x** adjuster on the LM20 to locate the portion of the beam traversing the chamber. Now using the **z** adjuster focus the instrument until the user can see a clear image of the particles. At this point the user should be in the correct focal plane in the centre of the transverse laser beam.



Using the **y** adjuster move along the beam towards the point where the beam enters the chamber, i.e. side of the chamber closest to centre of the LM20 viewing unit. This can be recognised as a static elliptical patch adjacent to the point from which the beam can be seen to emerge to one side.



NanoSight LM20 Nanoparticle Analysis System & NTA 1.4 Analytical Software



Class 1 Laser Product

Operating Manual

Part 2

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1 Basic Introduction

The Class 1 laser device (LM Sample unit) comprises a small Al metal housing (92x66x47mm) containing a solid-state, single-mode laser diode (<20mW, 635nm) configured to launch a finely focussed beam through the 500µl sample chamber. An upper optical window mounted in a detachable stainless steel top-plate through which the sample is viewed by the camera defines the chamber. The base of the chamber comprises a specially designed metallised or opaque optical flat above which the beam is caused to propagate in close proximity to the surface. Particles in the liquid sample which pass through the beam path are seen as small points of light moving rapidly under Brownian motion. A sample (suitably diluted if necessary) is simply introduced into the chamber by syringe via the Luer fittings and allowed to equilibrate to unit temperature for a few moments. For samples containing a high concentration of very large contaminants/aggregates, some pre-filtering may be employed.

The light scattered by the particles could be conventionally modelled by Mie theory (Kerker, 1969; Bohren and Huffman, 1983) though the determination of particle size by light scattering intensities using this device would require significant a priori knowledge of the optical properties of the particle, solvent, collection optics and camera sensitivity and performance. A more attractive alternative, given the ability of the NanoSight LM20 system to visualise nano-scale particles in real time and in liquids, is to dynamically analyse the paths the particles take under Brownian motion over a suitable period of time (e.g. 10's seconds). Despite the rapidity with which particles move (in the sub-100nm size range in particular), such motion can be readily tracked using conventional CCD cameras. Mounted within the LM20 instrument body and operating at 30 frames per second (fps), such cameras can be used to capture video clips of particle suspensions when present in the approximately 80µm wide laser beam within the device. It should be appreciated, however, that the particles are not being imaged. For the nano-scale particle range to which the LM20 system is best suited, the particles act as point scatterers whose dimensions are far below the Rayleigh or Abbé limit, only above which can structural information and shape be resolved. Such videos can then be analysed using a specially designed analytical software programme and from which the size of each particle can be separately determined and accurate particle size distribution profiles derived accordingly.

The video images of the particle's movement under Brownian motion are analysed by a single particle tracking programme (Nanoparticle Tracking Analysis [NTA] 1.4 software). The video can be either captured directly from the camera through the programme or imported as a separate *.avi file. The first frame of the 8 bit video sequence can be user-adjusted in terms of image smoothing, background subtraction, setting of thresholds, removal of blurring etc. to allow particles of interest to be tracked without interference from stray flare or diffraction patterns which can occasionally occur with non-optimum sample types.

Having selected suitable image adjustment settings, the remainder of the video is similarly treated allowing particles to be identified and located on a frame-by-frame basis. Through use of specific selection criteria, movement of individual particles can be followed through the video sequence and the mean squared displacement determined for each particle for as long as it is visible. The user can further select for trajectories whose lifetimes are sufficiently long to ensure statistically accurate results, ignoring those which are so short (e.g. below 5 or 10 frames) that the estimation of diffusion coefficient is statistically inaccurate. Similarly, the occurrence of trajectory cross-over can be accounted for thereby minimising error. From these values, the diffusion coefficient (Dt) and hence sphere-equivalent, hydrodynamic radius (rh) can be determined using the Stokes-Einstein equation:

$$D_t = \frac{K_B T}{6\pi\eta r_h} \quad (1)$$

where K_B is Boltzmann's constant, T is temperature and η is viscosity.



Given that each and every visible particle is separately tracked, it is possible to generate particle size distribution profiles that reflect the actual number of particles thus seen and which is a significant advance on those distributions that are obtained by other dynamic light scattering techniques such as Photon Correlation Spectroscopy (PCS) in which a large ensemble of particles are collectively analysed and from which only a z-average particle mean is obtained as well as a polydispersity quotient indicating the width of the particle size distribution (Pecora, 1985). As all particles are measured simultaneously in PCS, it is frequently the case that a relatively small number of highly scattering larger particles (e.g. contaminants or aggregates) can effectively obscure the presence of the bulk of the smaller particles that may be present (hence the limitation to an intensity weighted, z-average). It is possible, however, through the application of various de-convolution algorithms, to extract particle size distribution structure (e.g. a bimodal distribution) from the results obtained but this approach is reliable only if the two populations are not too polydisperse themselves or too close together in size.

The light scattered by the particles follows conventional light scattering Mie theory, though at the smaller particle dimensions accessed by the device (<100nm), the particles act as Raleigh scatterers in which light is scattered isotropically i.e. they act as point scatterers with no variation in scattered intensity with angle. Similarly, no image of particle structure can be obtained at these dimensions. Of course, if the sample contains larger 250nm+ particles, conventional Mie scattering principles apply and some evidence of interference effects within the light scattered from a particle will be seen.



2 Sample Preparation and Dispersion

2.1 Introduction

As a particle sizing system based on the scattering of light, the NanoSight device measures all particles visible in the sample and each separate light scattering centre is seen as an individual particle, whether or not it is made up of a number of small particles, *i.e.* is an aggregate or agglomerate of primary particles. Should the user be interested in the detection and analysis of such aggregates, sample preparation is restricted simply to effecting a suitable dilution for analysis.

Similarly, should the number of aggregates be low (compared to the number of primary particles within the sample population) the unique capability of the NanoSight technique to simultaneously analyse each and every particle on an individual basis means the presence of low numbers of aggregates will not interfere with the analysis of the bulk of primary particles.

If, however, the presence of aggregates (or agglomerates or flocs) is undesirable in so far as they are present at such a high number that they interfere with or dominate the analysis and result, steps should be taken to remove or disperse them to allow the primary particle population to be sized more accurately. Aggregates can be dispersed in a number of ways using physical processes such as ultrasonication or the application of high shear forces (see later).

2.2 Suspending solvent

The standard LM viewing unit is configured to operate optimally in aqueous-based samples. For routine analysis of samples in which particles are suspended in non-aqueous solvents, it may be necessary for the manufacturer to adjust the device to accommodate solvents of refractive indices outside the range 1.30 – 1.36. While the device can be used to visualise particles in any solvent type, the suspending medium must be sufficiently non-absorbing, non-index matching and gas free

a) Transparent Solvent

For the particles to be visible, it is necessary for the solvent in which they are present to be sufficiently transparent to the illumination beam.

b) Non index matching

Similarly, the refractive index (R_i) of the solvent must be sufficiently different from that of the particles otherwise they will be effectively index matched and therefore invisible. The light scattered by the particle (I_s) varies as a function of the square of the refractive index ratio (d) between the solvent and the particle ($I_s \propto d^2$). It is clear therefore, that high refractive index particles (inorganics, metals etc.) present in low R_i solvents will be more visible (*i.e.* detectable at smaller sizes) than weakly scattering systems such as low molecular weight biological macromolecules.

c) Formation of bubbles

The device contains a high-powered laser and can get warm over extended periods of use. This may cause any dissolved gasses in a sample to produce bubbles which may degrade image quality. In such cases it is recommended that the sample be degassed prior to use.



2.3 Particle Size Range Applicable

The NanoSight system allows particles as small as 10-20nm to be visualised (depending on particle and solvent type) and will allow particles as large as a micron to be seen. However, large particles ($>1\mu\text{m}$) will scatter significant amounts of light and may mask the presence of smaller particles if present at too high a concentration. Similarly, very large particle aggregates ($>10\mu\text{m}$) may affect image quality or might block the sample inlet/outlet ports and should be removed by filtration or centrifugation before the sample is analysed.

2.4 Particle concentration

For particles to be resolved on an individual basis, it is necessary for the sample to be diluted to a particle number concentration of $<10^{10}$ per ml though this limit will vary with sample type. It is best to adjust sample concentration until a clear image is obtained of a population of at least 100 particles in the scattering volume. The laser beam is focussed, on manufacture, to generate a beam waist of approx $100\mu\text{m}$ width of which a length of beam of between $100\mu\text{m}$ is observed.

2.5 Dilution

The sample must be diluted to a number concentration of between 10^6 and 10^{10} particles per millilitre (depending on particle type) and not contain particles larger than $10\mu\text{m}$ diameter. Such particles will degrade image quality and may sediment in the sample chamber necessitating frequent cleaning of the optical surfaces.

The choice of diluent is important and should not adversely affect particle dispersion, conformation, or sample stability *i.e.* it should not induce unwanted flocculation, aggregation or disassembly. Should the sample require dispersion before analysis, gentle agitation can be used, although the introduction of bubbles into the sample will degrade image quality (bubbles are very effective light scattering centres). Small quantities of dispersant, or a suitable detergent, may be employed, but formation of foam should be avoided.

Higher energy dispersion methods such as sonication may be employed. Note however that such vigorous methods frequently cause aggregation if carried out for too long, or at too high an energy input.

2.6 Physical Removal of large particles

Physical removal of large particles can be effected simply by filtration or centrifugation of the sample or allowing the sample to settle over a suitable time period.

2.6.1 Filtration

If filtration is selected, care must be taken to ensure the sample does not adhere to the filter to an unacceptable level. It should also be borne in mind that removal of large numbers of such particles will result in significant loss of material. The limited number of available filter cut-off values can restrict the resolution with which the sample can be fractionated.

2.6.2 Centrifugation

Centrifugation is an alternative to filtration in which samples can be separated according to size and density on a potentially finer separation resolution. This is achieved by varying the centrifugation speed or centrifugal (*g*) force to which the sample is subjected. Note, however, that a particle's buoyant density is an important parameter and can, on occasion, severely limit the ability to remove one class of particles from another. Furthermore, centrifugation can, for sub-micron particles, be a time consuming process.



2.6.3 Settling

It is often advantageous to allow the sample to settle over a suitable time period, during which larger particles are allowed to settle under gravity. Allowing the sample to stand undisturbed for a period of a few hours or, for instance, overnight can often result in larger, potentially interfering particles settling to the bottom of the sample chamber, allowing the user to extract an aliquot for analysis from the upper layer of the sample container.

While any particle which settles out rapidly (e.g. within an hour) represents a particle size which is probably too large for the NanoSight technique to analyse with accuracy and should be allowed to clear the sample, it should be borne in mind that, over extended periods of settling, increasingly smaller particles will settle and the region from which a sample aliquot is extracted from the sample container will reflect such changes in particle population.

It is advisable that the user determines, through experience, which of the above methods are optimal for the sample under analysis.

2.7 Dispersion and Deglomeration

The optimum method of dispersion will depend strongly on the nature of the primary particle, the mechanism by which the aggregate formed and the nature of the solvent in which the aggregate is suspended. Clearly, choosing the correct dispersing agent is central to the formation of a stable dispersion, and the identification and selection of a suitable dispersant requires a significant understanding of the sample and its interaction with its suspending solvent. The agent should not interfere with the measurement itself (*i.e.* for an optical/light scattering technique it should not optically absorb or index match the sample particle), it should be able to contact (wet) the primary particle without causing it to dissolve and it should (perhaps through the addition of a secondary/additional stabilising agent) prevent re-aggregation of the primary particle.

It is frequently the case that, in the absence of an established dispersion protocol for any given sample type, empirical methods are the only way by which optimum dispersion protocols can be arrived at. By way of example, the following table shows the kind of initial choices available to the user wanting to develop a dispersion protocol for their sample:

2.7.1 Typical Examples of Dispersants

For non-aqueous (oil or solvent-based systems), the following dispersants may be considered:

- Phosphate ester
- Block copolymers (acid/base)
- EO/PO polymers (plurionics)
- Phospholipids - fats, oils
- Polyhydroxy stearic acid
- Hydrogenated polyisobutene
- Silicone phosphates

For aqueous (water-based systems):

- (poly)phosphates (oxides in general) e.g. ZnO-NaHMP, TiO₂-TSPP
- (poly)silicates for silica, quartz, clays
- Polyacrylates, polysaccharides

Similarly, the series water → organic acids → alcohols → simple alkanes → long chain alkanes/alkenes represent solvents with decreasing polarity, a major factor in choosing the correct solvent in which to attempt dispersion.



For further guidance on the development of a suitable dispersion protocol we would recommend the user obtain a copy of **ISO 14887:2000** entitled “**Sample preparation -- Dispersing procedures for powders in liquids**” (see www.iso.org or your own national equivalent for details). This International Standard was developed to help particle size analysts make good dispersions from powder/liquid combinations with which they are not experienced. It provides procedures for:

- wetting a powder into a liquid;
- deagglomerating the wetted clumps;
- determining if solution composition can be adjusted to prevent reagglomeration;
- selecting dispersing agents to prevent reagglomeration;
- evaluating the stability of the dispersion against reagglomeration.

This International Standard is applicable to particles ranging in size from approximately 0.05 to 100 μm . It provides a series of questions on the nature of the powder and liquid involved. The answers are used with charts that guide the user to generic dispersing agents that are likely to be suitable for dispersing the powder in the liquid. This International Standard applies only to the preparation of simple, dilute dispersions (less than 1 % by volume solids) for particle size analysis. It does not deal with the formulation of complex and commercial mixtures highly loaded with solids, such as paints, inks, pharmaceuticals, herbicides and composite plastics.

3 LM20 Usage

3.1 Assembling the LM Sample Unit

The LM sample unit can be assembled as follows:

- 1) Switch off unit and remove power lead.
- 2) Clean all necessary parts (see Section 2.6 below).
- 3) Lay cleaned glass element into unit.
- 4) Ensure the 'O'-ring is bedded correctly in its channel in the top plate of the device.
- 5) Lay top plate on glass element and adjust gently until plate locates.
- 6) Drop in four screws and finger tighten loosely alternately in opposite diagonal pairs.

The LM sample unit is now ready to inject a sample.

3.2 Injection into the LM sample Unit

Load the sample into the sample chamber using a syringe (without needle). The Luer fittings are designed to accept standard syringe bodies of all sizes. Note that high pressures can inadvertently be generated when narrow bore (e.g. 1-2 ml) syringe bodies are used.

To avoid generating pressures which might result in the sample bypassing the seals in the sample chamber or damaging the window, care must be taken to **introduce the sample slowly**.

Similarly, care should be taken to avoid the introduction of bubbles at this stage. The LM sample unit should be tilted such that the syringe is **injecting vertically upwards**, allowing the chamber is filled slowly against gravity.

! Occasionally a bubble may be present in the chamber and in the path of the laser beam. This might cause some degree of specular reflection of the laser beam off the bubble surface. The bubble must be removed before viewing through the microscope.



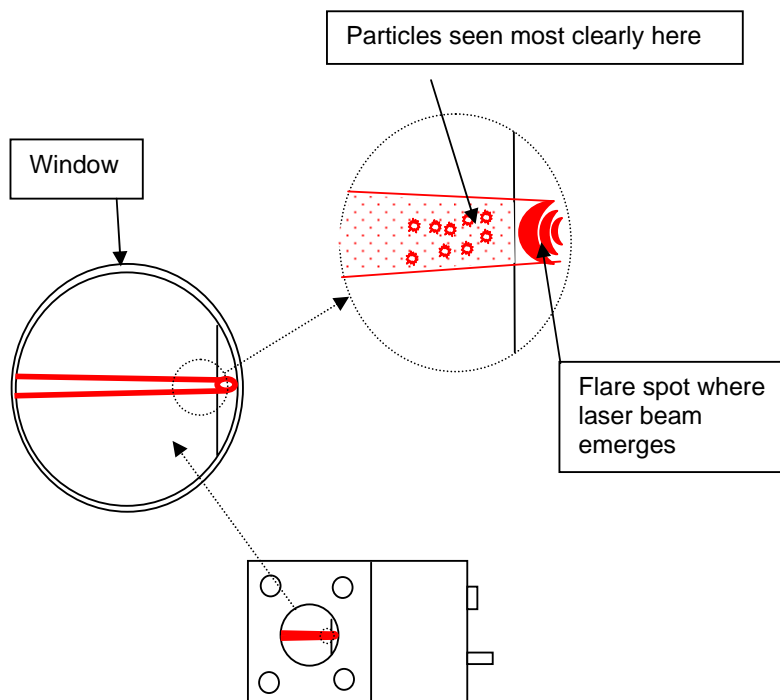
A properly prepared and loaded sample will appear as a clear sample through which the laser beam can be seen as a thin line passing through the sample chamber. If the beam appears very bright and appears to 'bloom' within the sample, the sample is too concentrated and must be further diluted before viewing via the microscope.

3.3 Adjusting the LM20

Once the sample is loaded and checked as above, place the LM unit onto the microscope stage and, if necessary, adjust the position and height of the microscope objective to obtain a clear image of particles present within the beam.

! Extreme care must be taken to ensure the tip of the objective does not impact or touch the window of the NanoSight device which may break or crack if this is allowed to happen.

The laser beam exhibits some divergence as it passes through the chamber. Accordingly, beam width will vary depending on which part of the beam is located. Similarly, looking at different regions of the beam may necessitate a slight refocusing of the microscope taking care to avoid contact between the window and the objective.



3.4 View or Capture Images

Once an image can be seen by the camera, a movie can be recorded using suitable camera settings as discussed in Section 3 below.

3.5 Loading a New Sample

To remove a sample, simply extract using a syringe and rinse the chamber using fresh particle-free water or solvent prior to loading with a different sample. Between samples which differ significantly in terms of solvent, particle type or particle loading, it is advisable to disassemble the top plate and rinse and dry thoroughly the optical element and top plate as described below.

3.6 Cleaning

Depending on sample type and length of use, it may prove necessary to clean the chamber and optical surface of the LM sample unit.

! Before cleaning the system, the laser MUST be switched off and the power disconnected. Failure to take these precautionary steps may expose the user to direct Class 3B radiation.



Once the power has been disconnected, the unit may be cleaned as follows:

1. Empty the sample chamber by extracting all residual fluid by syringe via the Luer ports.
2. Unscrew the nuts securing the top plate (housing the viewing window) and lift off the top plate.
3. Clean the inside of the window by gently wiping it with a cotton bud or tissue wet with either ethanol, acetone or other solvent required to remove all traces of the sample being analysed.
4. Clean and dry both surfaces of the window with optical grade lens cleaning tissue.
5. If available, an air stream (e.g. from a can of compressed air) can be used to blow any residual liquid from the input and output channels through which the sample is introduced into the chamber.
6. Carefully remove the glass optical flat seated in the upper surface of the main device housing. This can usually be achieved by inverting the unit so the optical flat drops out (ensuring it is caught on a soft surface from a small height). Never use a sharp instrument to prise the flat out of its seating or irreparable damage may occur.
7. Carefully wipe all surfaces of the optical flat taking special care with the upper surface of the optical element onto which the top plate is located and the bevelled edge through which the laser beam is launched into the system. This can be done as before with a lens tissue or cotton bud damped with solvent.
8. When all optical surfaces have been cleaned and are dry, reassemble (taking care to ensure the Viton gasket ('O'-ring) on the underside of the top plate is correctly located in its channel), using only finger-tight pressure to clamp down the top plate using the metal screws and tighten with the tool on the LM unit power supply.
9. DO **NOT** OVERTIGHTEN.

! Under no circumstances must liquid be allowed to enter the housing of the LM unit at any time. This will cause irreparable damage to the laser mounted within the unit.

Take care not to abrade or scratch the optical flat surface, or introduce particulates or contaminants onto the surface. If necessary, other cleaning agents may be employed such as dilute mild detergents, or even dilute acid washes prior to a final solvent cleaning step. The NanoSight device is normally supplied with NanoSorb B optical flats which are relatively robust. However, please treat all optical surfaces with the same care as would be employed with equivalent surfaces on your microscope. The optical flats are a consumable part of the instrument and may need replacing if scratched or damaged. Replacement NanoSorb B optical flats can be supplied by NanoSight (pricing on request). If in doubt about the choice of solvent and its compatibility with any part of the NanoSight device, please contact us for further information.

3.7 Storage

Between uses of the NanoSight LM unit, or for longer term storage, the unit must be cleaned as described above and stored with no fluids in the chamber.



4 NTA Software Usage

4.1 Aim

The aim of this Section is to take the user step-by-step through the NTA (Nanoparticle-Tracking Analysis) software from image capture to the production of a particle size distribution chart. The system allows for a degree of user interaction in the analysis and hence this document will allow the user to make appropriate decisions and gain consistent results.

4.2 System Requirements

The software is designed for optimal use with a system operating on Windows XP Professional. It is recommended that the system used to run the software should have at least 1GB of RAM, 80GB of free hard disc space (for video storage) and the equivalent of a 3GHz Pentium processor or higher.

It is not possible to test the software with the full range of computers on the market. Therefore NanoSight strongly recommends the use of the PC that they specify directly and is included with your order.

The software requires a blue security dongle which should be inserted into the USB port on the computer and installed prior to using the software. Information regarding installation of the software and the dongle can be found in the installation document.

4.3 Start-Up

The software comes preinstalled on NanoSight supplied computers and can be loaded either from the desktop (NTA 1.4) or from the start menu (under All Programs → NanoSight → NTA1.4).

4.4 Image Capture

This function can be initiated using the 'Capture' button on the main interface.

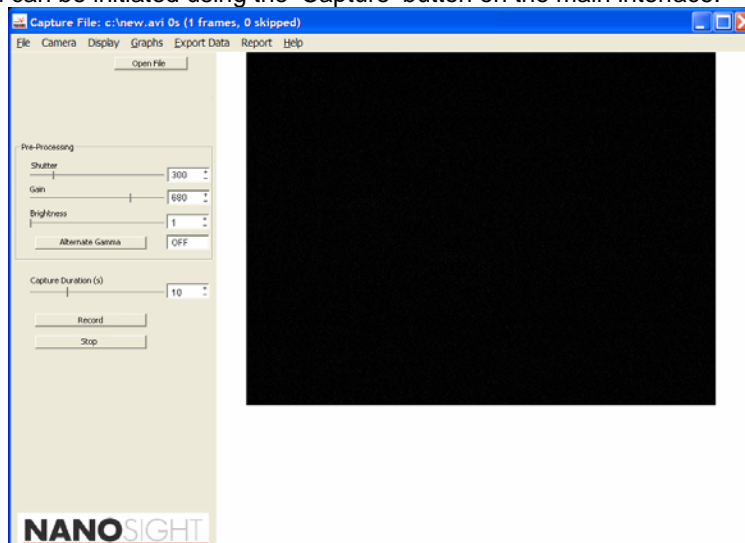


Figure 1: Video capture interface.



Step 1: Shutter – Determines the length of time the shutter is open for and therefore how much light is captured from the particles. Dim particles (associated with small particles or particles with a low refractive index) will need to be captured with a longer shutter opening time. Too avoid over exposure, bright particles (associated with larger or high refractive index particles) should be captured with a shorter exposure time. Due to the fact that the system is dynamic, in the period in which the shutter is open, the particles will move and hence produce a degree of image smudging, or blurring. This may adversely affect the centering of the particles and hence the estimated particle distribution profile. Therefore, to increase the accuracy of the system, the images should be captured using the lowest shutter speed compatible with visualising the particles.

Step 2: Gain – Acts to multiply the intensity reading from each pixel in the array. The gain should generally be set high to allow the shutter to be reduced to a minimum in order to reduce particle blurring.

Step 3: Brightness – The Marlin camera used to capture the image has a dynamic range of 256 gray scale increments. Altering the brightness shifts the intensity of this 256 grey scale range. Setting the brightness so as to detect very dim particles, may result in bright particles in the same image having an intensity which may exceed the maximum of the 256 grey scale (saturation). By varying the brightness of the 256 grey scale values to allow for bright particles, dim particles may now be below the detection threshold of the system. Generally the brightness should be left as zero (the default value).

The brightness, gain and shutter can be used in combination to produce an optimal image for the analysis suite. Particle centering is performed by finding the centroid of the intensity profile for each particle. If all of the pixels within the footprint for a particular particle are over exposed, then the software has no intensity profile from which it can find the particle centre and a simple geometric centering is performed. Therefore to achieve accurate particle sizing, the camera settings should be manipulated so as to produce images which have an intensity profile with few/none of the pixels 'topping out' (saturating) in terms of the 256 grey scale. Also, as mentioned previously, the shutter should be kept to a minimum to avoid particle blurring during the exposure period.

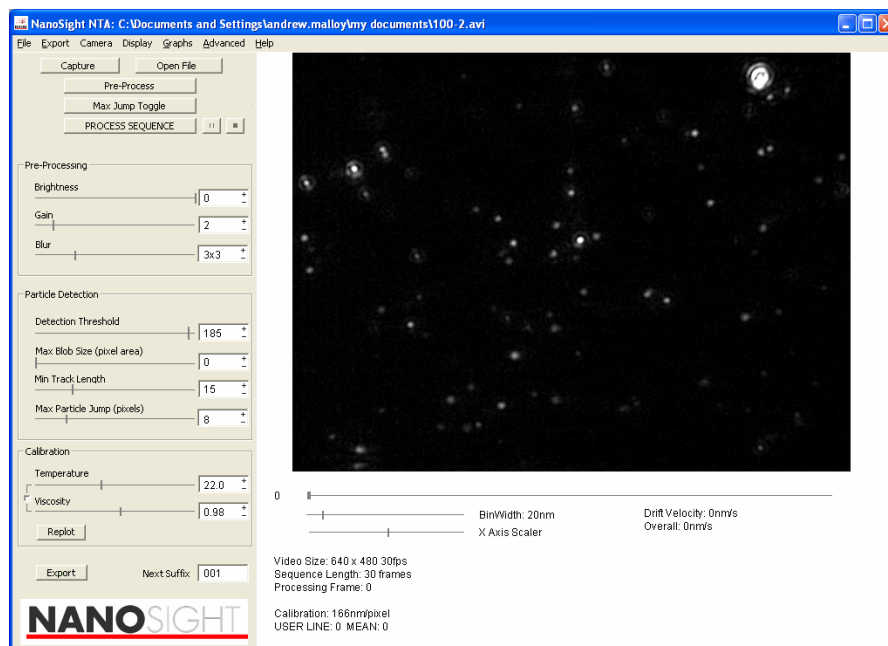


Figure 2: Example of an optimal image to allow accurate particle centering.

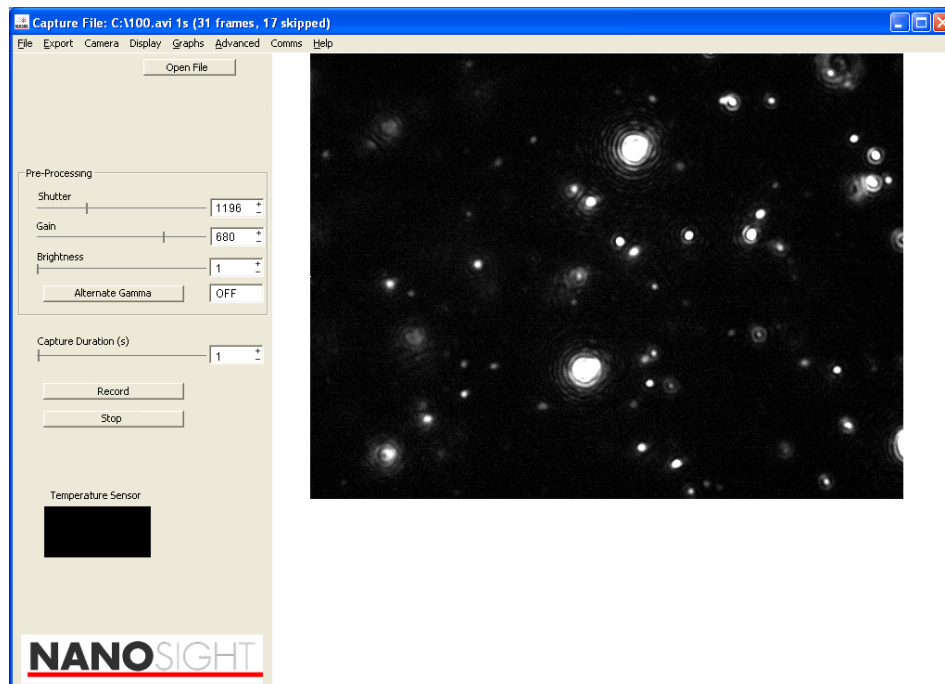


Figure 3: Example of poor image for analysis suite.

As can be seen from Figure 3, the particles appear as over-exposed bright white discs. Each pixel within the footprint of the particle will have a saturated intensity of 255, and hence it is very difficult to centre the particle. In addition, the camera settings are such that the image suffers from increased background noise.

Step 4: Alternate Gamma – This function alters the relationship between the intensity recorded on the camera CCD array and that which is displayed by the computer monitor. Without gamma correction the relationship is that of straight line graph (linear). With the correction switched on, the camera preferentially amplifies the signal from the dim pixels whilst maintaining a straight line relationship for brighter pixels. This allows both small and larger particles to be visualised in the same image without the brighter particles dominating the image. Use this option when very small particles are present and with polydisperse systems.

Step 5: Set Capture Duration – This determines the length of video which is captured. Longer videos allow more accurate particle distributions to be produced. The camera operates at 30 frames per second (fps), therefore a 20 sec clip comprises 600 frames. Adequate capture duration is largely dependant on the size of the particles present in the sample and their concentration. Small particles have a more exaggerated Brownian motion, hence the rate of particle refreshment is increased and sufficient numbers of particles can be included in the particle size distribution. With low concentrations of larger particles, longer capture durations should be used to allow a sufficient number of particles to be included in the particle size distribution.

Step 6: Record – Starts the video capture. The video can be stopped at any time by using the 'Stop' button. Figure 1 shows the camera capture interface. As can be seen at the top of the image, the software informs the user if any frames are dropped from the camera upon capture. Dropped frames are usually associated with a slow processor speed, hard discs which are nearly full, or with high computer activity. It is important to record a video clip with no dropped frames as this will affect the calculated particle sizes. If the computer drops (skips) frames whilst the video is capturing, the preview screen can be switched off in order to help reduce the computer workload. This option is located in the 'Camera' section of the



toolbar under 'Display Video During Capture.' At the end of the video capture the user is prompted to choose the destination where the file is saved.

4.5 Image Processing

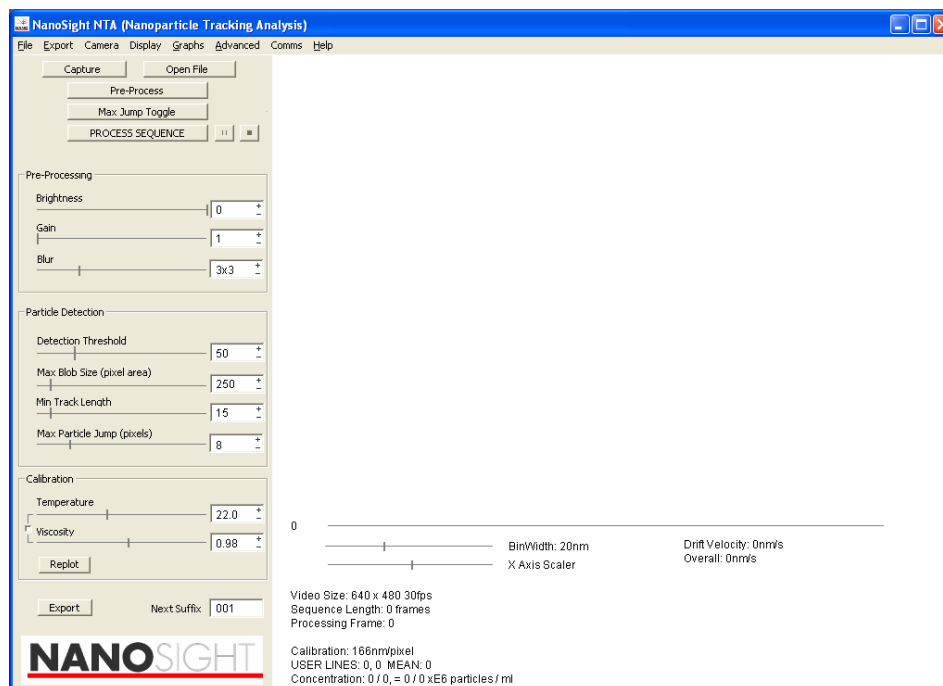


Figure 4: Image processing interface.

Step 1: Open File – Allows the user to load a previously captured video clip into the analysis suite. Upon opening the file the software will automatically place red crosses on the image denoting particle centres; the position of these centres is determined by the default filtration settings. These settings may not be appropriate for all sample types and hence the following steps can be used to vary the filtration. The aim of this section of the analysis suite is to filter the image and set the detection thresholds so that as many particles as possible are identified with a particle centre, but without areas of background noise being falsely identified as a particle.

Step 2: Brightness – This is the same function as in the camera capture section, although it varies the captured image brightness and not the live image.

Step 3: Gain – Again, the same function as in the camera capture section. The gain can be used to multiply the intensity value for each pixel, bringing dim particles above the minimum value that the software accepts as a particle (see Detection threshold – Step 5). High gain values may also increase background noise to a level at which it may also be falsely deemed a particle. Generally, gain and brightness are used antagonistically. Gain can be used to increase the numerical difference between the particle intensity and that of the noise. The brightness can now be used to drop the intensity of the background noise below the detection threshold.

Step 4: Blur – The blur function allows the user to smooth the intensity profile for each particle. This allows accurate centering of the particles and also for the user to remove false centres from the analysis. Due to the manner in which the particles scatter light, they may appear to have a ring structure around the particle (due to diffraction). With incorrect filtration settings these may have false centres located on them. In these instances, blurring can be used to merge the rings into the main region of the particle and hence create a footprint with



one centre and no false centres. Blurring can also be used effectively to remove false centres located in regions of noise. Note, however, that 'false' centres are frequently very transitory and short lived. As such no such centre is likely to survive for more than a low number of frames and therefore is unlikely to be analysed by the programme given an adequate 'minimum track length' setting described below.

Step 5: Detection Threshold: This value determines the minimum intensity required for an area of light intensity to be determined as a particle. In practice, the user should aim to adjust this setting such that all clearly recognisable particles have been centred with a red cross and there are few, if any, false centres marked by red crosses where there are no clearly recognisable particles.

Step 6: Maximum Blob Size – This determines the maximum size (in terms of number of pixels) of a particle for it to be deemed a particle. This feature can be used to remove bright particles from an analysis. Large, high refractive index particles will scatter large amounts of light and hence will appear to have a larger footprint than a dimmer particle. In a mono-dispersed sample this feature can be used to discount contaminant particles. Alternatively this feature can be used in a poly-dispersed sample to discount the larger population of particles. This allows a two-step approach of sample analysis allowing accurate, independent measurement of two particle populations within the same video clip.

Step 7: Minimum Track Length - This determines the minimum number of frames a particle must be tracked for to be accepted as a valid particle. Brownian motion is a random process whereby a particle may move a large or small distance over a time period (e.g. within the 33msec between frames at a frame rate of 30fps). On average however, a particle of a specific size will move a fixed and reproducible distance if enough individual movements are averaged. Therefore the minimum lifetime can be used to ensure enough particle steps are taken for an adequately accurate average of the particle movement to be produced. Increasing this value will increase the confidence in the results, but will decrease the number of particles analysed. Larger particles can be analysed using a longer minimum lifetime than smaller particles because larger particles have a more limited Brownian motion than smaller particles and will remain in the detection volume for longer. As particle movement becomes more restricted the accuracy with which the size can be determined by Brownian motion is reduced; this can be offset by only allowing particles in the analysis which the software has tracked for extended periods of time. Smaller particles should be analysed using a shorter minimum lifetime as these particles may only exist within the field of view for short periods of time, due to their increased speed of Brownian motion and correspondingly greater distances travelled.

Step 8: Maximum Particle Jump: The value the user selects here determines the maximum number of pixels from its position in frame 1 that the software will look for a particle in frame 2. It also establishes an exclusion zone around a particle. If another particle enters this exclusion zone then the software excludes the information from both particles. This prevents the software erroneously joining the tracks from two different particles.

It is important to set this value correctly as it has the potential to influence the estimated particle size distribution. By setting this value too large, larger exclusion zones are created around a particle and a lot of valid information is lost through a reduction in number of tracks analysed. By setting it too small, the particle may jump out of the zone and its movement will not be picked up by the software. In order to accurately determine the maximum distance a particle jumps in the sample, the 'Max Jump Toggle' button should be used. This feature zooms in on a point at which a particle can be seen and which the user selects with the mouse and toggles between two successive frames. This allows the user to estimate the distance each particle jumps; the crosshair can be used as an aide to estimate the jump distance in pixels. The size of the crosshair alters as the user changes the 'Max Particle Jump' slider. The user should sequentially zoom in on several particles to estimate the maximum distance jumped by the particles and 1 or 2 extra pixels should be added to the



value to allow a margin for particles which may uncharacteristically jump slightly larger distances. This value should then be input as the Maximum Particle Jump.

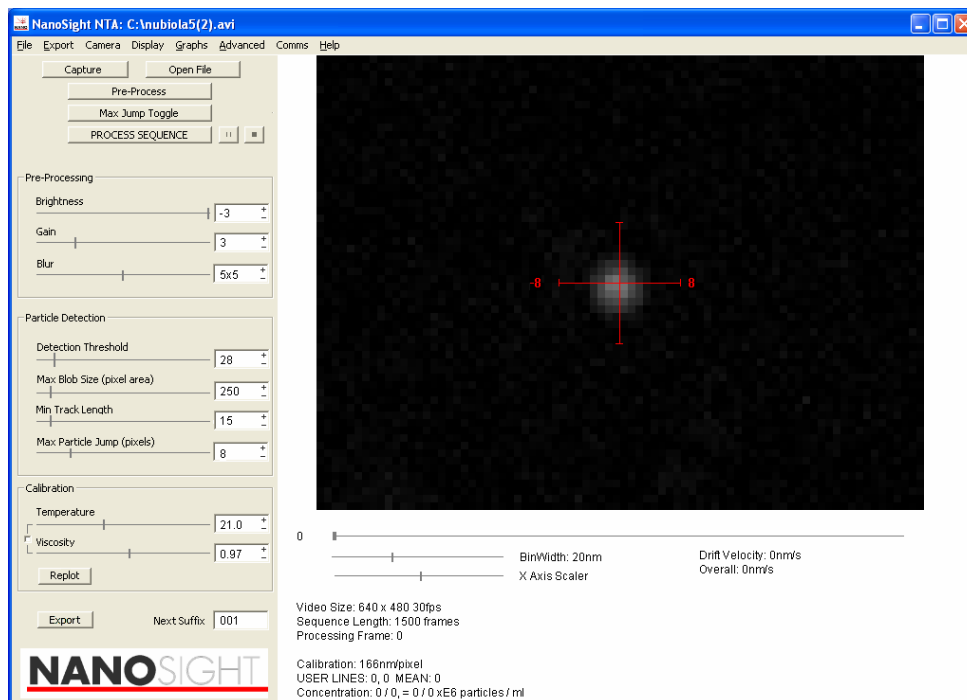


Figure 5: Image 1 of a zoomed-in image showing the position of a particle in two successive frames.

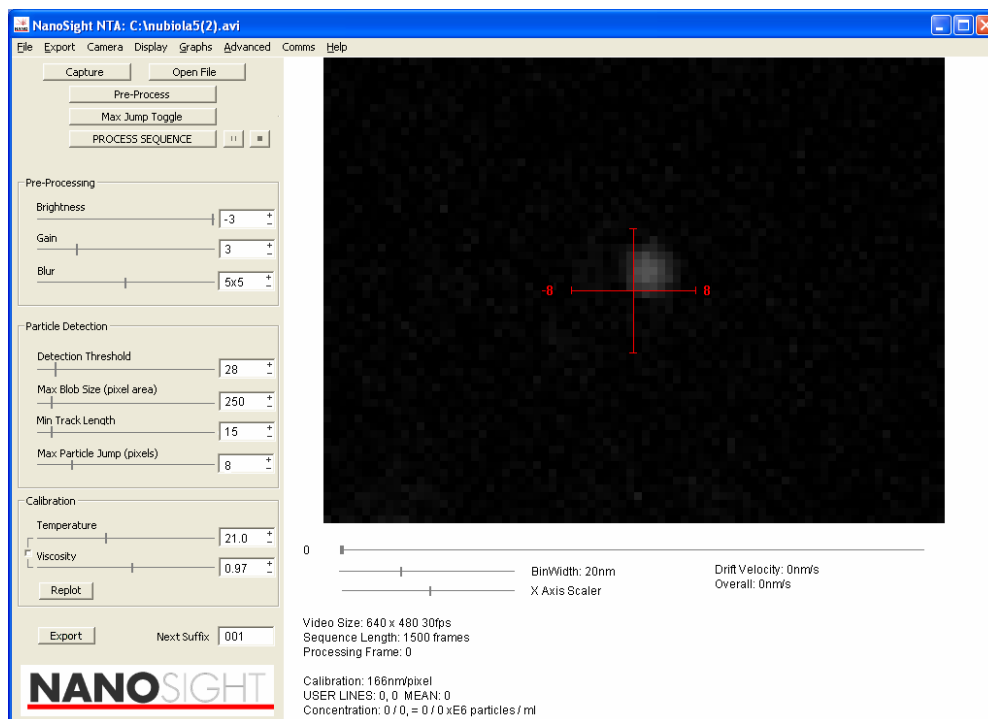


Figure 6: Image 2 of a zoomed-in image showing the position of a particle in two successive frames.



Step 9: Pre-Process – This button will take the user back to the interface showing the first frame of the video allowing the user to alter the image settings further.

Step 10: Temperature and Viscosity – The Brownian motion of a particle is influenced by the temperature and the viscosity of the fluid in which they are suspended. These values therefore need to be input by the user for accurate calculation of the particle sizes. By clicking the tick box next to the temperature slider bar, the temperature and viscosity are linked. If the box is red, the temperature will automatically be linked to the viscosity of water. When the user changes the temperature, the viscosity changes automatically for samples dispersed in water. For samples not dispersed in water, un-tick the box (it will appear white) and enter the viscosity value for the solution at the indicated temperature.

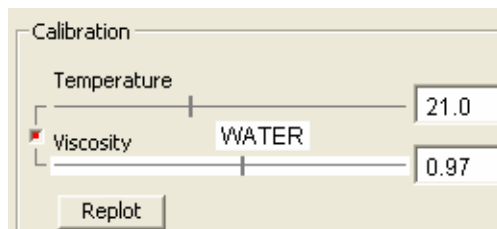


Figure 7: Diagram to show linked values of temperature and viscosity for water.

Step 11: Bin width adjustment and X Axis Scaling and off-setting– These two slider bars allow the user to determine the plotting parameters of the particle distribution graph. The bin width allows the user to vary the size of the histogram bin in nm. The X Axis slider allows the user to vary the maximum particle size in nm of the particle distribution. The X axis can also be 'picked up' (by clicking on it with the mouse) and shifted or offset from side to side.

Step 12: Process Sequence – Starts the analysis. The analysis can be paused, restarted and stopped at any point. When paused, the analysis can be incremented one frame at a time by pressing the forward arrow on the keyboard. This can be useful for following the analysis of any given particle in more detail. Note, however, the analysis cannot be run backwards using the back arrow.

Step 13: Excluding An Area From The Analysis: During video capture, unwanted light scattered from a static object, such as dirt on the optical flat or chamber window, may be included in an analysis. The field of view can be modified to exclude these static or very slow moving regions of scatter by clicking using the mouse on the field of view and dragging an exclude box over the region to be excluded. Multiple areas can be excluded. By right-clicking on the mouse all the exclusion areas are removed from the analysis.

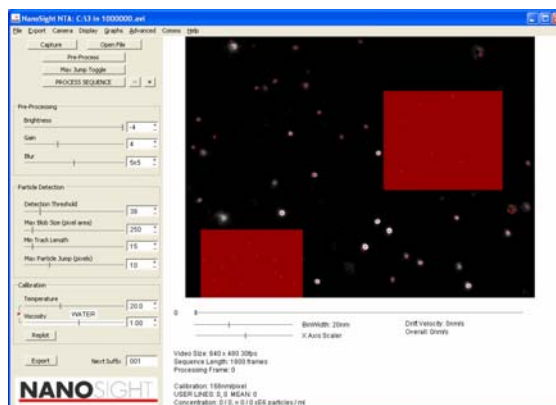


Figure 8: Diagram to show how the field of view can be restricted to remove areas of static scatter.



Step 14: Background Extraction: This function can be used to remove constant background noise from the analysis. Some solvents such as toluene scatter light even when there are no particles present in the sample chamber. The light scattered from the toluene can be removed using the background subtraction so that only light scattered from the particles is included in the analysis. This feature can be found in the '**Advanced**' drop-down menu.

Step 15: Varying the Experimental Parameters during analysis– During the analysis the **Bin width** and **X axis** value can be altered at any point without affecting the final analysis. All other filtration values can be altered as the analysis proceeds but the changes will only effect the remaining frames *i.e.* changing the viscosity half way through the analysis will not influence the calculated values in the first half of the analysis.

By pressing the 'Replot' button at the end of the analysis, mid-analysis changes in **Temperature** and **Viscosity** can be recalculated over all frames.

Changes in **Brightness**, **Gain**, **Blur**, **Detection Threshold**, **Max Blob Size**, **Max Particle Jump** can not be corrected for using the Redraw function. Whilst these values can be changed mid-analysis, their effects are only seen in subsequent frames. If necessary the analysis should be restarted using the new parameters.

Step 16: Drift Correction

Drift is generally associated with heating of the fluid and resulting convection currents; it manifests as a tendency for all the particles to move with a common vector. There are two components to the drift indicator. The WHITE box indicates the instantaneous drift measured in any specific frame whilst the RED box indicates the overall drift averaged out over all the frames analysed to that point.

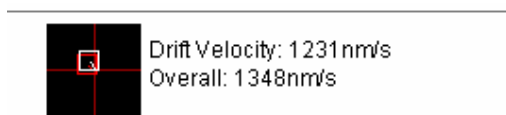


Figure 9: Enlarged image of drift correction.

Drift within a system will result in the particles appearing to be smaller than they actually are. Drift correction can be automatically removed by clicking the 'Auto Drift correction' in the 'Advanced' drop down menu on the tool bar. The drift correction will remove any over propensity for the particles to move in a specific vector and will recalculate particle sizes taking into account its removal.

Step 17: Changing the Display

By clicking on the buttons in the bottom-right corner of the interface (zoomed in above, Figure 10) the user can alter the image displayed on the software. The software defaults with the particle distribution overlaid on the captured image.

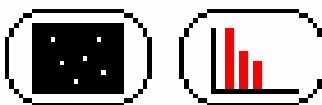


Figure 10: Enlarged image to show how user can change display from video clip to particle distribution.



Clicking the left button will display the video of the particles only (Figure 11 (a)). Clicking on the right screen will bring up the particle distribution again (Figure 11 (b)) and then clicking on the right screen for a second time will bring up the particle distribution only (Figure 12).

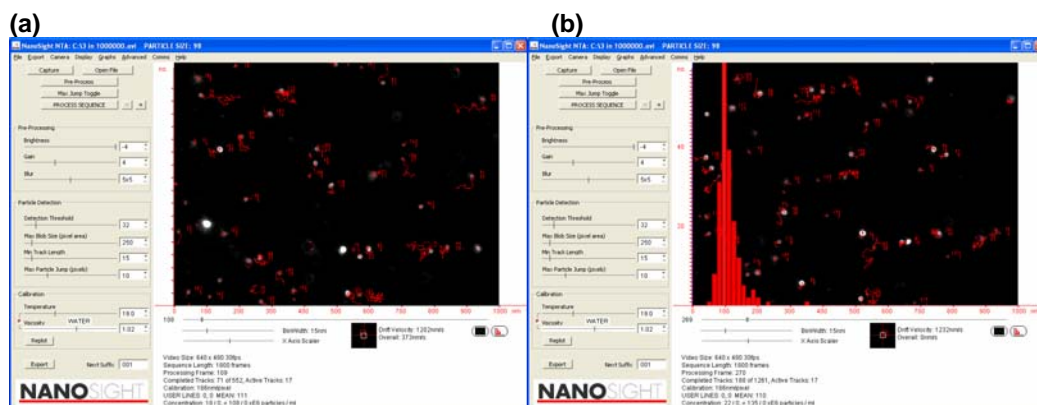


Figure 11: (a) Video only (b) Video and data graph.

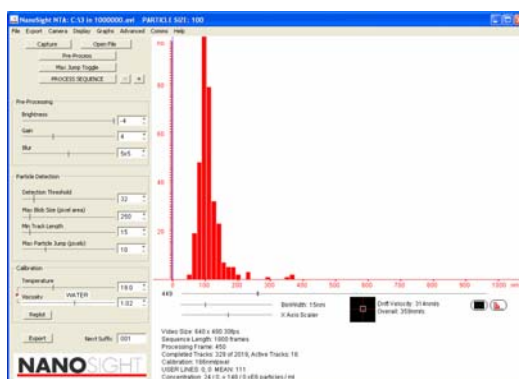


Figure 12: Data graph only.

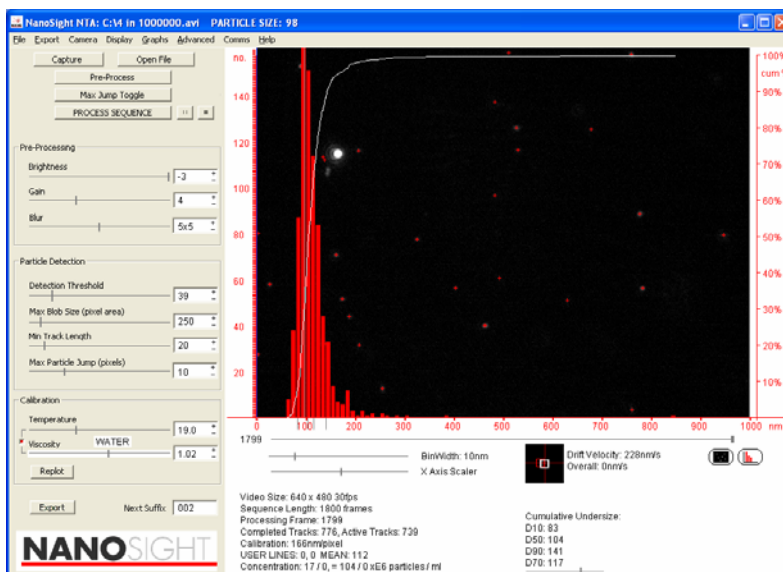


Figure 13: Typical particle distribution produced by the software.



As can be seen the software will automatically build the particle distribution over the video clip as the analysis proceeds. The software draws the path the particle has taken during its lifetime and calculates the average step jump distance it has taken along this path, from this average the hydrodynamic radius of each particle within the field of view is calculated. As can be seen in , the software produces a mode value for the distribution (in this example 98nm), the user can click on any point in the graph to produce a user line for comparison to the modal value.

Each particle is labelled with two numbers (see). The top number represents the calculated particle size at that instant and the bottom number represents the number of frames the particle has been tracked for and on which the instantaneous size estimation is based. These values can be switched on and off at any point through the 'Display' section of the toolbar.

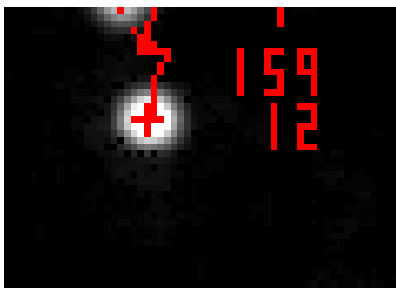


Figure 14: Particle labels.

4.6 Batch Capture and Process

The software can be configured so that it will automatically capture and analyse a sequence of video clips. This feature may be important when the user wishes to characterise an aggregation or dissolution process.

4.6.1 Batch Capture

The batch capture facility can be found under the 'Advanced' section of the toolbar. The image on the following page shows the interface used to set the batch capture process:

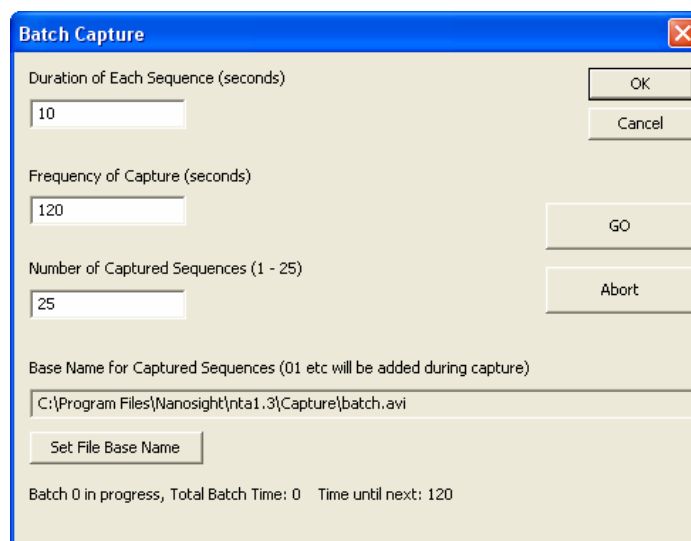


Figure 15:Interface used to control the batch capture process.



The duration of video capture can be determined (**Duration of each Sequence**), the time between the start of each capture (**Frequency of Capture**) and the number of cycles to be captured (**Number of Captured Sequences**). For each cycle the video is captured at the start of the cycle and then written to disc. For long video clips *i.e.* 60 seconds, the software can take 60 seconds to write the information to disc. Hence for a 60 second video clip the frequency of capture should not be set to less than 120 seconds to avoid the computer trying to capture video whilst writing the previous information to disc. In such cases many frames would likely be dropped as the computer struggles to keep up with the demand.

The sequence captured can be processed using common software filtration parameters. The sequence can be loaded using the interface as is shown below *i.e.* found under the '**Advanced**' toolbar.

4.6.2 Batch process

Any sequence of files captured, either as part of a batch capture run (as described in the section above) or individually, can be processed using common software processing parameters. The sequence can be loaded using the interface as is shown below *i.e.* found under the '**Advanced**' toolbar. Either locate and load the separate files individually or click the 'Find Sequence' button and locate the first movie of a batch. This will load in all files in the sequence for analysis. When the user clicks '**go**' the movies will be processed in sequences using the parameters that are stored in the process text file (see export files, below) for the first movie. Therefore it is necessary to analyse the first movie by the normal method in order to set the parameters that should be used for the rest of the analyses.

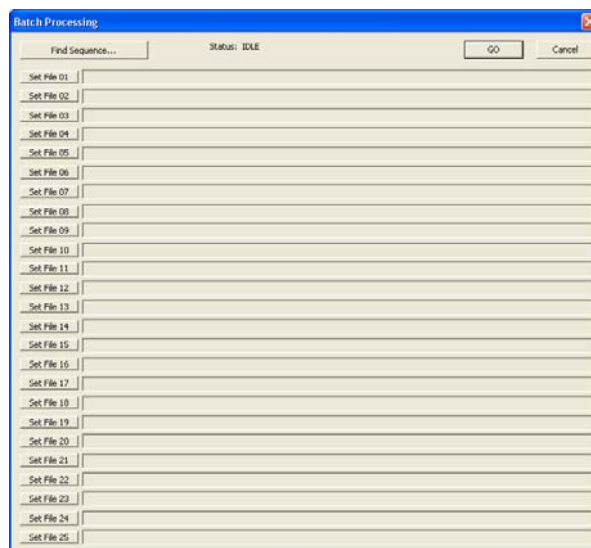


Figure 16: Interface to load video clips into the Batch Process facility.

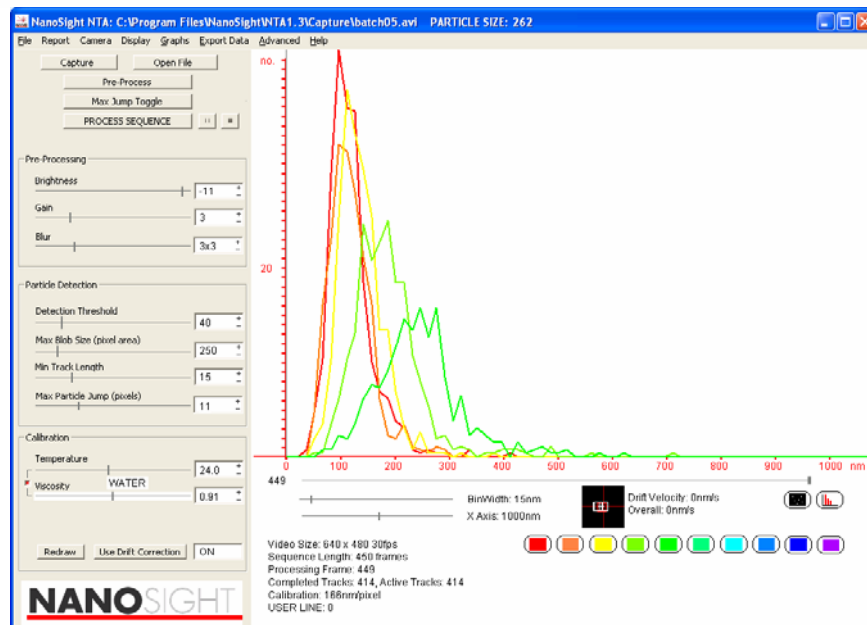


Figure 17: Typical image produced from batch capture process.

As can be seen from Figure 17 the software will automatically overlay distributions produced from the batch capture process. Each line can be added or removed by clicking on the coloured button for that line.



4.7 Output Parameters

4.7.1 Screen display

Some of the most important parameters are displayed on the screen. These are outlined below:

4.7.1.1 Modal size

The modal size of the particle is displayed in the blue line along the top of the screen.

4.7.1.2 User lines

To identify secondary peaks or to measure concentration between two lines (see below) two user lines are available to the user. These can be accessed by clicking the left/right mouse button on the bar graph display. The x-axis value at which the user line is positioned is displayed in the text at the bottom of the screen.

4.7.1.3 Mean Size

The average of all particle sizes is listed at the bottom of the screen.

4.7.1.4 Concentration

An estimation of the concentration is given in the following format at the bottom of the screen:
 $10 / 5 = 0.4 / 0.2 \text{ E8 particles /ml.}$

- The 10 refers to the average number of particles per frame that are being sized.
- The 5 refers to the average number of particles per frame that are sized within the range set by the two user lines (if the user lines are not activated this value will read 0).
- The 0.4 means that on average 0.4×10^8 particles per ml are sized.
- The 0.2 means that on average 0.2×10^8 particles per ml are sized in the size range between the user lines.

4.7.2 Graphical Output Scales

The particle sizing data output can be displayed as either a number plot or a concentration plot (under the 'Graph' section of the toolbar). For a number plot, the graph plots the particle size against the number count of particles tracked. However, since smaller particles move faster under Brownian motion, they will diffuse in and out of the field of view faster than larger particles, leading to more small particles being counted and over-estimating the number of small particles present compared to larger ones. The concentration plot weights the data to ensure that a true reflection of the size distribution is presented based on the concentration of particles of different sizes (this is the default setting).

Other options that are available are:

4.7.2.1 Surface area and Volume.

Here the frequency is simply multiplied by the square/cube of the radius respectively to give relative proportions of surface area or volume contributed by each size range.

4.7.2.2 Diffusion coefficient

Plots the diffusion coefficient of particles instead of size on the x-axis.

4.7.2.3 Draw Bars

Toggles the standard bar-chart.

4.7.2.4 Draw Multigraphs

Toggles the lines from batch process.

4.7.2.5 Log scale

Rescales the x-axis on logarithmic scale.



4.7.3 Cumulative Undersize and Oversize

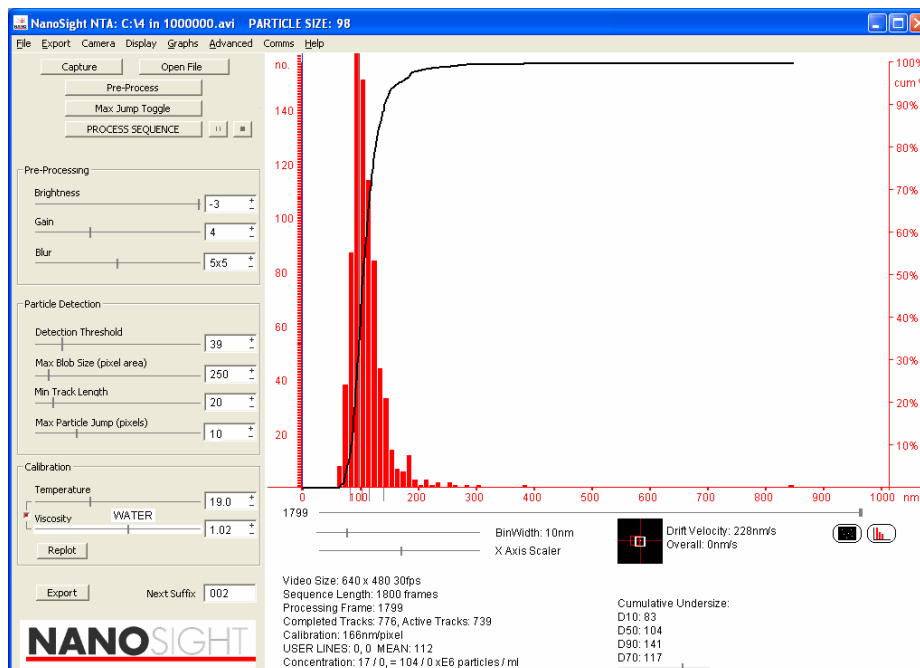


Figure 15: Distribution showing cumulative undersize for analysis.

As can be seen from above the D10, D50 and D90 values can be calculated for the sample by using the cumulative undersize function. This function can be found in the 'Graphs' dropdown on the toolbar.

4.7.4 Report

The software can be used to produce a standardised sample report sheet. This feature is found under the 'Export' section of the toolbar. By clicking on 'Set Report Details' a report template appears into which the user can fill in the relevant details. The user can select which graphs and images they would like to appear on the report. Four different images can be selected to appear on the report. The user can select which images appear on the report in the 'Export' section of the toolbar. By clicking 'Use current Display as Fig 1,2,3 or 4' the user can select which images will be displayed on the report print out, which can be directly printed from the software.

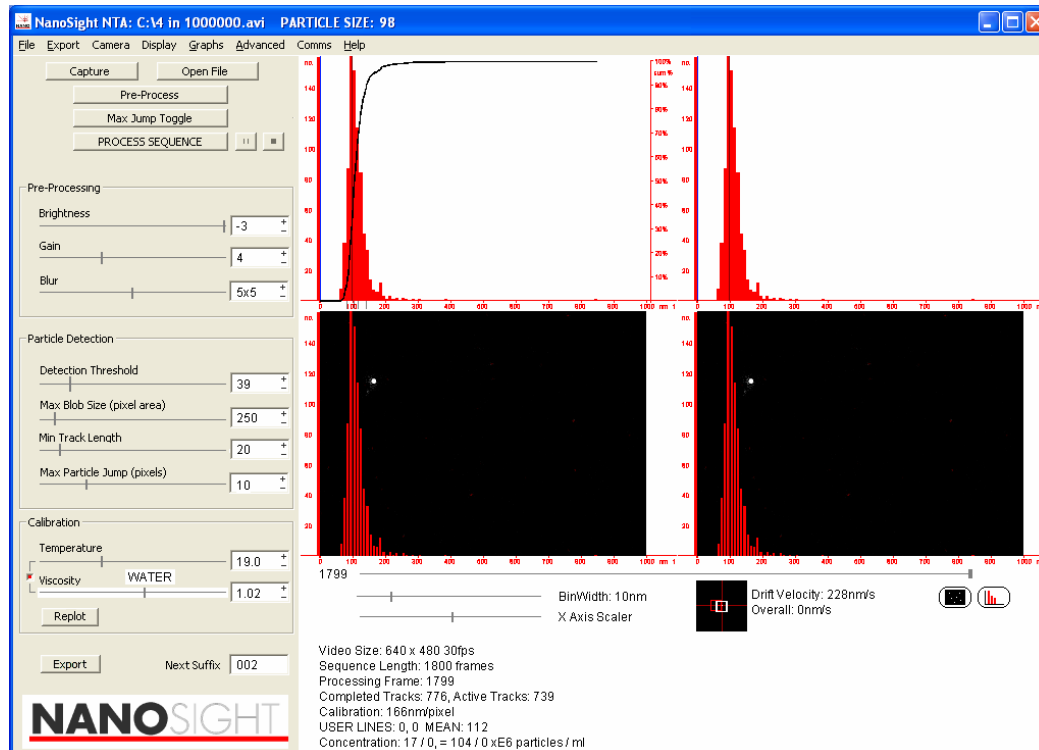


Figure 16: Preview of Report Diagrams.

4.7.5 Export Data

The software can be used to export data to various comma separated values (csv) files depending on the level of detail of results required. These options are found under the 'Export' section of the toolbar.

Summary File

The following comma separated values (.csv) file is generated via the Export File facility within the programme and is named "xxxx-001-summary.csv". It is saved to the same location in which the video file (xxxx.avi) is saved (user-selectable) and has the same name (e.g. xxxx as the video. Note that multiple analyses of the same video will be saved separately with an auto-incrementing number to distinguish the different analyses, e.g. *usertest-001-summary.csv*, *usertest-002-summary.csv*, etc.. These files can be loaded to/by Excel for further manipulation.



[ResultsSummary]

Operator=

SampleDescription=

Diluent=

Concentration=

PreTreatment=

Comments=

TotalFrames=1800

ProcessedFrames=1799

CompletedTracks=776

OverallDrift=228.489557

X-Drift=55.969406

Y-Drift=-221.528560

Calibration=166

Mode=98

Mean=112

UserLine=0

Sample description as entered by the user in the Report page

Total no of frames
in video

Number of frames
used in analysis

Number of
particles sized

Drift automatically compensated for
unless option deactivated

Fixed

Value (size in nm) of highest bin [weighted by values of adjacent bins]

Sum of particle size values for all particles ÷ number pf particles

PercentileUndersize	size
1	67
2	72
3	73
4	74
5	76
96	174
97	184
98	191
99	233
100	382

Percentile undersize values at 1% resolution

BinCentre	Count
5	0
15	0
25	0
35	0
45	0
55	0
65	8
75	38
85	87
95	161
105	51
1975	0
1985	0
1995	0

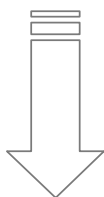
Values at centre of each bin (including zero values) at
user selected bin width (in this case, 10nm)



Result file

The following comma separated value (.csv) file is generated via the Export File facility within the programme and is named "xxxx-001-result.csv". It is saved to the same location as the video file (xxxx.avi) is saved (user-selectable) and has the same name (xxxx...) as the video. Note that multiple analyses of the same video will be saved separately with an auto-incrementing number to distinguish the different analyses, e.g. *usertest-001-result.csv*, *usertest-002-result.csv*, etc.. These files can be loaded to/by Excel for further manipulation.

Size (nm)	Count
60	1
63	1
64	1
66	3
67	1
69	1



218	1
223	1
235	1
238	1
256	2
268	1
282	1
303	1
382	1
848	1

Values for each bin containing a non-zero value, i.e. empty bins are not displayed.

Note that the raw data stored in this table at a 1nm resolution while the particle size distribution graph displayed on the screen or in the report will depend on the bin width selected by the user.



Analysis File

The following comma separated values (.csv) file is generated via the Export File facility within the programme and is named "xxxx-001-analysis.csv". It is saved to the same location in which the video file (xxxx.avi) is saved (user-selectable) and has the same name as the video. Note that multiple analyses of the same video will be saved separately with an auto-incrementing number to distinguish the different analyses, e.g. *usertest-001-analysis.csv*, *usertest-002-analysis.csv*, etc.. These files can be loaded to/by Excel for further manipulation.

[Settings]

Pulldown=3

Gain=4

Blur=2

DetectThresh=39

MaxBlob=250

MinTrackLength=20

MaxParticleJump=10

Temperature=19.0

Viscosity=1.0227

BinWidth=10

Parameters set for processing video (also printed out in report)



Alltracks File

The following comma separated values (.csv) file is generated via the Export File facility within the programme and is named "xxxx-001-alltracks.csv". It is saved to the same location in which the video file (xxxx.avi) is saved (user-selectable) and has the same name as the video. Note that multiple analyses of the same video will be saved separately with an auto-incrementing number to distinguish the different analyses, e.g. *usertest-001-alltracks.csv*, *usertest-002-alltracks.csv*, etc.. These files can be loaded to/by Excel for further manipulation.

Particle ID	Track Step	X1	Y1	X2	Y2	Diffusion Co	Particle Size
1	2	100.4584	3.868786	95.32211	3.192722	361.9166	117.0273
1	3	95.32211	3.192722	98.14208	2.710382	361.9166	117.0273
2	2	160.4091	2.396104	164.0134	2.3125	172.6027	232.7189
2	3	164.0134	2.3125	165.9162	2.021739	172.6027	232.7189
4	2	99.38546	16.35463	101.3246	22.68264	517.314	78.79213
4	3	101.3246	22.68264	103.5821	23.76014	517.314	78.79213
7	2	450.9534	100.4378	455.8448	99.74518	453.3191	92.52557
7	3	455.8448	99.74518	458.1433	101.3496	453.3191	92.52557
7	4	458.1433	101.3496	454.4592	101.8439	453.3191	92.52557
7	5	454.4592	101.8439	454.0251	100.6831	453.3191	92.52557
7	6	454.0251	100.6831	454.7009	105.0745	453.3191	92.52557
7	7	454.7009	105.0745	457.2028	104.3766	453.3191	92.52557
7	8	457.2028	104.3766	457.1436	107.8561	453.3191	92.52557
7	9	457.1436	107.8561	456.2762	104.8696	453.3191	92.52557
7	10	456.2762	104.8696	456.6437	103.0476	453.3191	92.52557
7	11	456.6437	103.0476	459.4194	101.2233	453.3191	92.52557
7	12	459.4194	101.2233	458.6222	106.3778	453.3191	92.52557
7	13	458.6222	106.3778	454.9494	109.9508	453.3191	92.52557
7	14	454.9494	109.9508	454.9501	104.5261	453.3191	92.52557
7	15	454.9501	104.5261	451.5	96.5	453.3191	92.52557
7	16	451.5	96.5	452.7451	97.71292	453.3191	92.52557
7	17	452.7451	97.71292	451.9704	95.22328	453.3191	92.52557

! Note: A print-out of this raw data file can be extremely long for analyses containing many hundreds of particles. Thought should be given to requesting a hard copy.

The data which forms the basis of the particle distribution can be exported in a '.csv' file which can then be imported into an Excel spreadsheet. This is exported automatically at the end of each process. This function is found at the bottom left of the main screen. The data is exported to a file located in the same folder as the original video clip with the suffix displayed added to the file name.



4.8 Worked Examples

4.8.1 200 nm Latex Reference Spheres

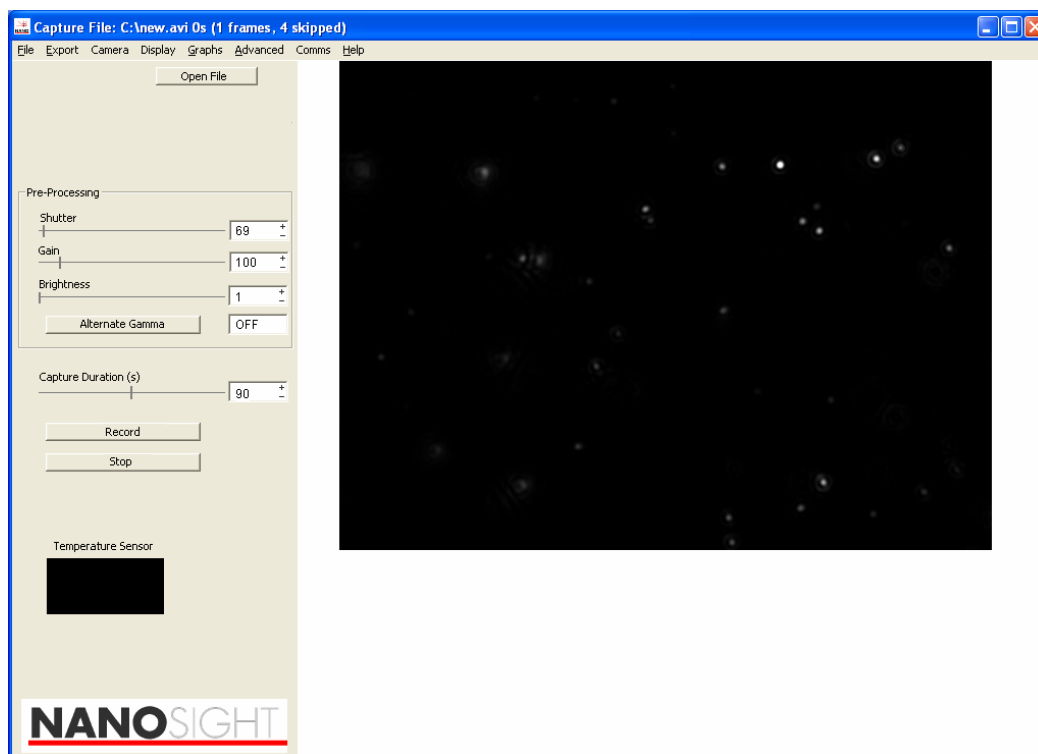


Figure 17: Diagram showing the ideal camera settings to capture a video of 200 nm diameter latex particles.

The above diagram shows an ideal image of 200 nm latex reference spheres. Notice the shutter speed is very low, combined with a low gain. The shutter is kept low to avoid particle blurring as explained previously. The gain is kept low in order that the particles retain their grey scale image and thus allowing accurate centering of particles. Note the alternate gamma is not used as this option is generally used for very small particles and poly-dispersed systems. For very accurate reading a 60 second analysis will produce a stable particle distribution.

The sample has a relatively low concentration and hence could be introduced at a higher concentration, or alternatively, a longer video clip captured to allow statistically relevant numbers of particles to be included in the particle size distribution.

- Shutter: 90
- Gain: 100
- Brightness: 1
- Alternate Gamma: No
- Time of Capture: 60-90 Seconds.

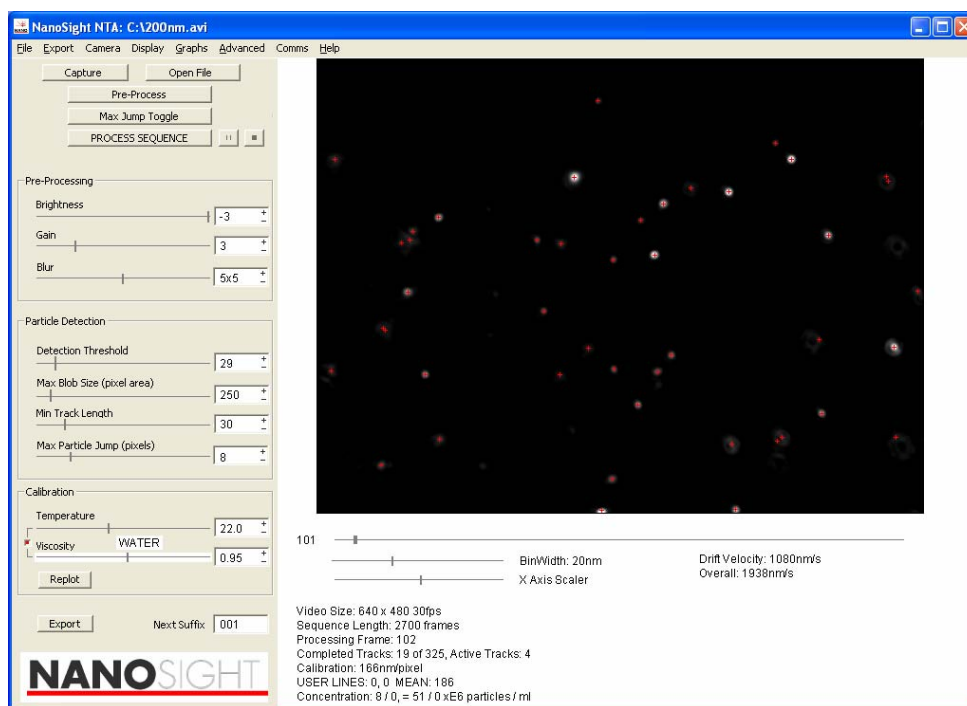


Figure 18: Shows the settings used to analyse 200 nm latex reference particles.

The above shows the ideal settings to analyse the 200 nm latex particles:

- Brightness: **-3** to remove any background noise.
- Gain: **3** makes the dimmest particles visible.
- Blur: **5x5** removes any false centres located between 2 particles which are close together or on the diffraction patterns around the brighter particles.
- Detection Threshold: **29** This allows the dimmest particles to be included in the analysis.
- Max Blob size: **250** Min Track Length: **30** With long analysis times such as 60 seconds, a min track length of 30 can be confidently set, producing a data set which is sufficiently large that the modal size can be accurately quoted. Large minimum track values ensure that all particles included in the analysis have been tracked for a sufficiently long time that a good average of their Brownian motion has been produced. 200 nm particles are large enough that their motion is slow enough that the software is able to track the majority of particles for longer than 30 frames. 50nm particle movement is so rapid that very few particles would exist in the field of view for 30 frames.
- Max Particle Jump: **8** By using the max jump toggle the longest particle jump can be estimated.

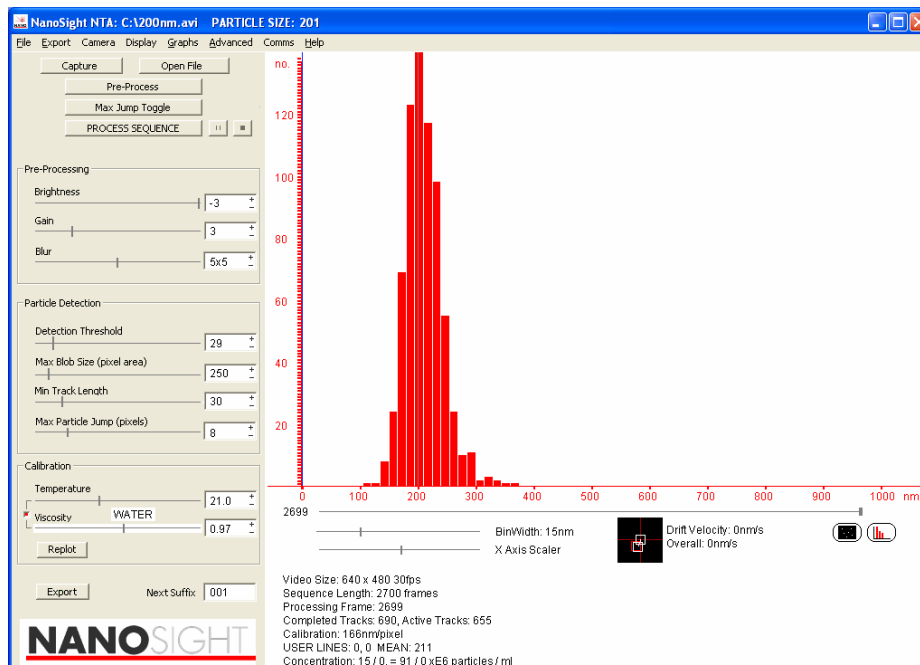


Figure 19: Image to show an optimal analysis of 200 nm latex reference particles.

With a sufficiently large number of particles included in the analysis, changing the width of the bins will not change the modal value calculated. The detail shown in the distribution can be altered according to the users requirements. By having insufficient particles included in the analysis, changing the bin width will likely change the modal value by virtue of changing which particles are included in which bin.

4.8.1.1 Recognizing and Dealing with Non-Optimal Conditions

4.8.1.1.1 High Gain Upon Capture

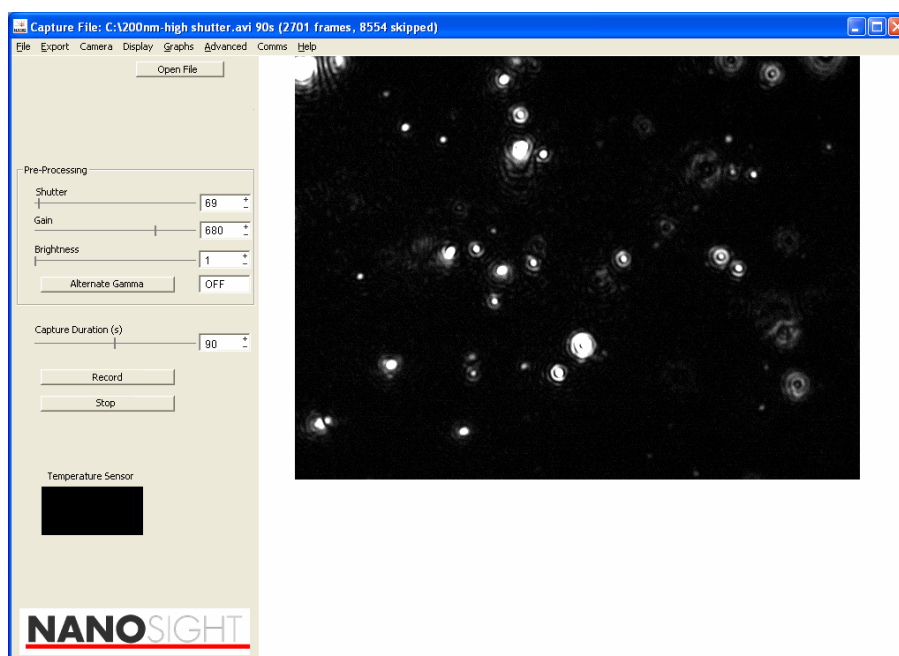


Figure 20: Image showing a high gain image.



As can be seen from Figure 20, a high gain image can suffer from a large amount of background noise and the diffraction patterns can clearly be seen around a number of particles. Although the original image is noisy, the software filters can be used to deal with the image.

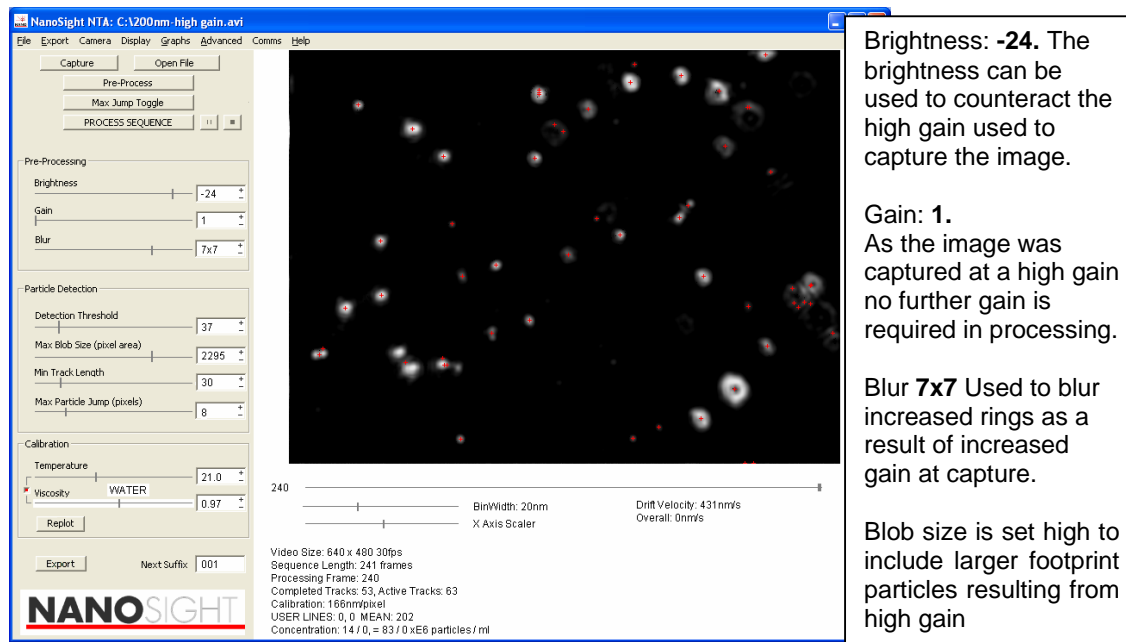


Figure 21: Image showing the software filters necessary to deal with a high gain image.

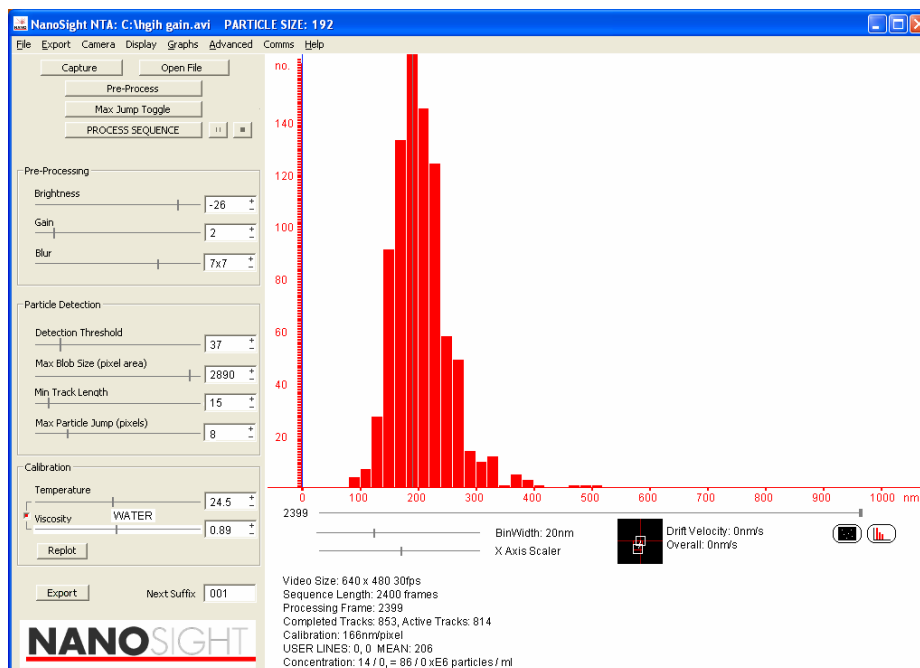


Figure 22: Image showing the analysis produced from the high gain image.



4.8.1.1.2 High Shutter Upon Capture

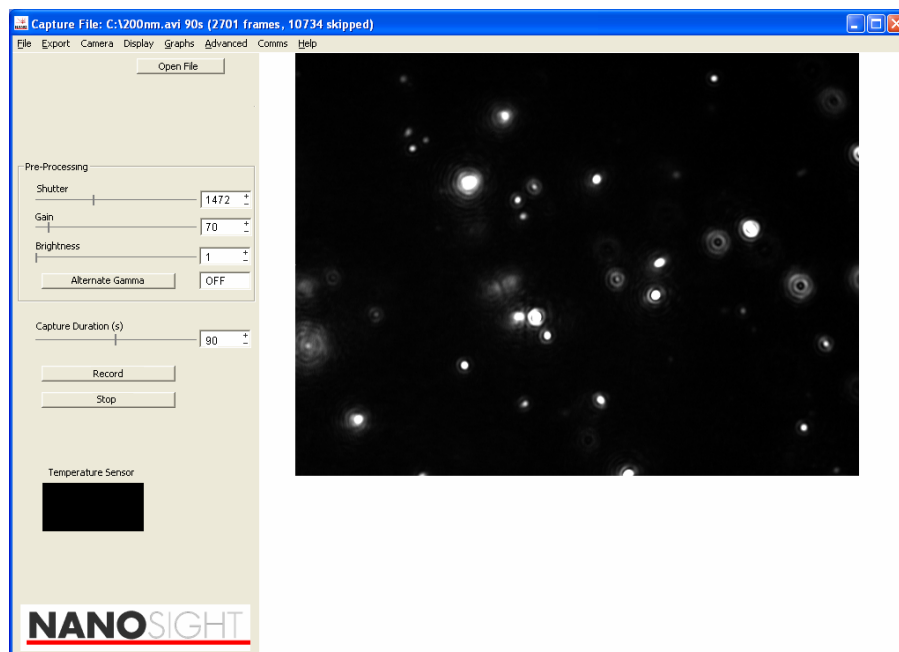


Figure 23: Image showing high shutter image of 200 nm latex reference spheres.

As can be seen from Figure 23, an image captured from a high shutter image produces a blurry image. As is explained in **Step 1**, long shutter times can result in problems with particle centering due to blurred particles.

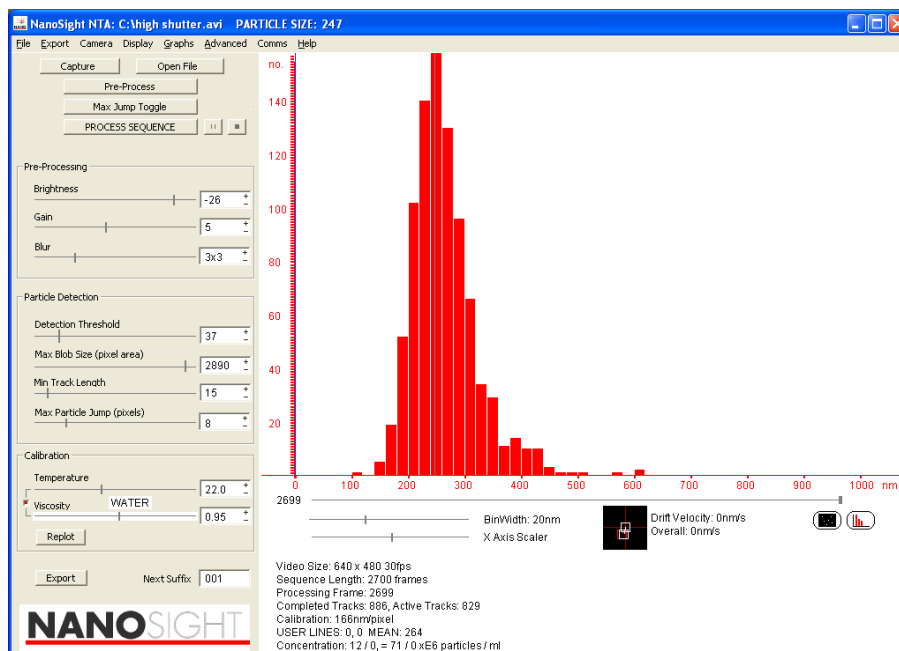


Figure 24: Particle distribution produced by a high shutter capture.

As can be seen from Figure 24 the modal value calculated by the analysis is too high due to the excessive shutter used to capture the image. As a general rule, the shutter should be



kept to the minimum value to allow visualisation of the particles but minimising particle blurring.

4.8.1.1.2.1 Incorrect Sample Concentrations

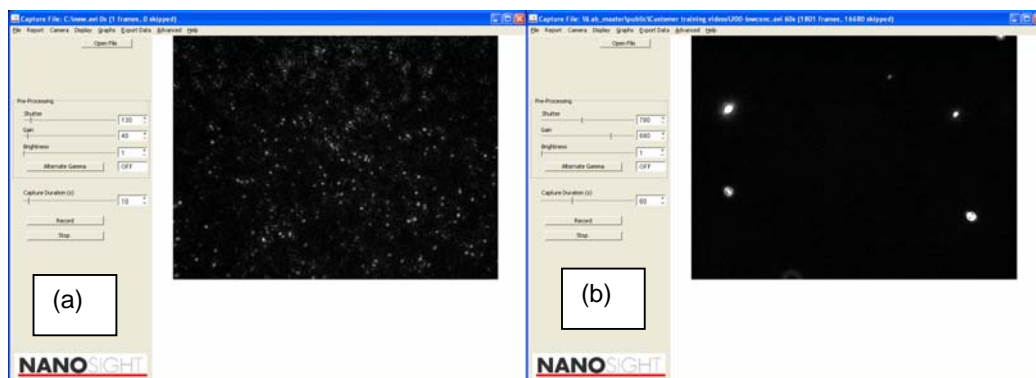


Figure 25: (a) Too high concentration of particles (b) Too low concentration of particles.

As can be seen from Figure 25, the system has certain requirements regarding sample concentration. Ideally the sample should contain between 10^6 and 10^8 particles per ml dependant upon the size of the particles. Figure 25 (a) represents an over concentrated image and represents a sample concentration of around 10^{10} particles per ml. Such images represent a problem in that the rate at which the particles cross over is unacceptable, hence there is a possibility that the software will erroneously join tracks from adjacent particles. Figure 26 (a) shows the resulting distribution from an over concentrated sample. As can be seen the modal value produced is slightly lower than should be expected – due to the increased cross over rate of the particles.

Figure 25 (b) shows a sample with too few particles are represents a sample concentration in the order of 10^6 particles per ml. Figure 26 (b) shows the resulting analysis from such a sample. As can be seen, insufficient particles were analysed to produce a stable distribution of particle sizes. If possible, such a sample should be increased in concentration or the analysis run for an extended period of time in order to increase the data set.

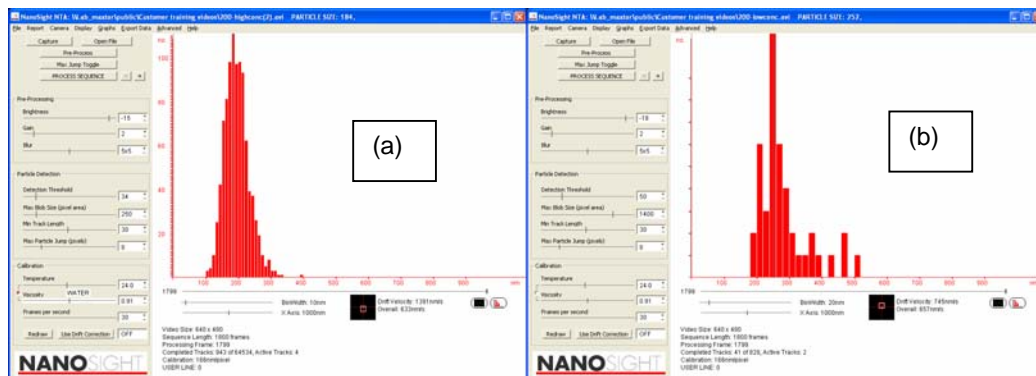


Figure 26: Particle size distribution of 200 nm Latex spheres (a) too concentrated sample (b) too dilute sample.



4.8.2 100 nm Reference Latex Spheres

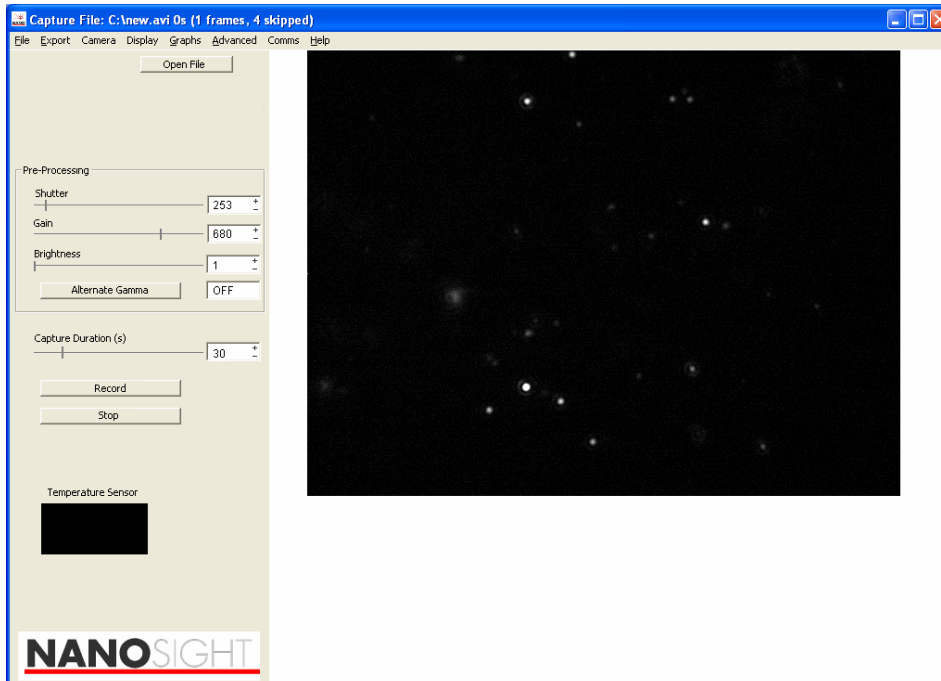
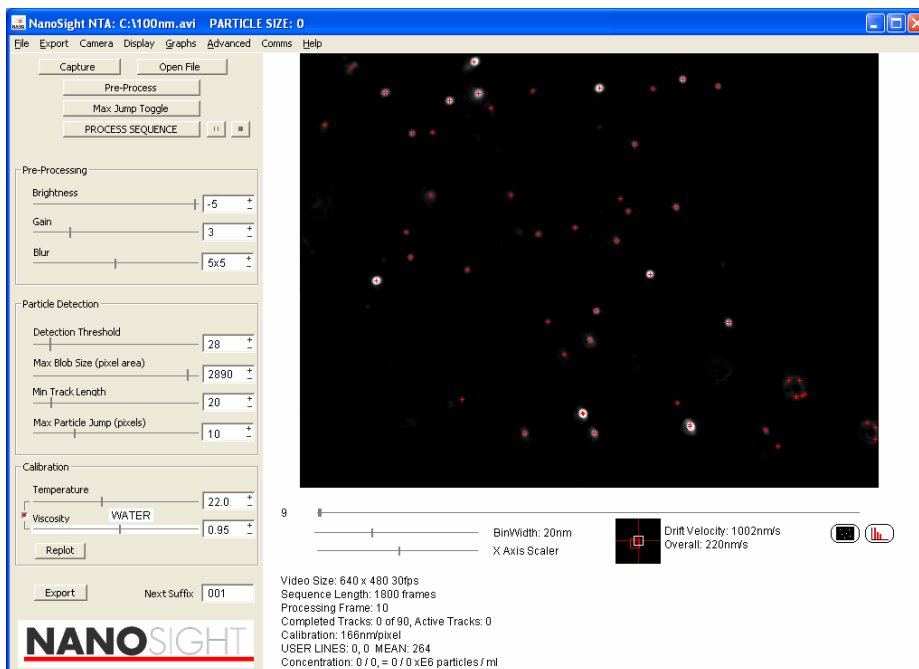


Figure 27: Ideal image of 100 nm Latex Reference Particles.



Brightness: **-5**. The brightness can be used to counteract the gain used to brighten image.

Gain: **3**. Used to increase difference between dim particle and noise

Blur **5x5**. Few rings therefore little blur needed

Max Jump **10** as particles have quite large steps through Brownian motion

Figure 28: Ideal setting used to analyse 100 nm latex reference spheres.

The software filters should be altered in the order in which they are listed going down the screen. The brightness should be reduced to a point so as any background noise is removed from the image. Following this, the gain should be used to increase the brightness of the very dimmest particles. Next the blur should be applied in order that the false centres which are located around the brightest particles are removed.



At the start of the analysis the detection threshold should be increased to maximum to remove all centres from the particles. This allows the user to better determine the effect of the software filters as they are changed without interference from the red centres. Having varied the brightness, gain and blur, the detection threshold can be reduced down to a level where the dimmest particles are included in the analysis and not the background noise. The max blob size should then be increased to a level so that all of the largest particles are included in the analysis. For particles in the region of 100 nm the minimum track length should be set between 15 and 25. As 100 nm particles have a fairly rapid Brownian motion, few particles will exist in the field of view for longer than 25 frames. Hence by setting a minimum track length longer than this will exclude the majority of particles from the distribution and prejudice the analysis towards the large particles.

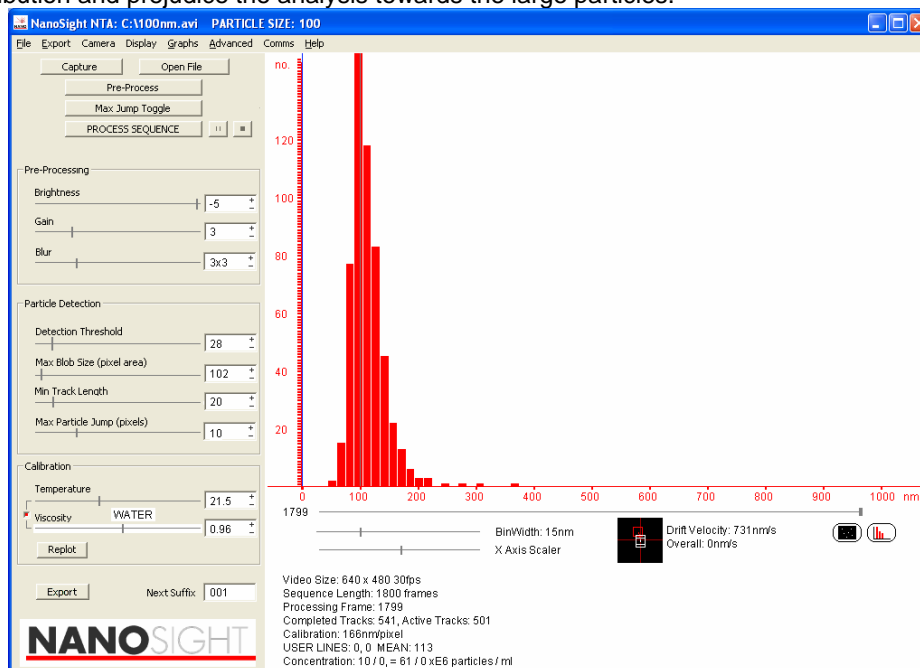
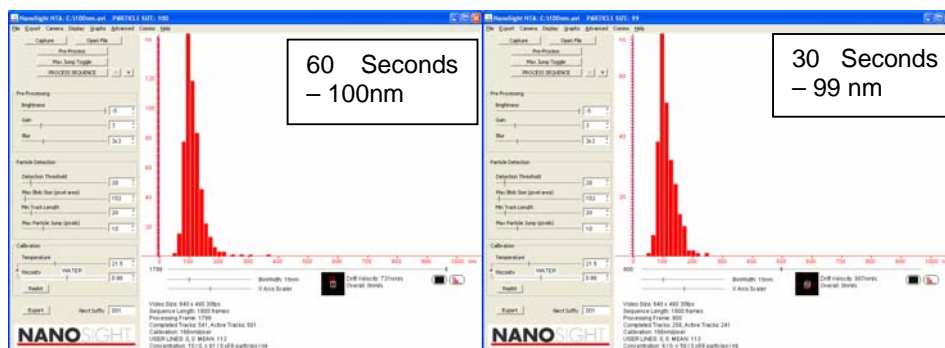


Figure 29: Particle size distribution produced from ideal image of 100 nm reference spheres.

4.8.2.1 Effect of Time of Capture on 100 nm Particles



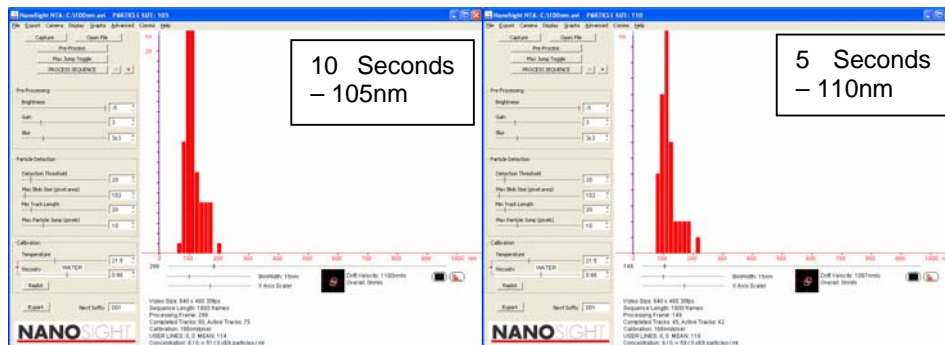


Figure 30: Images to show how length of capture influences the calculated size for 100 nm latex spheres.

As can be seen from Figure 30, the length of capture can influence the particle size distribution should not enough particles be analysed. As can be seen the difference between the 60 second analysis and the 30 second analysis is not appreciable as the distribution has reached a stable point by 30 seconds. Dropping the analysis time down to 10 seconds has had a detrimental effect on the results as the value starts slipping away from 100 nm. The analysis of the 5 second clip demonstrates that should an insufficient numbers of particles be analysed, the calculated value starts to suffer. 100 nm particles of a similar concentration as is displayed in Figure 28, can be analysed for 20 seconds with out the calculated value suffering. As the particles move considerably under Brownian motion, the particles move in and out of the field of view regularly, thus increasing the size of the data set. Hence a 20 second analysis will be sufficient for 100 nm particles where as for 400 nm particles (of the same concentration) may require a 90 second analysis in order that the same number of particles are analysed.

4.8.3 300 nm Reference Latex Spheres

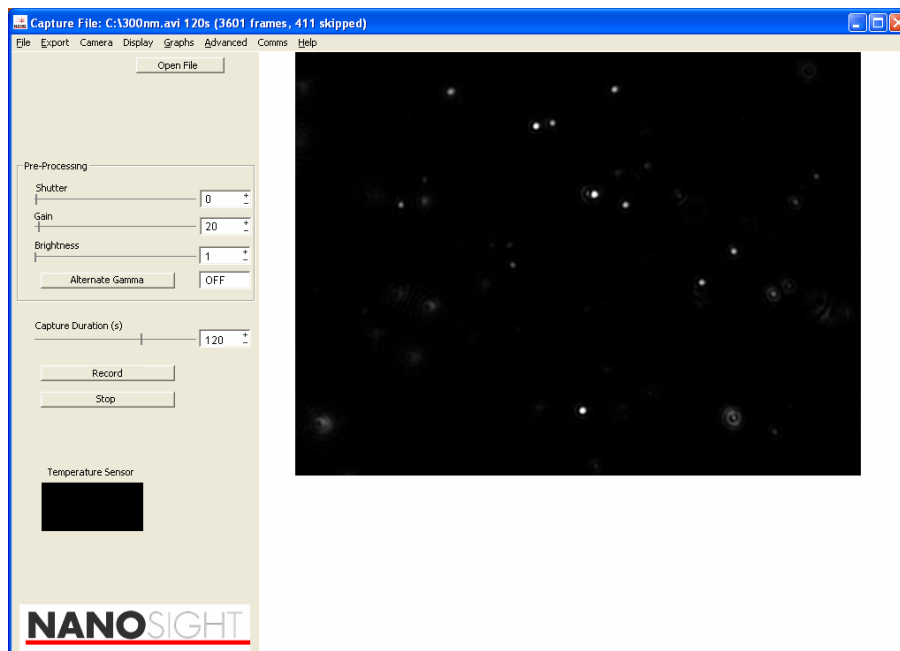


Figure 31: Ideal image of 300nm Latex Reference Particles.

As can be seen from Figure 31, 300 nm particles can be made to look similar to the signal produced by 100 nm and 200 nm particles by controlling the shutter speed and gain of the



camera upon capture. All images, independent of their size should optimally be made to look similar to that shown in Figure 31. The particles are not over-exposed with a grey scale from which the particles can be centered. The gain and shutter speed should be kept very low for large bright particles to reduce what would be an over-exposed image back to the image that is seen above.

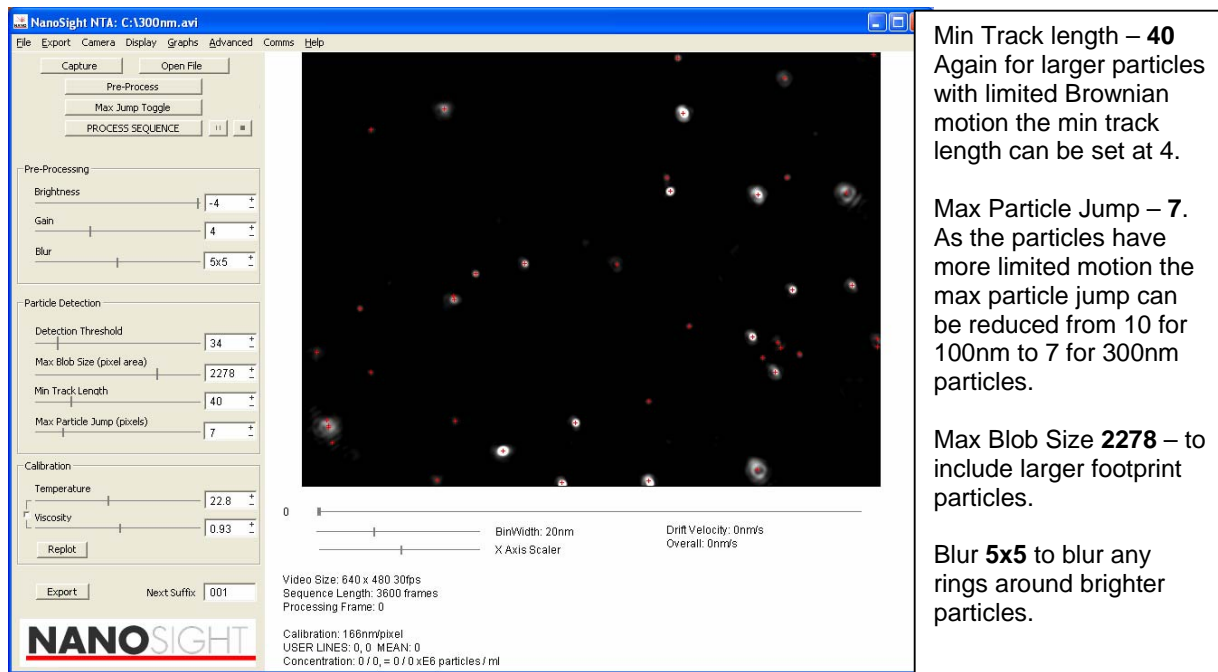


Figure 32: Image showing the ideal software setting used to analyse 300 nm particles.

As can be seen from Figure 32 the settings used to analyse the image produced by the 300 nm particles are largely similar to those used to analyse the 20 nm particles. This is largely due to the fact that the camera controls are used to produce, similar, optimal images across the range of particle sizes. There are a number of parameters which need to be altered when comparing analyses from 20 nm and 300 nm particles, see above right.

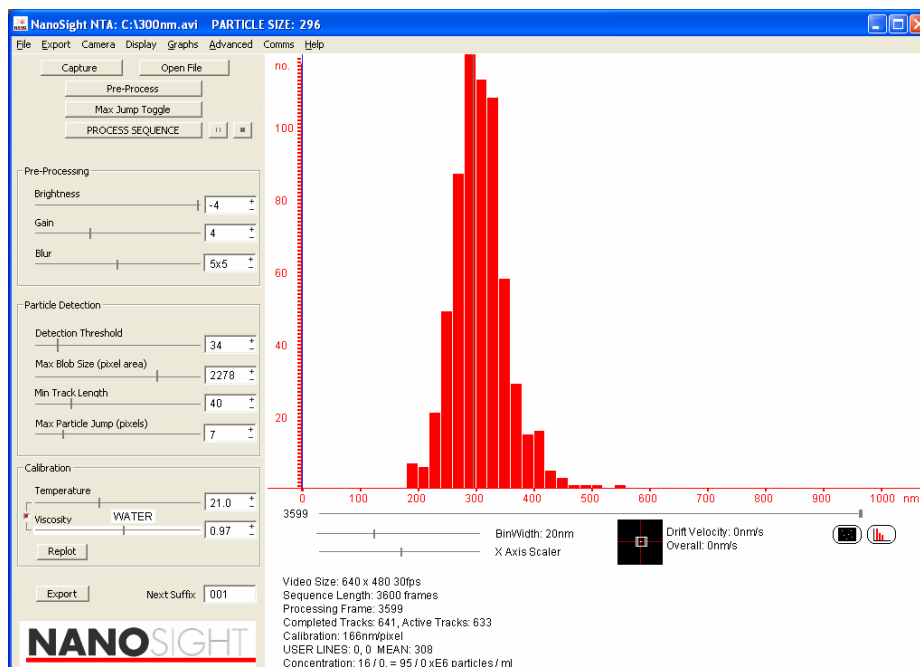


Figure 33: Particle size distribution produced from ideal image of 300 nm reference spheres.

4.8.3.1 Effect of Time of Capture on 300 nm Particles

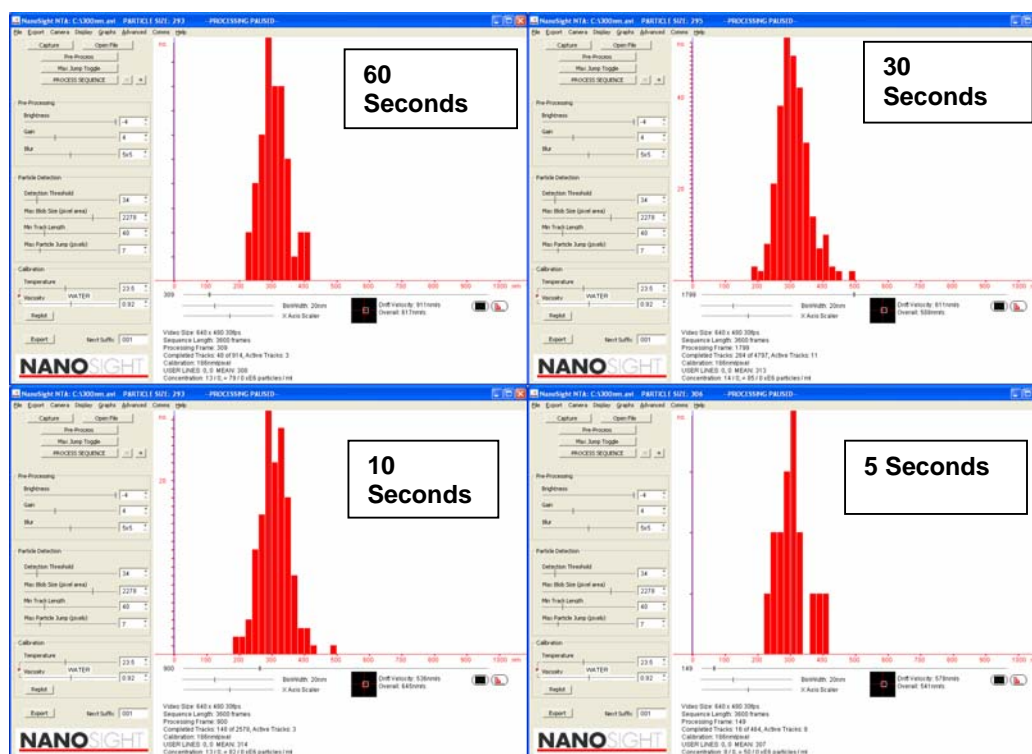


Figure 34: Images to show how length of capture influences the calculated size for 300 nm latex spheres



The effect of analysis time can be clearly seen in Figure 34. The distribution stabilizes at 60s and when using short analysis times the distribution has very few particles and hence the modal value may not be representative of the particles size.

4.8.5 400 nm Reference Latex Spheres

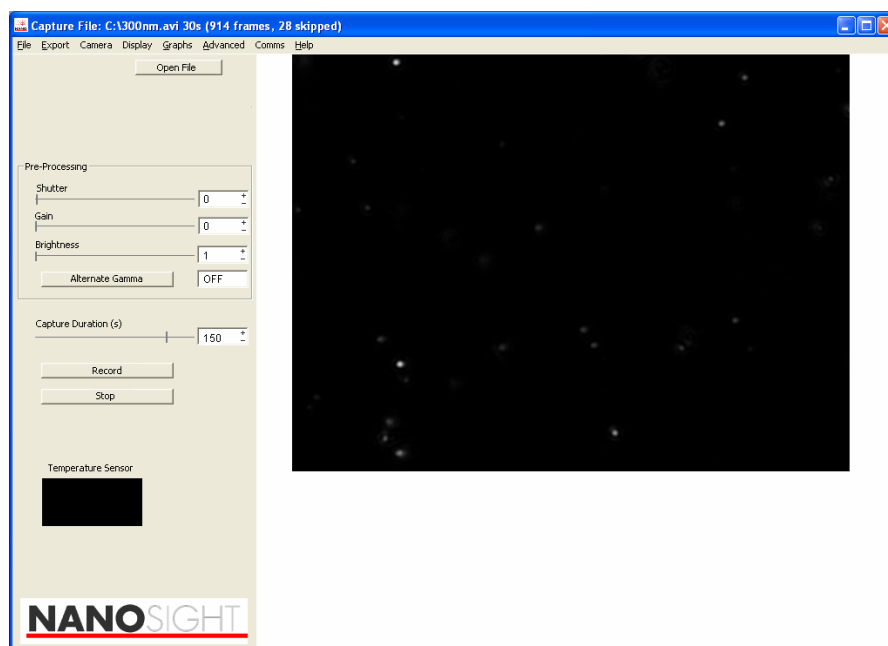
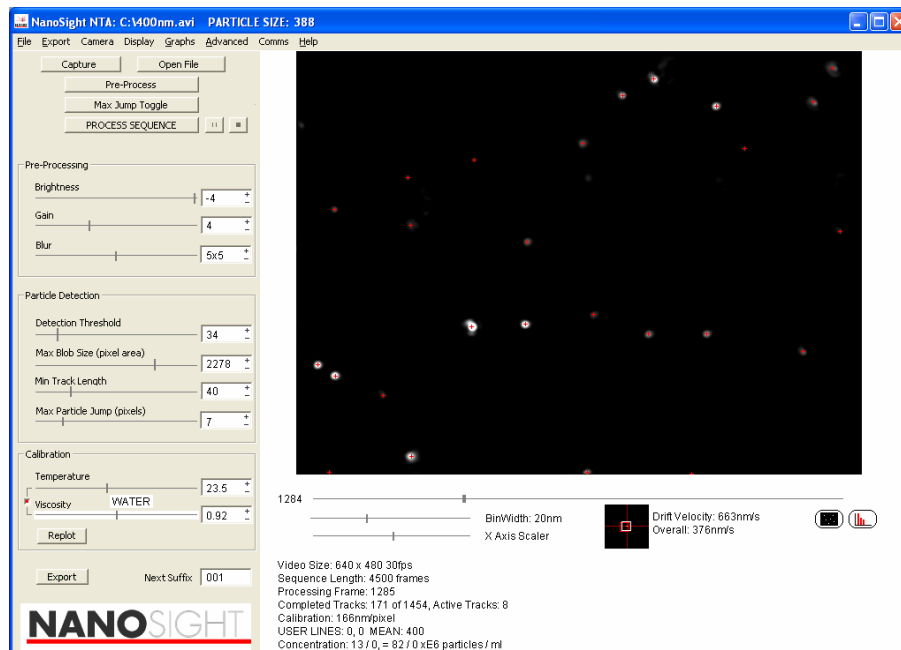


Figure 35: Ideal image of 400 nm Latex Reference Particles.

By using the camera settings the particles can be reduced to small points of light which can be centered very easily. The shutter speed and gain is set extremely low and a long analysis time is used. By using a long analysis time we are ensuring that the distribution produced is stable and accurate for slow moving 400 nm particles.



Min Track Length – 50.

Max Particle Jump – 5

Figure 36: Image showing the ideal software setting used to analyse 400 nm particles.

The settings used to analyse an optimal image are similar to those used to analyse an optimal 300 nm image. The only factors which need to be varied are the maximum particle jump and minimum track length.

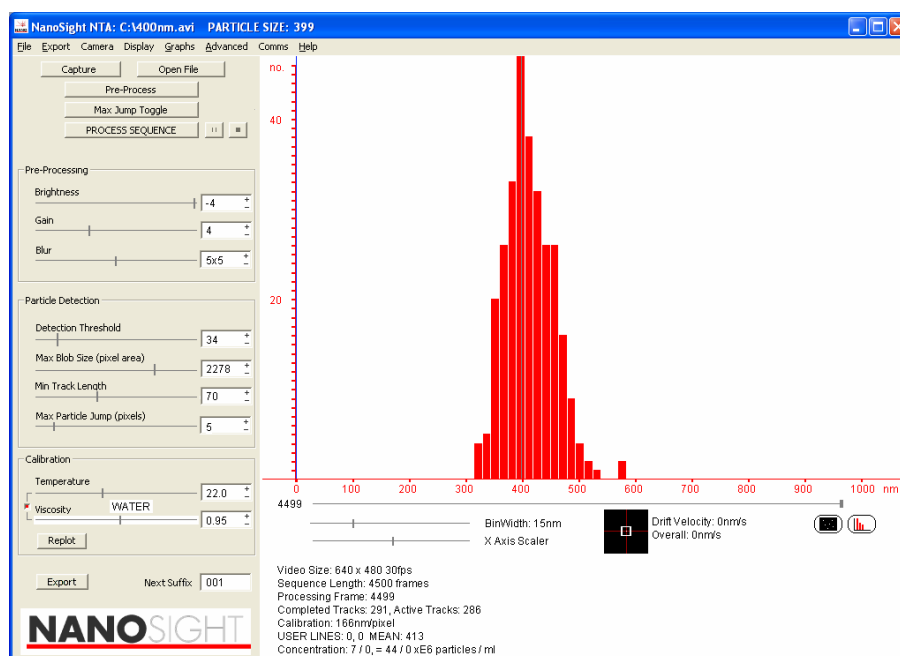


Figure 37: Particle size distribution produced from ideal image of 400 nm reference spheres.



4.8.5.1 Effect of Time of Capture on 400 nm Particles

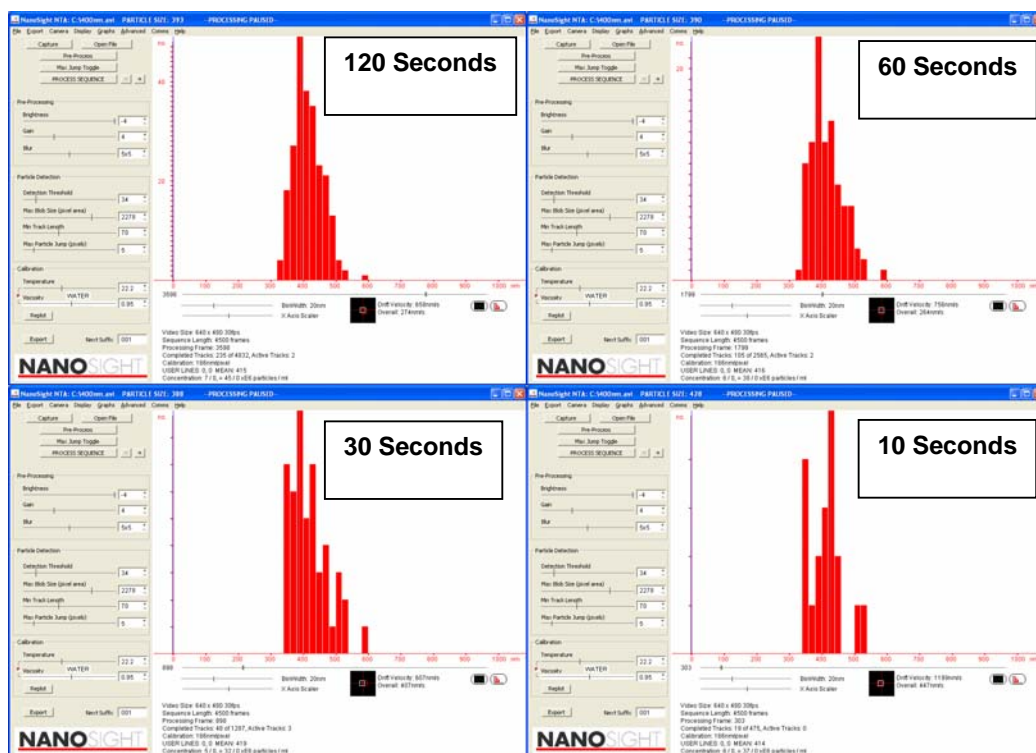


Figure 38: Images to show how length of capture influences the calculated size for 400 nm latex spheres.

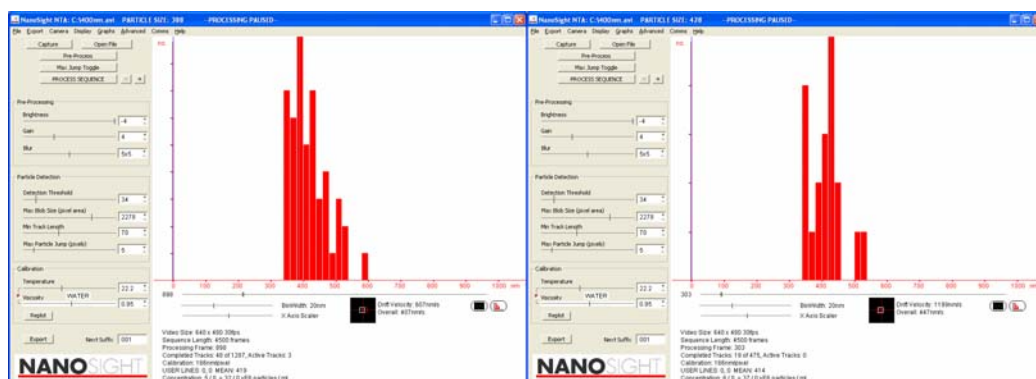


Figure 38, 400 nm particles are more sensitive than smaller particles to a reduction in analysis times. At 10 second and 5 second analyses, although the modal values are inaccurate and the distributions start to become meaningless due to the extremely limited number of particle being analysed.

4.8.6 30 nm Gold Colloid

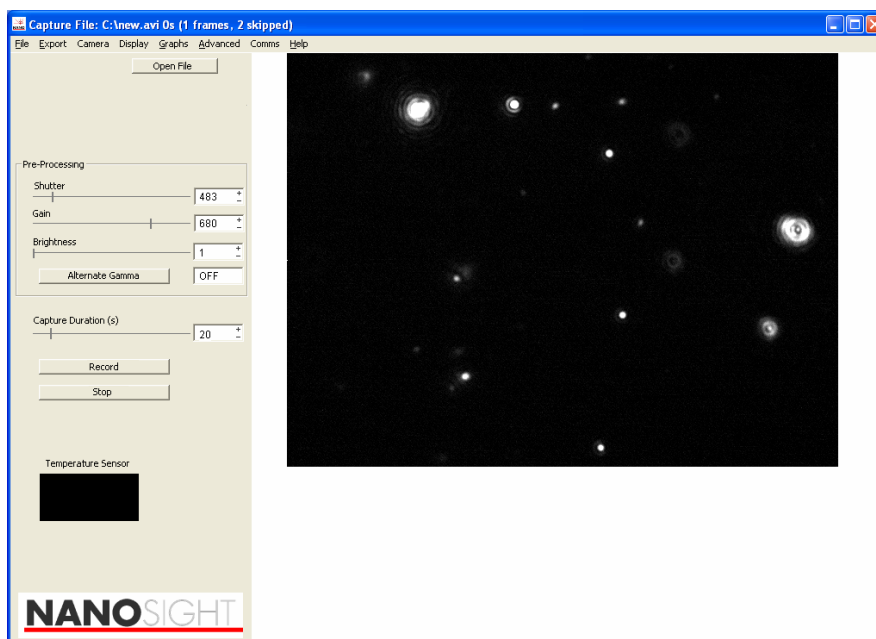


Figure 39: Typical image that would be seen when analysing 30 nm gold colloid.

As can be seen from the above image particles in the order of 30nm scatter light very poorly and as a result the image produced is very dim. Gold is an effective scatter of light and hence gold particles will appear brighter than a similarly sized particle of a low refractive index. Using the camera controls the particles can be made to appear brighter than in the image above, however the accuracy of the results may suffer. The above image has been captured at maximum **Gain (680)** and a moderate **Shutter** speed (**480**). By increasing the shutter to maximum the particles could be made to appear brighter however particle blurring would occur and the result would suffer as in Figure 24. The image should therefore be captured such that the particles appear fairly dim, as above, and then the image can be gained in the image processing part of the analysis.

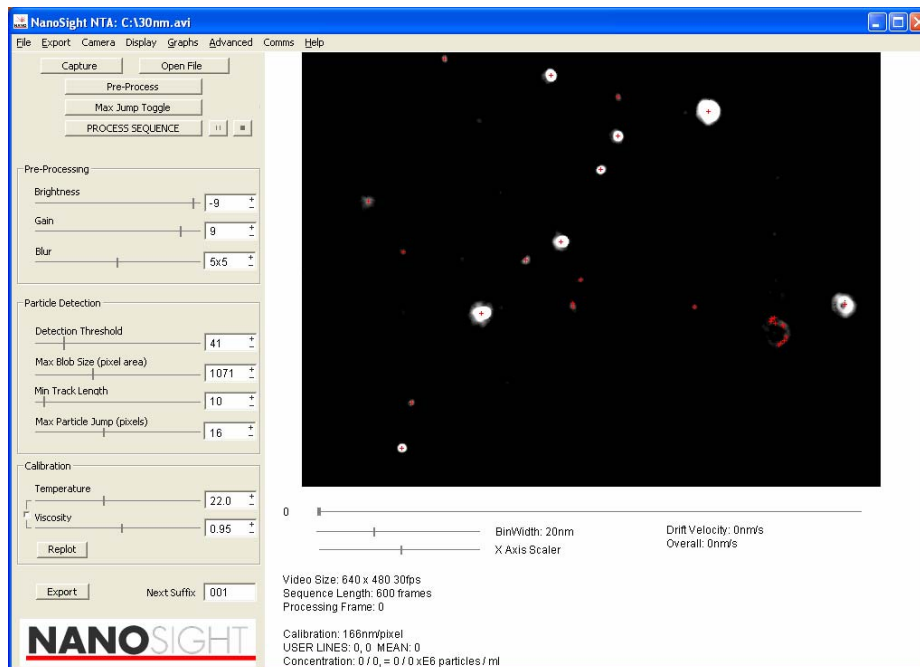


Figure 40: Optimum settings to analyse 30 nm gold colloids.

A **Gain** of **9** has been used to allow better visualisation of the dimmest particles. As a result of gaining the image, the background noise will also be gained. Reducing the **Brightness** to **-9** will remove the background noise from the analysis. The **Detection Threshold** should be reduced (**41**) to include the dimmest particles in the analysis and the **Maximum Blob Size** increased (**1000**) to include the largest particles in the analysis.

As the particles have very rapid movement under Brownian motion they will not exist in the field of view for extended periods of time. As a result if a long **Minimum Track Length** is set, no particles will be included in the analysis. As a result a short duration must be set *i.e.* **5-10 frames**. By using the **Maximum Jump Toggle**, the largest particle jump can be estimated by looking at a single particle movement over two successive frames. Particles in the order of 30 nm will have large movement over this time period and therefore this distance is set at **16 pixels**.

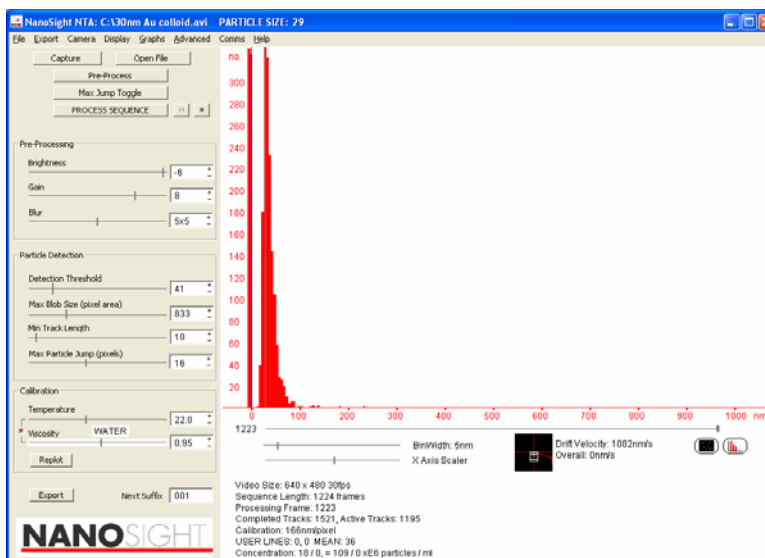


Figure 41: Particle size distribution for 30 nm Gold Colloid



4.8.7 Poly-Dispersed Sample of 200 nm and 400 nm Polystyrene Reference Spheres

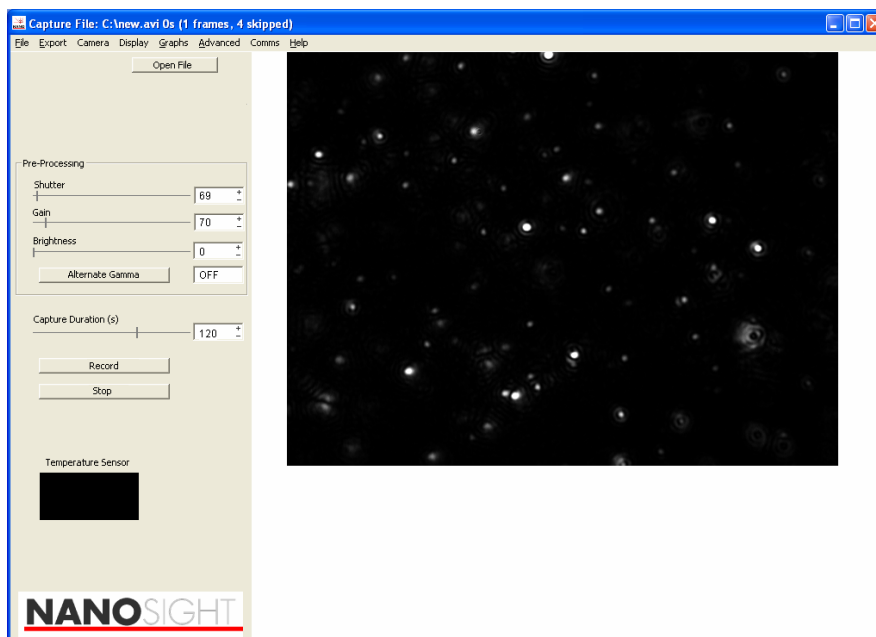


Figure 42: Ideal image of 200 nm and 400 nm poly-dispersed sample.

As can be seen from Figure 42, the sample has been treated as if it were a 200 nm sample. The camera controls have been used to reduce the 200 nm particles to dim discs as in Worked Example 1. As a result the 400 nm particles appear much brighter in the image and hence have been captured in non-optimal conditions. The limitations of the technique (when analysing poly-dispersed samples) depends largely upon the scattering efficiencies of the two particle populations. If the two populations of particles have vastly different scattering characteristics, *i.e.* 100 nm and 600 nm, then the user will not be able to capture both particle populations at optimal settings. If the camera settings are used to optimally capture the 600 nm particles then the 100 nm particles will not be visible, conversely, if the camera controls are used to optimally capture the 100 nm particles then the image produced by the 600 nm particles will be oversaturated and difficult to centre. In situations such as these, two-step analyses will produce the most accurate results. The system should be used to optimally capture an image from the 600 nm particles and an analysis performed. The sample should then be physically filtered to remove the large particles and a second analysis performed on the small particle population.

Poly-dispersed systems which have particle populations that are closely matched in size present a different problem. Now the broadness of the distribution is the limiting factor when trying to resolve particle peaks. The distribution produced by this technique is slightly broader than a distribution produced by techniques such as PCS. Since Brownian motion is a random process, the software tracks the particles over a number of frames and calculates the average distance it has moved over that time period. If the particles were tracked to infinity, the average distance moved by a particle would be accurately related to its size. The particles tracked in this technique however are tracked for a finite time and hence there is the possibility that the particle size produced for a single particle may be slightly larger or smaller than its actual size had it been tracked to infinity, and hence the distribution produced is broadened. As a 'rule of thumb', particles which are no larger than 4 times the size of another population of particles can be resolved (limited by the quality of the image produced) and particles no smaller than 1.5 times the size of another population of particle can be resolved (limited by the broadness of the distribution).

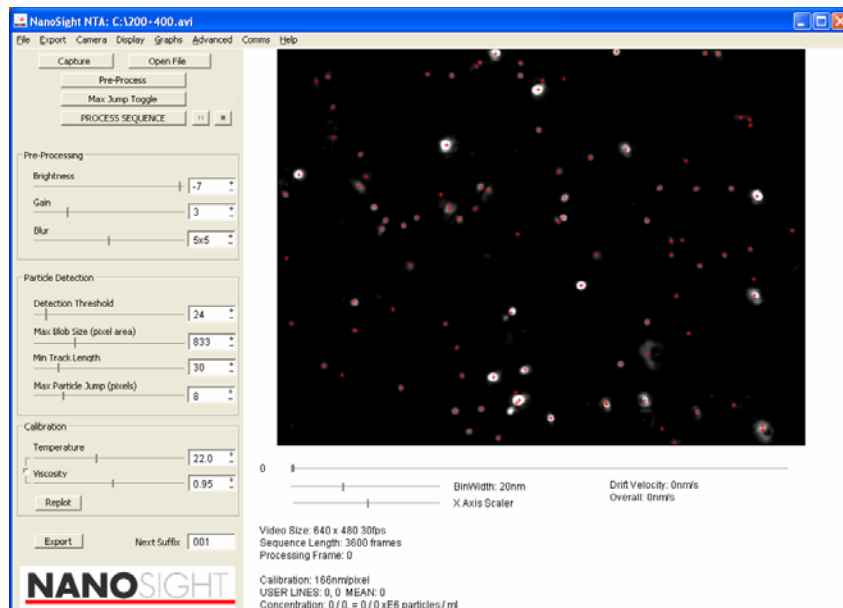


Figure 43: Ideal settings used to analyse 200 nm + 400 nm mixed sample.

- Gain 3: Makes the dimmest particles more visible – depending upon the image this may not be necessary as it also gains the larger particles.
- Brightness: -7 – Removes any background noise and flare around the particles.
- Blur: 5x5 Allows the centres of the largest particles to be accurately found.
- Detection Threshold: 25 – To pick up the dimmest particles.
- Max Blob Size: 1000 Increased to include the largest particles in the analysis
- Min Track Length: 30 – Set moderately low to include the relatively fast moving 200 nm particles in the analysis. Having a very high value here would prejudice the distribution towards the 400 nm particles as they are more likely to exist in the field of view for longer (as they are brighter and move more slowly).
- Max Particle Jump: 8 – Set to encompass the fast moving 200 nm particles.

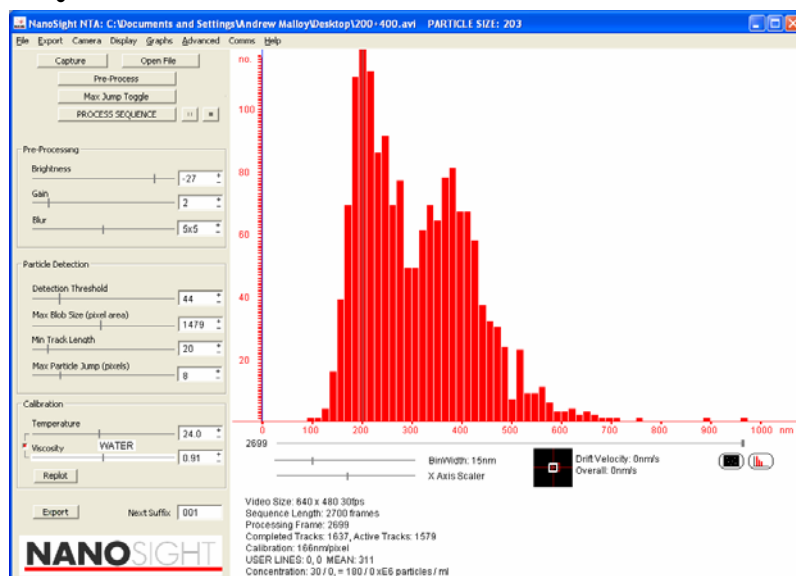


Figure 44: Particle size distribution produced for sample of 200 nm and 400 nm particles.



4.9 Summary

Ideal camera settings for optimal image acquisition:

Particle Size	Shutter	Gain	Brightness	Alternate Gamma	Capture Duration
30nm	500	680	1	Possibly	20s
100nm	250	680	1	No	30s
200nm	70	100	1	No	60-90s
300nm	0	20	1	No	90-120s
400nm	0	0	1	No	120-150s
200+400nm	90	280	1	Possibly	90s

Once an optimal image has been acquired using the above settings (or similar to) the following settings can be used to produce an optimal analyses.

Particle Size	Brightness	Gain	Blur	Detection Threshold	Max Blob Size	Min Track Length	Max Particle Jump
30nm Gold	-9	9	3x3	40	~250	5-10	16
100nm	-5	3	5x5	30	~250	15-25	10
200nm	-3	3	5x5	30	~500	30-50	8
300nm	-4	3	5x5	30	~1000	30-50	7
400nm	-4	3	5x5	30	~1000	40-60	5
200+400nm	-27	2	5x5	45	1450	20	8



5 Frequently Asked Questions

5.1 Fundamental questions

5.1.1 How much light is scattered by a particle?

The particles that we size are generally in the Rayleigh or Rayleigh-Debye-Gans scattering regime. As an approximation, the Rayleigh regime will be briefly discussed. In this case the efficiency of light scattering is given by:

$$\sigma_s = \frac{2\pi^5 d^6}{3 \lambda^4} \left(\frac{n^2 - 1}{n^2 + 2} \right)^2$$

Therefore the factors affecting the amount of light scattered are:

- d is the particle diameter. If you double the particle size you increase the power of light scattered by a factor of 64.
- λ is the wavelength of light. If you half the wavelength of light you increase the scattering by a factor of 16.

- n is the ratio of the particle refractive index to the solvent refractive index. For

example for $\left(\frac{n^2 - 1}{n^2 + 2} \right)^2$ latex particles (ref. ind. 1.59) in water (ref. ind. 1.33), n is 1.195 and the factor is 0.0157.

However for the case of gold (ref. ind. 0.14-3.7i) in water n is $0.1053 + 2.7820i$ and

$\left(\frac{n^2 - 1}{n^2 + 2} \right)^2$ is 2.3, a factor of 147 greater than for the latex particles.

The amount of light scattered is also directly proportional to the intensity of incident illumination.

5.1.2 How long is a sample stable for? Will the nanoparticles aggregate?

The science behind dispersion chemistry is not simple and we do not claim that we can stop your particles aggregating. We often use a little ultrasound but usually look to the customer to provide a protocol to disperse and stabilize the system. We also refer to ISO 14887 Sample Preparation – Dispersing procedures for powders and liquids.

However if the sample is at suitable viewing concentration then the injection and time to the start of the measurement can be less than 30 seconds and a measurement can take as little as 10 seconds. Therefore our system can be used to monitor aggregation if the process is sufficiently slow (e.g. takes longer than 5 minutes).

Note also that if a sample settles to a significant degree over an hour or two, this strongly indicates that it contains particles in the μm range and which are therefore not true colloidal nanoparticles and therefore may be at the extreme of our analytical capability.



5.1.3 The particles are only being imaged in two dimensions, but they must be moving in three dimensions. Is this a problem?

No. The Stokes-Einstein theory that is used to calculate the particles size from the particle motion can take into account whether two or three dimensions are tracked (see equations below).

$$\overline{(x, y)^2} = \frac{2kTt}{3\pi a\eta} \quad \overline{(x, y, z)^2} = \frac{kTt}{\pi a\eta}$$

5.1.4 How does a larger particle (such as an advanced agglomerate or contaminant) affect the measurement?

Unlike other light scattering techniques we are not strongly affected by large contaminants. This is because we visualise individual particles. Whilst an unusually large particle will scatter far more than the primary particles, its scattered light will be confined to and tracked only in a discrete region of the image.

5.1.5 Can I use different solvents in your device?

Yes. Whilst the system is typically optimised for water as the solvent we have got good results from other solvents. As an indication solvents with a refractive index up to a value of approximately 1.45 can be analysed. For solvents with a higher refractive index please consult NanoSight. Water is 1.33 and a water-optimised LM unit will work up to about Rf of 1.4. Above this, a unit optimised from 1.40 should work up to 1.52, but best way is to try this.

5.1.6 What is the upper size limit of particles that can be sized by your system?

The upper size limit of 500nm that we suggest is an approximation and dependent on particle type. The NanoSight NTA technique analyses the movement of a particle undergoing Brownian motion by tracking in time the spot of light it scatters. For large particles:

1. The diffusion of the particle becomes increasingly limited making the accurate determination of its mean displacement progressively less accurate.
2. For dense (metal or metal oxide) particles, sedimentation rates begin to exceed the limited Brownian motion.
3. The spot of light becomes so large and its edges so variable between frames, that identifying its centre becomes increasingly difficult leading to centring errors which are as large as the particle's movement.
4. the numbers of particles that can be viewed at any one time decreases (because of the amount of light they scatter)

These effects get progressively larger as particle size increases so there is no specific limit to our upper size range. This upper limit will depend on the nature of the system. In practice, for low density and low Ri particles such as polystyrene microspheres their buoyancy and weaker scattering allow them to remain Brownian movers with easily identifiable centres and the upper size limit is 600-800nm or above.

For dense, high Ri particles such as gold, their rapid sedimentation rate and excessive scattering restricts the upper size at which they can be analysed to approx <200nm.

5.1.7 What is the smallest particle that can be analysed?

The minimum size of particle that is detectable depends on the scattering being sufficiently strong. To understand this question better see 'How much light does a particle scatter?'. As a general guide:

- Low Ri (e.g. biological/polymer) will be detectable down to approximately 30-40nm.
- Higher refractive particles (e.g. ceramics, metal oxides) will be detectable down to approximately 15-25nm.
- Exceptionally high Ri particles (e.g. Ag sol, Au colloid) can be detected at diameters as low as approximately 10nm.



5.1.8 Why do my particles twinkle and appear to diffuse asymmetrically?

Whilst currently we can not put quantitatively answer this, our current understanding is that this is due to the particles being significantly aspherical (e.g. oblate or prolate)

Please periodically check the NanoSight website for future developments in the area..

5.1.9 What is the best particle concentration to use?

The NanoSight system relies on the measurement of each and every particle in the field of view over a period of time. The trajectory described by each particle is tracked for as long as it is visible or until it crosses with that of an adjacent particle. By determining the average step distance moved by any particle, and estimate of Δt and therefore sphere equivalent size can be determined.

Statistically, low numbers of particles measured for too short a time period will not generate data which is as good as that obtained by looking at higher numbers of particles for longer. Adequate statistics can be obtained from samples in which 50+ particles are visible at any given instant in the field of view. This equates to approx 10^6 particles / ml as an acceptable minimum. Of course, for low concentration samples, statistical accuracy can be improved by extending analysis time from, for instance, 10 or 20 seconds to 3-4 minutes.

Upper concentration ranges are defined by the acceptable proximity of particles to each other in the field of view. The trajectories described by small, fast moving particles very soon cross those of their nearest neighbour at which point tracking is terminated. This necessarily restricts the number of steps over which such particles can be tracked, reducing the accuracy with which the necessary average step length and therefore size can be determined. Therefore a maximum concentration of approximately 10^{9-10} /ml of these smaller particles is approached.

5.2 Validity of method

5.2.1 How accurately can particle size be determined?

For samples in which an adequate number of monodisperse particles are present in the sample (typically $>10^9$ /ml), the determination of particles size can be as accurate as that of equivalent dynamic light scattering techniques such as Photon Correlation Spectroscopy. For samples in which solvent viscosity and temperature is correctly entered, the diameter of a well prepared, monodisperse, calibration polystyrene microsphere can, for instance, be accurately measured to within 1-2 percent of nominal. Increasing analysis time (e.g. 1 to 2 minutes) will increase accuracy of measurement.

For polydisperse samples, the NanoSight system exhibits significant advantages over PCS in that the size distribution profile obtained is based on the separate but simultaneous analysis of each and every particle seen during the analysis period. This is in stark contrast to the data obtained by PCS in which a large ensemble of particles are measured and from which only an intensity weighted (z-average) is obtained.

5.2.2 Are there enough particles in my analysis?

For there to be enough particles in the analysis the only criterion that can reliably be used is that as more data is added to the histogram, the shape of the histogram does not change significantly. This is a function of the concentration of particles, the time of analysis, the tracking success of particles, the bin width and the polydispersity of the sample. As a guideline we would recommend that your first frame has greater than 10 particles (to keep the experimental time required to a reasonable length) and less than 100 (otherwise there is a significant risk of particle tracks being discarded due to ambiguity about their origin).



As a rough guideline a 30s movie clip of monodisperse 100nm particles will have sufficient number of tracks with a bin width of 20nm. Another guideline value to use is that the peak bin should have a frequency greater than 50.

5.2.3 Why do you not use an increased frame rate to obtain more data?

Whilst an increased frame rate (for example 60fps) would give more data, it also means that the exposure time for the camera is necessarily shorter which means weaker scattering particles may not be observed. Similarly, shorter sampling rates results in shorter distances being measured with a resulting loss in accuracy of estimation of motion. We have found that our current 30fps system is adequate for all particles visible in our system.

5.2.4 How well can different sizes be resolved from each other in a mixture?

Under ideal conditions (two very monodisperse populations of similar type and of similar number density, we have achieved resolutions of less than a factor of 1.25. Should either of these populations exhibit any significant breadth of distribution, their diffusion coefficients become too similar to differentiate easily and the size ratios at which they can be resolved will also increase accordingly.

In practice, for all but the most well prepared of calibration particles, resolution of mixtures of particles within a 1.5 diameter ratio can rarely be achieved with a high degree of confidence. This, however, represents a significant improvement on the resolutions routinely achievable with PCS which, in practice cannot resolve systems by greater than a factor of 2.

5.2.5 How reproducible is the result?

Given the statistical nature of analysis of a population of particles, reproducibility is a function of the numbers of particles measured over a given period of analysis. For optimum concentrations of monodisperse samples containing no contaminating aggregates, results reproducible to within approx 2-3% can be achieved in a comparatively short time (e.g. 10-20seconds). Clearly, for low concentrations of particles exhibiting a range of particle sizes, extended analysis periods will be required to obtain reproducibility of results. Note that analysis times need to be increased for larger particle diameters to achieve similar reproducibilities.

5.2.6 Can you use NanoSight system to obtain concentration measurement?

Whilst the NTA software suite does produce a number distribution, this is not an absolute function of the sample concentration. There are a number of reasons for this which are addressed in a number of the following questions.

5.2.7 Small particles are not tracked as long as large particles. Is this a problem?

The LM10/LM20 systems will size the particles that can be centred for a sufficient number of consecutive frames (min track length). Therefore if the particles are tracked for fewer frames than this they will not be counted.

The minimum track length should be set sufficiently low to track the smallest particles. The minimum track length is therefore set by the smallest particles in the sample. We suggest the following rough guidelines be followed; however this setting is also dependent on the particle type, concentration and analysis time being sufficient to obtain reasonable numbers.

Minimum particle size in sample (nm)	Minimum track length setting
<50	5
100	10
200	15
300	20
400+	25



5.3 User questions

5.3.1 How wide a range of particle sizes can be analysed at the same time?

When the range of particle sizes (and/or refractive indices or indeed relative number concentration) becomes too wide, no single choice of camera and software settings (gain, shutter speed, threshold, etc.) will allow all particles present to be analysed with equal accuracy. While a high number of small particles may be measured even when a certain number of larger particles (aggregates or contaminants) are present, when the number of such large particles becomes excessive, their high population and large light scattering footprints can begin to obscure the presence of the smaller population. Of course, the point at which the results become non-representative depends strongly on the relative numbers of each population and the size differential between them. For instance, in selecting a low camera gain setting which is optimal for the larger sized fraction, such settings may be inappropriate for the smaller size which accordingly, will be missed in the analysis. Similarly, a setting at which small particles can be visualised may well result in excessive signal from the larger population. In practice, for polystyrene microspheres, a mixture of 100nm and 400nm particles can be analysed if the numbers of each size are approximately 2:1. Conversely, 100nm particles will be increasingly difficult to analyse if 400nm particles are present at number ratios in excess of 1:2.

Visual inspection of the sample by the microscope can often allow the user to see the presence of lower numbers of smaller particles in a sample but at which exist at concentrations which become too low for the analytical programme to measure them accurately. This ability is, however, a significant advantage over other particle sizing techniques (such as photon correlation spectroscopy) in which the user is effectively working blind and frequently may be unaware of the presence of smaller particles. At this stage, physical removal of the larger sized fraction by either filtration or centrifugation is appropriate and will allow the smaller population to be reanalysed with far higher accuracy.

5.3.2 How do I calibrate my system?

Our method of particle tracking is absolute as it is based on dynamic measurements in the time domain. The only parameters that need to be known are the solvent temperature and the viscosity of the solvent at that temperature. Therefore there is no calibration required other than ensuring the field of view of the camera is correct. In all systems we supply this is factory set and not user adjustable. For estimation of particle number concentration, however, it is advisable that the user use another technique to determine the number concentration of a sample, using this value to calibrate the NanoSight system for counting purposes only.

5.3.3 How long does the sample temperature take to equilibrate?

The sample volume, at approx 500 μ l, is smaller than is typically used in other light scattering measurements. Therefore the time taken for the liquid temperature to equalise with that of the metal housing is relatively small. 30s should be adequate time for temperature equilibration.

5.3.4 I am still seeing a significant flow of particles, but have waited longer than 30s. Why is this?

There are three possibilities why this may be so and these are listed below in increasing likelihood:

- 1) Sample leakage. When setting up, it is imperative that the optical flat surface is correctly seated in the viewing unit, the sealing 'O' ring is correctly seated in the top plate and that the top plate has been brought into contact with the optical flat surface horizontally. Refer to the manual for a description of these processes.
- 2) The beam has become incorrectly aligned and light is being absorbed by the optical flat surface. If this is the case check that the flat was seated correctly in the
- 3) The particles are very large and very absorbing causing heating in the cell
- 4) The solvent you are using is very absorbing, which is causing convective currents.



5.3.5 Small particles are not as bright as large particles and I find it hard to track both at the same time, what should I do?

If after using the optimum capture settings, the processing settings can either not be adjusted such that the smallest particles can be seen or they can be seen but can not be successfully centred on, it may be necessary to carry out a two-pass experiment. This would involve first sizing the large particles then subsequently filtering the sample and recording a movie of the smaller particles. As a guideline, if the smallest particles are a factor of three smaller (in terms of diameter) than the largest particles these may be difficult to analyse if present in equal numbers.

5.3.6 I want to put my movies in a presentation but they are very large. Can I compress these?

Yes, the easiest way we have found to do this is using the Windows Movie Maker software that comes with, for example, Windows XP. The settings we use are all the default ones and this we find perfectly sufficient. Note, you will not be able to re-analyse video clips following compression

5.3.7 Is it possible I could have blocked the inlet outlet ports with particles?

Particles over 100 microns may block the inlet ports.

5.4 Specification questions

5.4.1 What are the space requirements of the system?

The system requires room for a desktop PC and (in addition) approximately 0.4 x 0.4m of bench space

5.4.2 What are the dimensions of the sample chamber?

Cell volume: Approximately 0.25 ml.
Cell depth: 0.9mm

5.4.3 How long is the lifetime of laser source in LM10 unit?

10,000 hours specified by manufacturer

5.4.4 How long can I leave a sample in the cell?

Providing that the system that you are looking at is suitably stable, the water will not evaporate over a period of many days. Note however, that some particle types will 'plate out' on the internal surfaces of the sample chamber (through, for instance, electrostatic interactions) and may increasingly contribute to noise within the system. Also, on re-analysis, there may be a significant reduction in particle number.

5.4.5 Is the system safe?

Yes. The system is a Class I Laser product if used correctly according to the manufacturer's instructions as it has a fail-safe mechanism.

5.4.6 What is the unit made of?

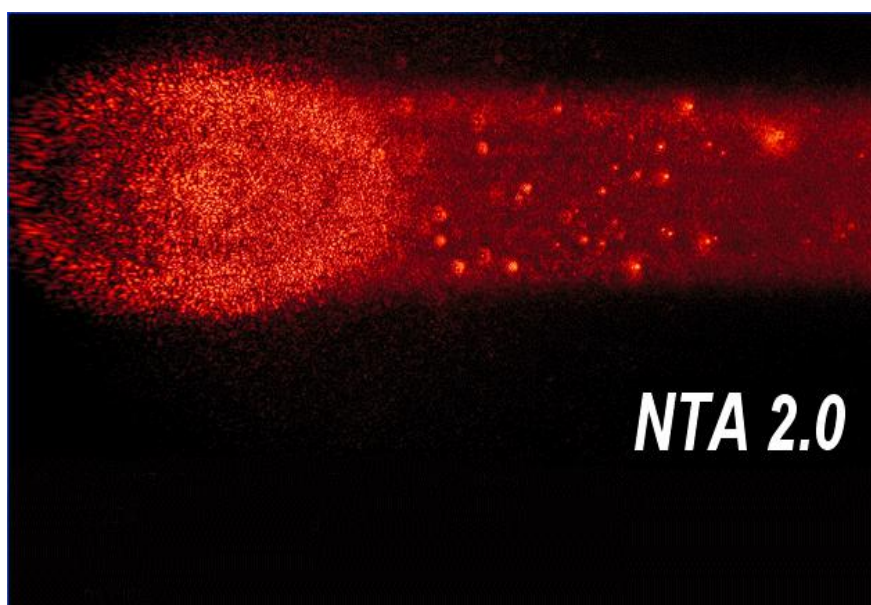
The parts of the LM10 unit in contact with the sample are made of glass, stainless steel and the plastic of the inlet/outlet ports. The seal is Viton.

5.4.7 Do I need an anti-vibration table for the system?

No. Any solid and heavy surface that is suitable for conventional microscopy will suffice though extended analyses will be affected by significant and continuous vibration.



NTA 2.0 Analytical Software



Operating Manual

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Introduction

NTA 2.0 is a new release of NanoSight's Analytical Software and is used for determining nanoparticle size through individually but simultaneously tracking and analyzing the trajectories described by multiple nanoparticles undergoing Brownian motion in a fluid.

NTA 2.0 adds significantly improved functionality compared to the previous NTA v.1.5;

- New screen layout.
- Extended Dynamic range allowing analysis of more polydispersed particle system.
- Improved analytical views including Particle Intensity vs. Size and 3D plot.
- Live display allowing extended capture durations for better statistical accuracy.
- Improved accuracy of tracking and sizing algorithms.
- Modelling to take into account track length.

System Requirements

The encrypted software is designed for optimal use with a system operating on Windows XP Professional.

The system requirements are:

Minimum Specification

- 1.83GHz Intel Core 2 Duo processor
- 2 GB 800MHz DDR2 RAM
- 160 GB 7200rpm SATA hard drive
- 1280 x 1024 Display
- IEEE 1394 port (Firewire) + 2 free USB ports

Recommended Specification

- 3.0GHz Intel Core 2 Duo processor
- 2 GB Ram 1333MHz DDR2 RAM
- 250 GB, 7200rpm SATA hard drive
- 1680 x 1050 Display
- IEEE 1394 port (Firewire) + 2 free USB ports
- 256Mb Video graphics card

As it is not possible to test the software with the full range of computers on the market, NanoSight strongly recommends, and only supports, the use of the PC that they specify and supply directly and which is included with your order.



Software protection

In order to run the program a software license key needs to be installed and then activated on the PC. This will come preinstalled on any PCs supplied by NanoSight, but will need contact with NanoSight (at support@nanosight.com) for details of how to obtain further licenses.

For capture of videos the NTA 2.0 software also requires a blue security dongle which should be inserted into a USB port on the computer and installed prior to using the software. Information regarding installation of the software and the dongle can be found in the 'NanoSight NTA2.0 Installation document' document.

Start-Up

The software comes pre-installed on NanoSight-supplied computers and can be run either from the desktop icon (NTA 2.0) or via the start menu (under All Programs > NanoSight NTA 2.0).

Basic Principles

The NanoSight LM10 or LM20 nanoparticle analysis instruments generate videos of a population of nanoparticles moving under Brownian motion in a liquid when illuminated by laser light. Within a specially designed and constructed laser illumination device mounted under a microscope objective, particles in the liquid sample which pass through the beam path are seen by the instrument as small points of light moving rapidly under Brownian motion.

The light scattered by the particles can be conventionally modelled by Mie theory (Kerker, 1969; Bohren and Huffman, 1983) though the determination of particle size by measurement of light scattering intensities alone by using this device would require significant a priori knowledge of the optical properties of the particle, solvent, collection optics and camera sensitivity and performance. A more attractive alternative, given the ability of the NanoSight system to visualise nano-scale particles in real time and in liquids, is to dynamically analyse the paths the particles take under Brownian motion over a suitable period of time (e.g. 10 seconds).

Despite the rapidity with which particles move (in the sub-100nm size range in particular), such motion can be readily tracked using conventional CCD cameras. Supported on a C-mount on the microscope and operating at 30 frames per second (fps), such cameras can be used to capture video clips of particle suspensions when present in the approximately 80 micron wide laser beam within the device. It should be appreciated, however, that the particles are not being imaged. For the nano-scale particle range to which the NanoSight system is best suited, the particles act as point scatterers whose dimensions are below the Rayleigh or Abbé limit, only above which can structural information and shape be resolved. Such videos can then be analysed using the NanoSight NTA 2.0 analytical software program described here and from which the size of each particle can be separately determined and accurate particle size distribution profiles derived accordingly.

The video can be either captured directly from the camera through the program or imported as a separate *.avi file. The first frame of the 8 bit video sequence can be user-adjusted in terms of image smoothing, background subtraction, setting of thresholds, removal of blurring etc. to allow particles of interest to be tracked without interference from stray flare or diffraction patterns which can occasionally occur with non-optimum sample types.

Having selected suitable image adjustment settings, the remainder of the video is similarly treated allowing particles to be identified and located on a frame-by-frame basis. The movement of each particle is followed through the video sequence and the mean squared displacement determined for each particle for as long as it is visible.



The user can further select for trajectories whose lifetimes are sufficiently long to ensure statistically accurate results, ignoring those which are so short (e.g. below 5 or 10 frames) that the estimation of diffusion coefficient is statistically inaccurate. This feature can be employed under manual or automatic control. The possible occurrence of measurement of trajectories which cross is automatically eliminated thus minimising errors in particle tracking.

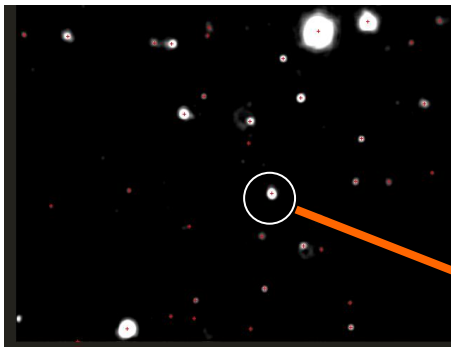
From simultaneous measurement of the mean squared displacement of each particle tracked, the particle diffusion coefficient (D_t) and hence sphere-equivalent, hydrodynamic radius (r_h) can be determined using the Stokes-Einstein equation:

$$D_t = \frac{K_B T}{6\pi\eta r_h}$$

where K_B is Boltzmann's constant, T is temperature and η is solvent viscosity.

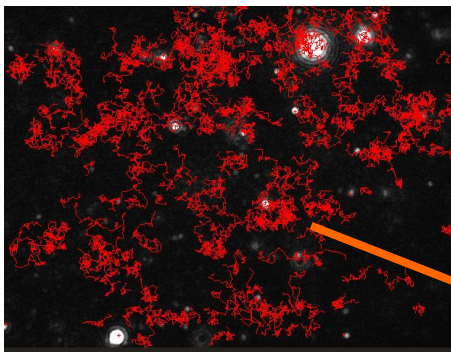
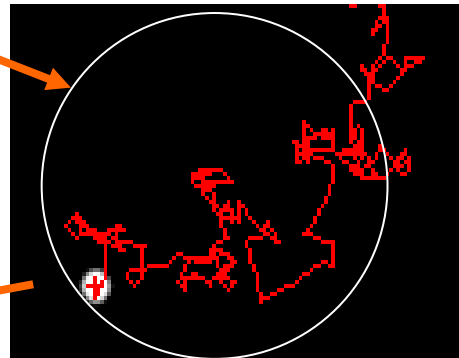


Principle of Operation of NTA 2.0 at-a-glance



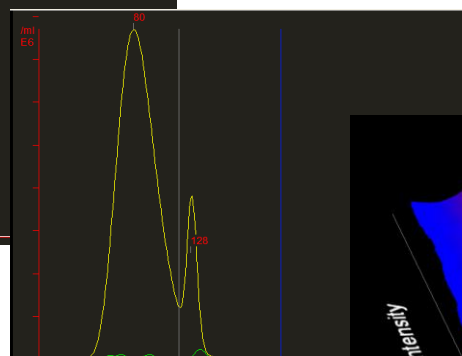
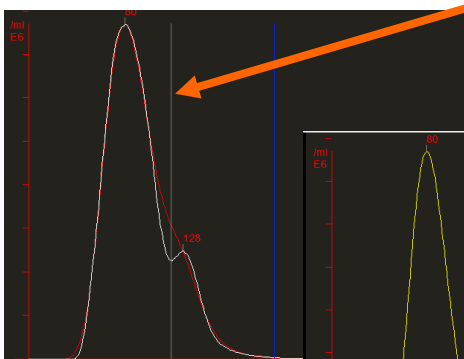
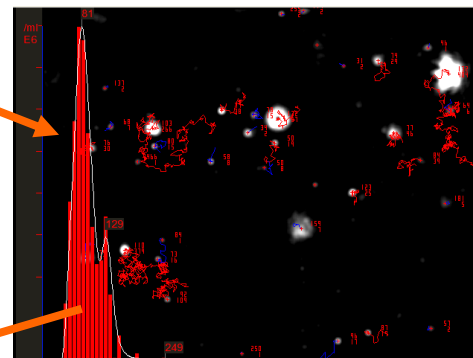
NTA 2.0 captures a video of particles moving under Brownian motion.

NTA 2.0 automatically locates and follows the centre of each and every particle and measures the average distance it moves per frame

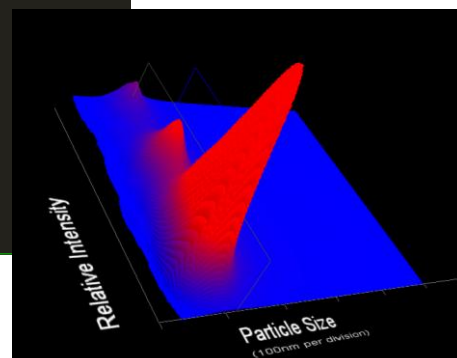


This is done simultaneously for all particles until hundreds or thousands of particles have been tracked

NTA 2.0 converts the distances moved into a particle size and plots accumulated results in real time as a particle size distribution profile.

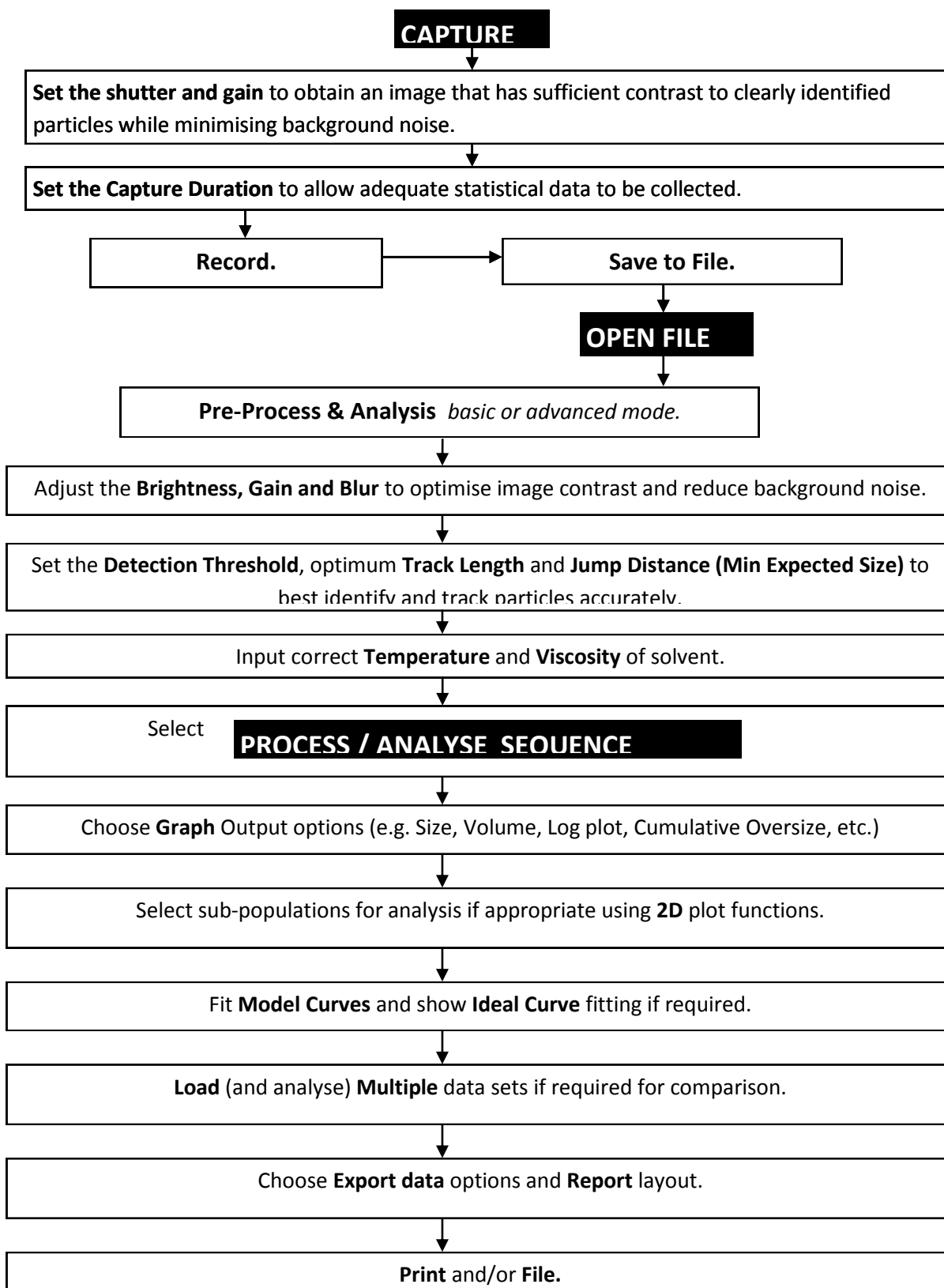


NTA 2.0 analyses the raw data, fits model distributions or displays different particle parameters (size vs brightness vs number) against each other.



NTA measurement process flowchart

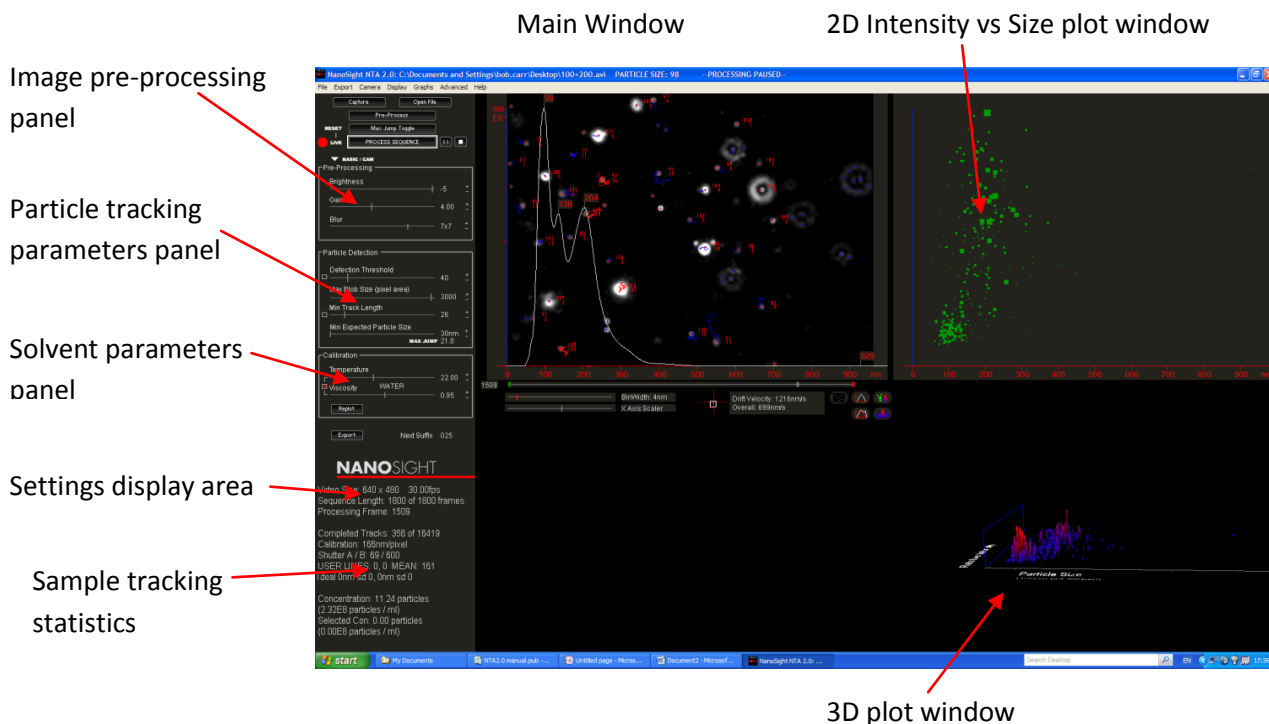
The flowchart illustrated below demonstrates a typical sequence of an experiment.





NTA 2.0 Opening Screen

On opening the NTA Program, the following screen appears*:

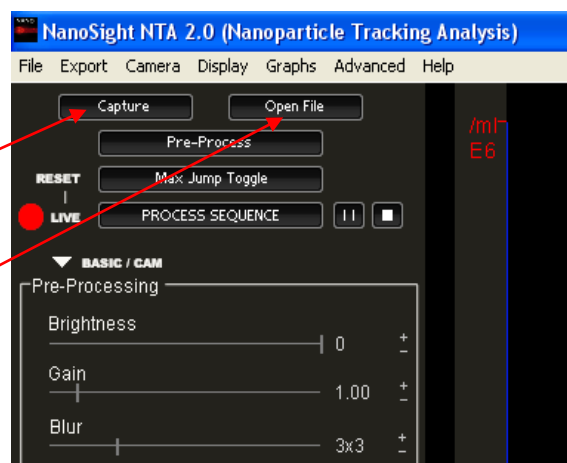


The user has two choices, either of which can be selected by clicking one of the two buttons found at the top left of the opening screen;

1. **Capture** a video from the LM10 or LM20 camera for saving or immediate analysis
2. **Open File** to load a previously captured video file for (re-)analysis.

Depending on choice, the program will either

1. go to the **Capture** Screen, or
2. **Load** a *.avi video file(where * denotes the file name chosen when captured and saved)



*NTA display on non standard resolutions.

Dependent on screen resolution, the NTA 2.0 Interface presents a slightly different layout to the standard 1680 X 1250 pixels. The differences are such as when displayed on a 1280 X 1024 screen are summarised as follows:

- Under Extended Dynamic Range (see page 16) the B screen is not fully visible at this resolution, so to view each screen use the image A/B button
- The Intensity scatter plot & 3D plot can be displayed by selecting the toggle 2D / 3D plot.
- The "Output parameters" interface is repositioned below the live display.



NTA 2.0 Capture Screen

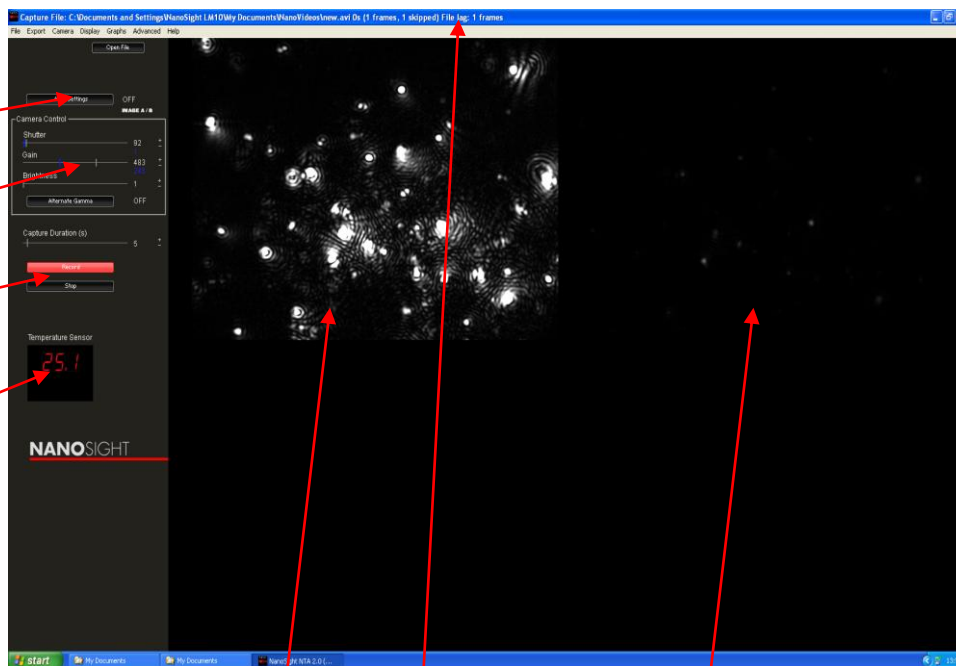
The Capture Screen illustrated below is broken into two different functional areas;

1. The control interface, which is responsible for setting the capture and analysis parameters
2. The display screen, which displays a screen of the live camera output.

Control Interface

Display Screen(s)

Auto-settings
button
Camera Control
panel
Capture
duration, start
and stop buttons
Temperature
display (for
LM20 only)



Live display
from camera

Area of second live display when
system running in Extended Dynamic
Range mode [see page]

Window header bar displaying;

D:\My Documents\NanoVideos\new.avi 0s (1 frames, 1 skipped) File lag: 1 frames

File name

Seconds of video captured

Frame number

Skipped frame count

File lag


File Lag—The Windows header bar shows, on the right hand side, the “File lag” value which represents the degree to which the rate at which frames are being written to disc from RAM is falling behind the rate at which they are being captured by RAM from the camera. If this value exceeds 150 (through slow disc write speeds arising from fragmented drives (lack of memory) or excessive disc activity (through the running of other background programs), the camera capture will be momentarily stopped until the RAM buffer clears enough to continue.

Video Capture

The video capture interface comprises the camera control interface and the live display. The control interface is responsible for setting the capture controls; shutter and gain to produce an image that has sufficient contrast to identify particles while minimising noise levels in the dark areas of the image. On pressing record a user predetermined number of frames will be recorded as an uncompressed video file (*.avi—where * is a file name chosen by the user). The following commands and settings are designed to be set and enabled in sequence.


Auto Settings

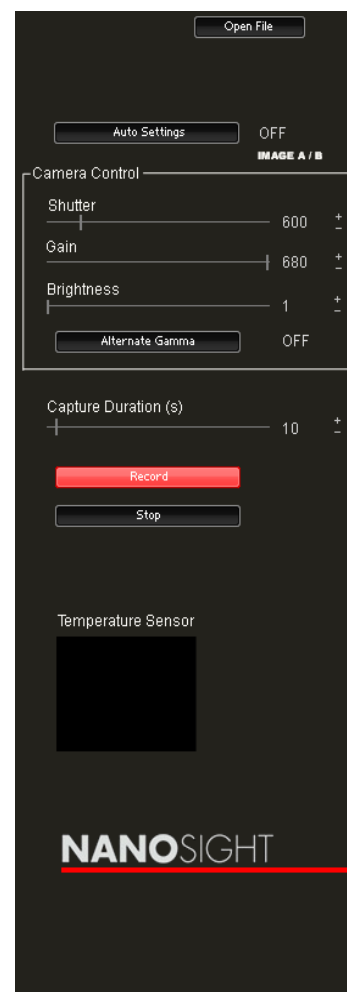
Toggling the Auto-settings button will switch this feature on and off. When switched “ON” this feature allows the software to automatically select appropriate shutter and gain settings (based on the intensity of the brightest particle present) for the sample being interrogated. When activated, the green indicator lines move automatically to ideal setting positions and should stabilise their positions within a few seconds.

 *Note : This feature should be used only for relatively monodispersed samples. If the sample contains low numbers of large particles, the numbers of which fluctuate significantly over time periods of a few seconds, the automatically adjusting camera shutter and gain settings will be seen to be continuously shifting in an attempt to accommodate these large fluctuations in scattered intensity. Accordingly, there is no single range of settings which will be optimum for such sample types. It is advised that the user switch this option off for such samples and manually adjust the settings such that the smallest particles present in the sample remain visible and therefore capable of being analysed.*

If the sample is very polydisperse, the user could alternatively engage the **Extended Dynamic Range** option available in this version of NTA. For details of the operation and usage of this option, see page 16.

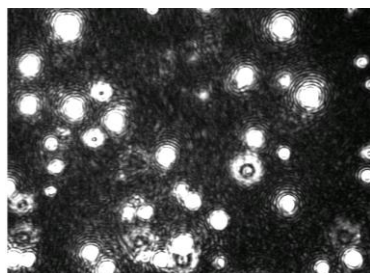
Shutter Determines the length of time the camera electronic shutter is open for and therefore how much light is captured from the particles. Dim particles (associated with small particles or particles with a low refractive index) usually need to be captured with a longer shutter time.

 *If the shutter setting bar turns red, the camera is being over exposed and the shutter setting will need to be reduced until the red warning bar disappears.*





Gain This setting increases the sensitivity of the camera and should be increased to allow the smallest particles present in the sample to be made visible and therefore capable of being tracked and analysed. This setting should be increased instead of increasing shutter speed for the detection of small particles.

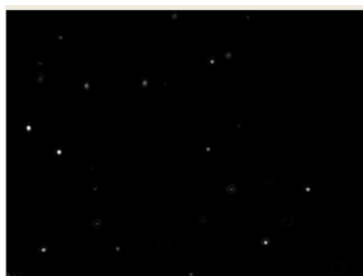


Example of overexposed image



In practise, it is generally a good idea, on looking at an unfamiliar sample, to momentarily increase the gain setting to maximum to ensure that all particles that exist in the sample are being visualised.

If no smaller particles appear on increasing the camera gain, the gain should be reduced to lowest setting compatible with retaining an image of the smallest particles present.



Example of an optimal image to allow accurate particle centering

Alternate Gamma Increases the value of low intensity pixels whilst applying reduced gain to the brighter pixels thus improving the detection of low intensity particles without causing larger, brighter particles to become saturated. Because background noise increases when this option is enabled, it should only be used for samples containing extremely small particle types at the limit of detection.

Capture Duration Determines the length of video that is captured. Longer videos allow more particles to be analysed therefore increasing statistical accuracy of determination of the particle size distribution profile. Adequate capture duration is largely dependent on the size of the particles present in the sample and their concentration.



Optimising capture duration

- *Too short a capture duration will result in insufficient numbers of particles being analysed to generate reproducible particle size distribution profiles. This, of course, will depend on the concentration of particles present in the sample.*
- *As a general guide, capture durations should be chosen to be long enough such that the particle size distribution profile obtained is reproducible on repeat analyses. For modestly high concentrations of monodisperse particles in which >50-100 particle centres are identified in any given video frame ($\geq 10^9$ particles/ml), adequate analysis times may be as short as 10 seconds. In general, however, 30-90 seconds capture is adequate for most sample types of correct dilution (e.g. 10^8 - 10^9 particles/ml).*



- *For samples containing lower concentration of particles (e.g. <10 particles in the field of view at any given instant) extended analysis times up to the maximum capture time of 215 seconds can be employed. Note, however, that longer capture times can result in very large video files occupying large amounts of disc memory space (e.g. 300MB/10seconds of video).*
- *An alternative to capturing such large videos is to employ the **Live Capture** option. In this case, the output of the camera is analysed simultaneously with video capture. When this option is engaged, no video is stored representing a significant saving on disc space usage.*

*Note however, because no video is collected, re-analysis of the sample is not possible. When run in **Live Capture** mode, samples can be run for **many hours** continuously thus accumulating data on a large number of particles and improving measurement statistical accuracy. For details of use of the Live Capture option, see page 30)*

Record Starts the video capture. The video can be stopped at any time by pressing 'Stop'.

Input temperature At the end of the video capture sequence, the user is prompted to input the temperature of the sample at the time of analysis. This can be obtained from use of the Temperature Probe that is supplied with the LM10 instrument (see relevant manual). In the case of the

LM20, the temperature is automatically read by the LM20 instrument and input into the analysis program. The temperature is displayed in the lower left hand side of the Capture Screen.

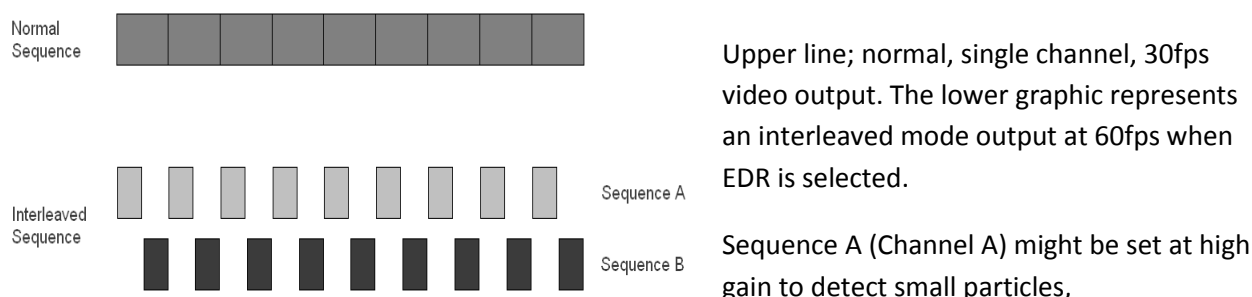
Save file On entering the temperature, the user is finally prompted to input a file name for the video to be saved and a location to which it can be saved. The default name is "new.avi" and the default save location is in c:\...\mydocuments\nanovideos*.avi. When saved, the program automatically opens the video in the **Pre-Process and Analysis** window if the **Process after Capture** option is enabled in the **Camera** menu

Extended Dynamic Range (EDR) capture

! Due to the high processing and disk access demands on the computer when running this NTA 2.0 option, it is recommended that all other applications are shut down.

Prior to operating in EDR mode it is expected that the user has become familiar with the use of NTA 2.0 running in standard mode. This is described in the previous section Video Capture and in the following section on Pre-process & Analysis (*Page 18*).

The Extended Dynamic Range (EDR) option described here offers additional functionality to the standard operating mode of NTA 2.0. It allows particles in very polydispersed or very heterogeneous samples (which may differ significantly in light scattering intensity) to be simultaneously visualised, tracked and analysed. This is achieved by doubling the camera frame rate from 30 frames per second (fps) to 60fps, and outputting two simultaneous, interleaved video streams each running under different camera settings. This process is shown below:



Sequence B (Channel B) might be set with a very low gain to enable large particles to be visualised and tracked without over-exposing the camera.

In EDR mode, alternate frames of the double-speed video output are set at different shutter and gain settings allowing one channel to be optimised for one part of the population, the other channel optimised for the remainder of the mixture.

Typically, EDR is enabled when analysing a bimodal sample containing two particle populations of significantly different light scattering power (e.g. of very different size (and/or refractive index) such as a mixture of 100nm and 400nm particles) and which would normally require different shutter and gain settings to be set for each population.

Activating the Extended Dynamic Range (EDR) Mode

! In order to reconfigure the camera an NTA restart is necessary.

Steps to engage EDR mode:

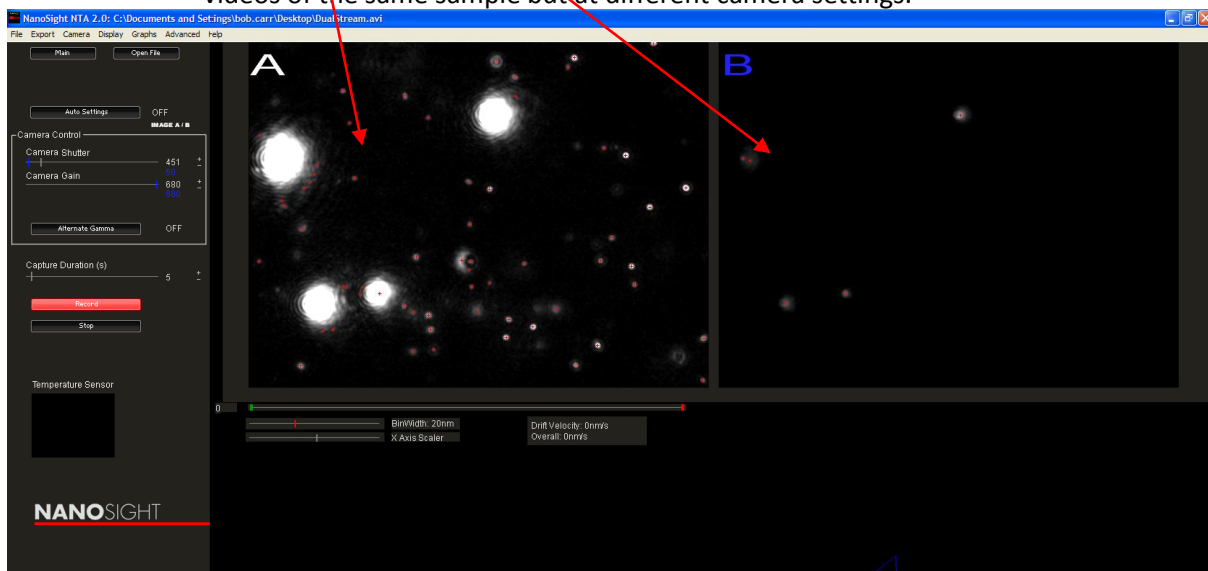
1. If in NTA already, exit the program and restart NTA 2.0 from the start menu or the NTA icon on the desktop.
2. Click on **Extended Dynamic Range** in the Pull down **“Advanced”** menu.
3. Select Capture mode by pressing the Capture button.
4. The EDR Capture screen will then be displayed.

! Note:

- Once the camera is initialised in 60fps EDR mode, it is possible to change back to 30fps normal capture mode. However, the program must always be restarted to enable 60fps EDR mode.
- Auto Capture Settings mode is disabled in EDR Mode

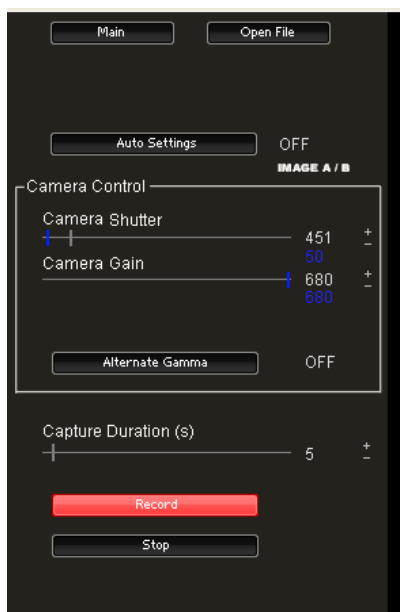
EDR Capture Screen

Channel A and Channel B screens representing interleaved and simultaneous videos of the same sample but at different camera settings.






Optimise exposure for capture



“A” Capture window

Using the *left* mouse button to move the white slider bars, increase the shutter and gain controls such that the smallest of particles present are made visible irrespective of whether the larger particles present may be significantly over-exposed (these larger particles will be captured in the ‘low gain’ B Channel).

 Remember to use the gain to increase particle visibility while keeping the shutter as low as is possible without losing the image.

“B” Capture window

Using the *right* mouse button to move the blue slider bars, reduce shutter and gain settings to the point where the larger particles in the B Channel screen are reduced to small spots which can be more accurately tracked. Even if, at such low settings, many or all of the smaller particles disappear, they will be tracked in Channel A.

! Note: Auto Capture Settings mode is disabled in EDR Mode

- In EDR mode the maximum shutter value is 700
- Shutter settings **must** either be **both above 180** or **both below 180** to avoid a camera configuration conflict.

Set **Capture Duration** and **Record** as normal

! NTA display on lower resolution screens.

- NTA 2.0 is designed to display on 1680 X 1050 pixel resolution screens. If the program is run on a lower resolution screen (e.g. 1280 X 1024), only one of the Channels can be *displayed* at any time even though both are capturing as required.
- To switch between Channel A and Channel B displays simply toggle the “IMAGE A / B” button.



Pre-process & Analysis

Analysis Screen main features

Pre-processing or Image control panel

Particle Detection - tracking control panel

Temperature and solvent viscosity input panel

Analysis parameters

Graphing and display options area

- **Pre-processing (Image Control Panel)**

Allows adjustment of the image to give optimal particle detection. The image is the first frame of the video and settings applied to this frame are then automatically applied to all the remaining frames in the video upon processing.

- **Particle Detection (particle tracking control panel)**

Allows optimum settings for tracking of particles

- **Calibration—Temperature and Viscosity Input**

Allows input of solvent temperature and viscosity

- **Analysis parameters**

Selected results from analysis

- **Graphing and display options**

Allows different graph and display options to be selected



- **Particle Size Distribution plot and Image screen**
Main screen for results plots optionally overlaid on sample image
- **2D Intensity vs. Size Plot**
Plot of particle brightness vs. particle Size in nm
- **3D Intensity vs. Size vs. Number plot.**
3D plot of Intensity vs. Size vs. Concentration

Pre-Processing

Having loaded video, the Pre-Processing section in image control panel allows the user to further optimise the image for subsequent particle tracking and analysis.

! The Pre-processing, Particle Detection and Calibration panels described below are designed to be used in sequence for ease of use and should be adjusted before starting the analysis.

Image Pre-Processing

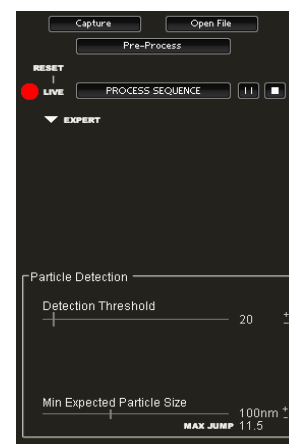
Basic mode

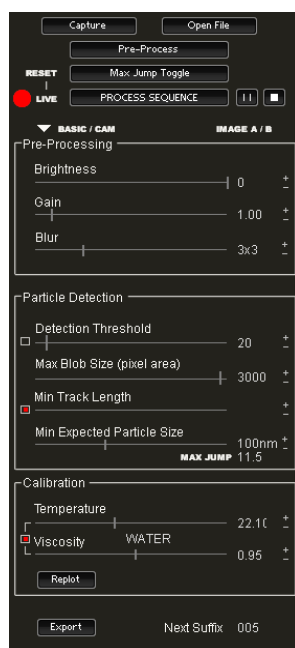
If a video was captured under Auto-settings, the Pre-processing panel under which the image can be adjusted is not required and is therefore not present. This is because video capture under Auto-settings mode automatically adjusts image quality for optimum analysis and there is no need to adjust the image. The Image Control Panel can, however, be activated by pressing the “**EXPERT**” mode button allowing manual override of the settings (see below). The remaining **Detection Threshold** and **Min Expected Particle Size** buttons and their use are described below.

! Note that Auto-settings mode should only be used for samples which are relatively monodisperse and do not contain larger, highly scattering particles.

Expert Mode

The following settings can be adjusted to allow as many particles as possible to be identified and successfully tracked throughout the video and to reduce background noise.





Basic/Cam Toggles back to the basic mode described above.

Brightness This varies the captured image luminance. It should be decreased by use of the slider bar to remove background scatter (i.e. not directly from particles) or increased grey-scale background associated with high camera gain settings and use of Alternative Gamma. Excessive use of Brightness reduction may result in scatter loss of smaller particle images.

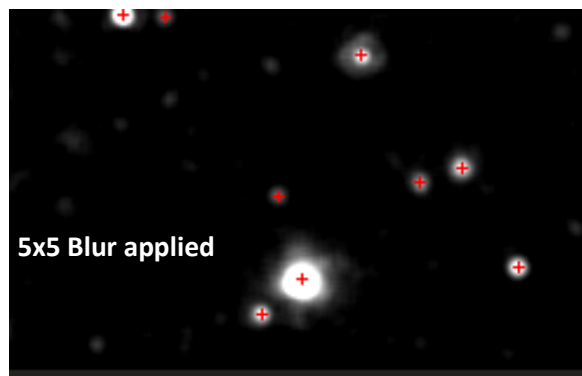
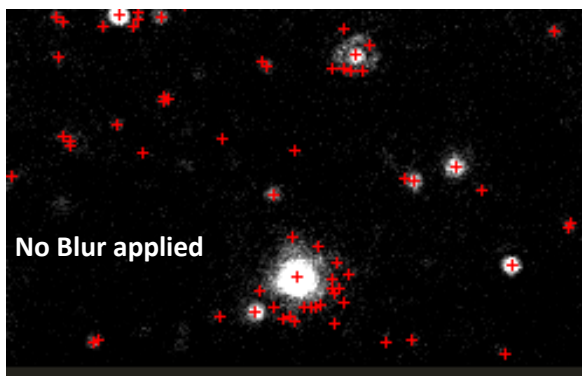
Gain The gain should be adjusted to allow all particles present to be visualised. The gain should, however, be increased only to the level where the smallest particles present are visible. Too high a gain setting for the sample will result in excessive saturation of the image which will cause problems in identifying and tracking the centre of particles.

i In practice, it is generally a good idea, on looking at an unfamiliar sample, to momentarily increase the gain setting to maximum to ensure that all particles that exist in the sample are being visualised. If no smaller particles appear on increasing the image gain, the gain should not be increased beyond this level.

i A successful combination of increased gain and reduced brightness acts to enhance the contrast of an image thus facilitating accurate particle tracking and analysis.

Blur – Acts as a smoothing function to eliminate noise (such as diffraction rings surrounding larger particles) from around or within a particle image or ‘blob’. A 3x3 blur integrates intensities over a 3x3 matrix. Higher blur values (5x5, 7x7 or 9x9) increase smoothing but become computationally expensive and cause the rate of analysis to slow. Furthermore, excessive blur may cause smaller particles to be smoothed to the point they fall below the detection limit.

As can be seen from the example images on the next page, the addition of a 5x5 blur allows a noisy image of particles to be smoothed such that particles/blobs are centred more accurately and false centres eliminated.



Note that 'false' centres are frequently short lived. As such, no such centre is likely to survive for more than a low number of frames and therefore is unlikely to be included in the analysis given an adequate 'minimum track length' setting described below. A minimum Blur setting of 3x3 is recommended and normally 5x5 is suitable. For polydisperse systems a Blur of 7x7 may be appropriate

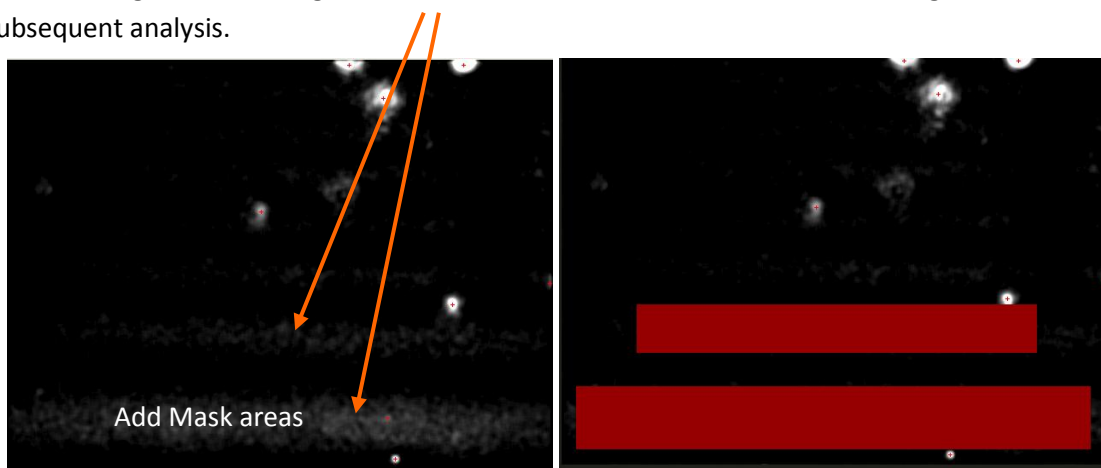
Masking/excluding an area from analysis - Occasionally, during video capture, an area of stray light or flare which is not associated with particles appears. This might arise from contaminant adhesion onto one of the optical surfaces, bubble formation in the sample, etc.. This area of static stray light may interfere with analysis and its effects should be eliminated. This can be done by **Masking** the area from analysis.

The user can insert a Mask/Exclusion Area onto the field of view by left clicking and dragging (from upper right to lower left only) the cursor when placed on the image. A red exclusion area is then

'painted' onto the image. Any area beneath it is excluded from subsequent analysis. Many areas can be introduced on the same image by multiple click-and-dragging in different places.

Right-clicking the mouse removes all Mask/Exclusion areas.

The following shows two regions of flare at the bottom of the field of view being masked out from subsequent analysis.

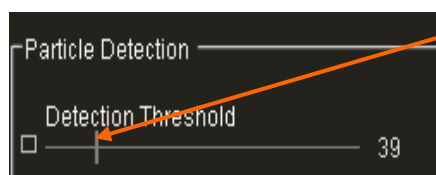
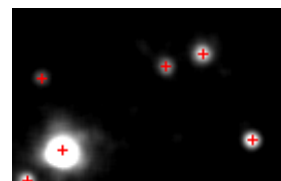


! Exclusion Areas cannot be set during processing


Particle Detection Settings

The following settings are used to optimise the tracking parameters by which particles visualised in the video as 'blobs' are sized.

Detection Threshold This value determines the minimum grey scale value of any particle image or 'blob' necessary for it to qualify as a particle to be tracked for analysis. When a particle has been correctly identified as a trackable object, a red cross will appear at the particle centre




Manual setting Use the slider bar to vary the minimum intensity level considered to be associated with a particle. In practice, the user should adjust this setting such that all clearly recognisable particles have been successfully centred with a red cross and there are few, if any, false centres (i.e. red crosses not associated with particles images).

 *Too high a threshold will result in loss of particles from the analysis. Too low a threshold may result in noise being incorrectly identified as particles.*



Automatic threshold setting. The user can activate the

Automatic setting for Detection Threshold by checking the box adjacent to the slider bar (red = on). The Auto-threshold option measures the size (in terms of pixel area) of each and every spot of light as scattered by a particle (a 'blob') on a frame-by-frame basis and adjusts the detection threshold to be optimum for that particle. One of four thresholds are allocated to each particle. These predetermined threshold settings are shown by the four green bars displayed when Auto-threshold is activated.

 *When **Automatic Threshold Detection** is enabled, the **Blur** setting that would normally be selected should be increased by a single step (e.g. from 3x3 to 5x5).*

Max Blob Size (pixel area) Allows the user to ignore particles or blobs larger than the value selected such that they are not included in the analysis and particle size distribution plot.


Min Track Length Because NTA 2.0 estimates the size of any given particle from measuring the average distance the particle moves, clearly the longer the time the particle is tracked for (the higher the number of steps taken), the better will be the statistical accuracy of the average and therefore of the particle size estimation. However, the time any given particle remains in the small scattering volume is limited, particularly so for small particles whose Brownian motion is rapid.

The average beam residence time for smaller particles is far shorter (perhaps as short as 0.2 seconds

[= 6 steps]) than for larger particles whose very limited Brownian motion means that they may, on average, remain in the scattering volume for many seconds (> 60 steps). Furthermore, for any given particle size, there may be a range of residence times depending whether the random three dimensional movement of the particle keeps it in the scattering volume for longer or shorter periods.




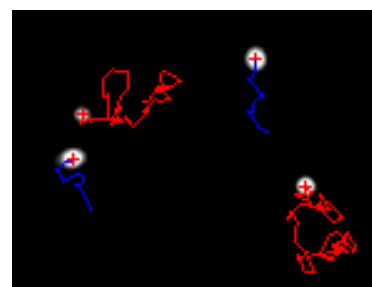
Manual setting Using the slider bar to enter a value for Minimum Track Length defines the minimum number of steps that a particle must take before its size value can be accepted for inclusion in the particle size distribution plot. Entering too large a value will bias the results to larger, slower moving particles in a mixture of particle sizes, or, for a more monodisperse sample, simply reduce the proportion of included tracks.

 *The combined effect of reduced particle numbers in the particle size distribution plot and a shift towards larger particle sizes can be easily seen either during or after analysis by increasing the Min Track Length value. A value of between 10-15 is adequate for most samples.*



Automatic Min Track Length setting. The user can activate the automatic Minimum Track Length facility by checking the small box adjacent to the slider bar (red = on). In this mode the program automatically selects and updates the optimum Minimum Track Length based on its calculated size at that instant.

 *.During analysis, the point at which a particle has reached the minimum number of steps required is shown by a change in colour of the particle trajectory from blue to red. Only once the trajectory turns red will this data will be incorporated into the results. This feature can be used to visually estimate whether an appropriate minimum track length setting has been selected. If few tracks ever turn red before the particle diffuses out of the scattering volume, the setting is too high for the sample and should be reduced.*

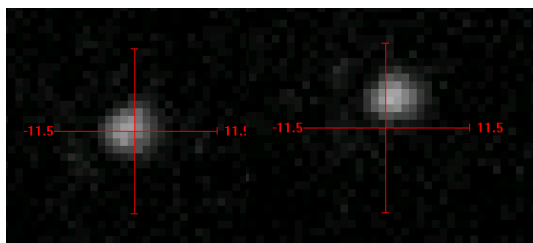
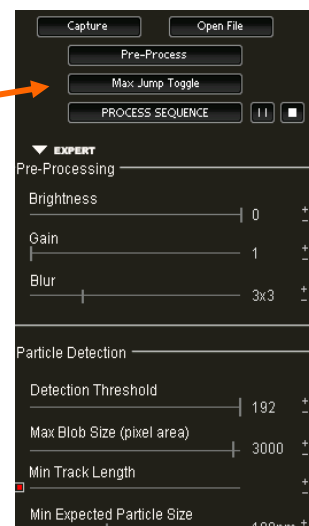


Min expected particle size The distance a particle moves between frames (i.e. within 30ms) will depend on its size (as well as the solvent viscosity and temperature). The value the user selects here determines the maximum distance (pixels) from the particle's position in a given frame that the software will search for a particle in the next frame (the "search area"). It also establishes an exclusion zone around a particle of the same distance. If another particle enters this exclusion zone then the software excludes the information from both particles. This prevents the software erroneously joining the tracks from two different particles. This value can be chosen and set in two ways:

Maximum Jump Toggle- Manual Setting


In order to accurately determine the maximum distance a particle jumps in the sample, the '**Max Jump Toggle**' button should be used.

When pressed, this button causes the program to toggle between Frames 1 and 2 of the video repeatedly. The user should then identify a particle image which appears to move the *greatest* distance between frames (a particle with a typically "Maximum Jump").



To 'select' the chosen particle, place the mouse cursor over the particle and click the *left* mouse button. The view then zooms into that particle and shows its movement between Frame 1 and 2 (as shown left).

This movement is overlaid with a crosshair showing the extent of the maximum particle distance selected (Note; the centre of the crosshair is zero). The size of the crosshair alters as the user changes the 'Min Expected Particle Size' slider. Using the slider, the user can adjust this distance such that the observed particle centres jump *within* the distance chosen.


 *It is advisable that the user adds at least two additional steps to the distance chosen to ensure a sufficiently large "search area". It is also good practise to repeat this exercise for a small number of particles.*

Maximum Jump Toggle– Automatic Setting



As an alternative to manually setting the Max Jump Distance as described above, the user can, if the Minimum **Expected Particle Size** is known, input this value using the slider bar shown below. The program will automatically

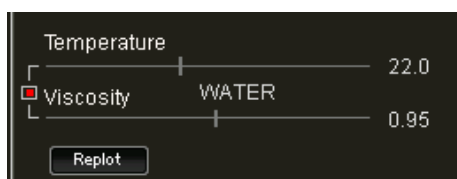
enter a 'max. jump distance' value which is known to be appropriate to that particle size.

 *This Max Jump distance automatically changes based on user selected values for Minimum Expected Particle Size for input values of viscosity, temperature, camera calibration and frame rate.*

Calibration

Temperature & viscosity

The Temperature and Viscosity of the solvent needs to be set before processing and analysis begins. This is either recorded automatically in the LM20 system or is prompted for by NTA 2.0 in the case of an LM10. These values are automatically input when a video is captured and are loaded with the video file.



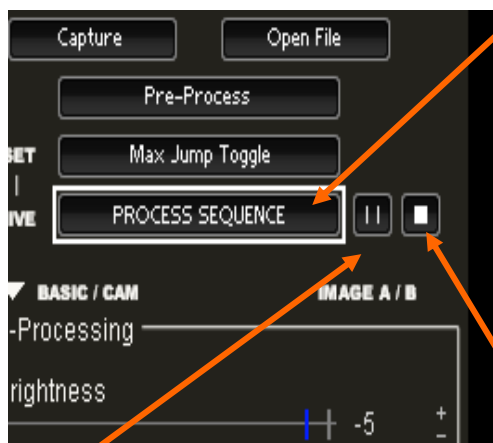
By clicking the tick box next to the temperature slider bar, the temperature will automatically be linked to the viscosity of water. For samples not dispersed in water, un-tick the box (it will appear white) and enter the viscosity value for the solution at the indicated temperature.

Replot If the values of sample temperature and solvent viscosities are changed, an adjusted particle size distribution profile is generated on clicking the Re-plot button.

Once the above settings have been adjusted to optimise the image of the first frame of the video and suitable detection and tracking parameters selected as described above, the rest of the video can be analysed and data accumulated.



Analysis



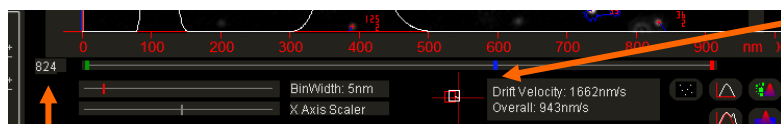
Process Sequence Starts the analysis of the Captured video sequence based on the Pre-processing settings previously selected.



Note that a previously captured video, loaded via *Open File*, will load the Pre-processing settings with which it was previously analysed or, if it has not yet been analysed, with the settings chosen for the last analysis undertaken.

Stop Stops the analysis and saves the results data accumulated to that point.

Pause Pauses the analysis until the button is pressed again at which point the analysis continues.



Progress Bar Shows the progress of the analysis through the video (grey bar). The green bar shows the start position (defaults to first frame), the red bar shows the finish position (defaults to last frame of video sequence)

Frame number

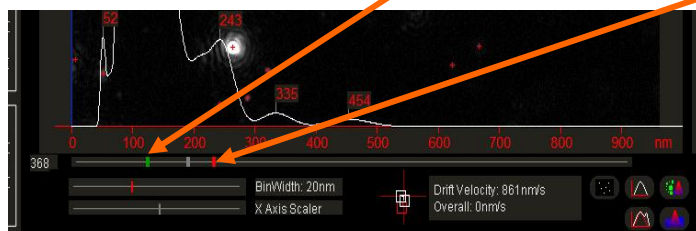
Shows the number of the frame being analysed at that instant.

Frame by Frame analysis

Pressing Pause and repeatedly using the 'right arrow' key on the keyboard allows the user to progress through the analysis on a frame-to-frame basis. Pressing Pause again allows the analysis to resume at normal speed.

Selecting a portion of the video sequence to analyse

At any time during the analysis, or when Pause is pressed, the user can select a specific region of the video to analyse. This can be done by placing the cursor to the left of the grey progress indicator bar and *right clicking the mouse button* to move the green start position bar. Similarly, moving the cursor to the right of the progress indicator bar and *right clicking*, the finish point (red bar) can be moved. It is necessary to allow the analysis (as shown by the grey progress bar) to analyse to a point *within* the region to be selected.



Pressing Process Sequence again restarts the analysis but only between the newly selected start and finish positions.

Pre-processing and Analysis in Extended Dynamic Range (EDR)

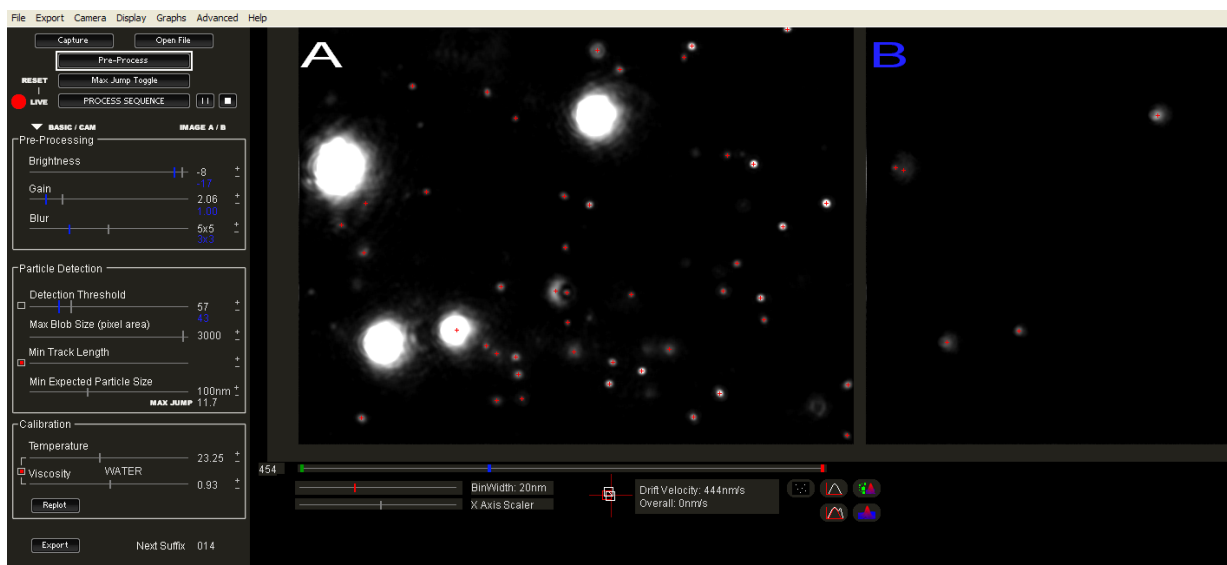
The Capture of a video under EDR has already been explained on page 16.

The Pre-processing and Analysis of an interleaved video is very similar to that of a single Channel video except that there are two Channels (A and B) whose settings have to be adjusted separately.

Pre-Processing

Therefore, **Brightness**, **Gain** and **Blur** and the setting of **Detection Threshold** are all adjusted as usual but using the

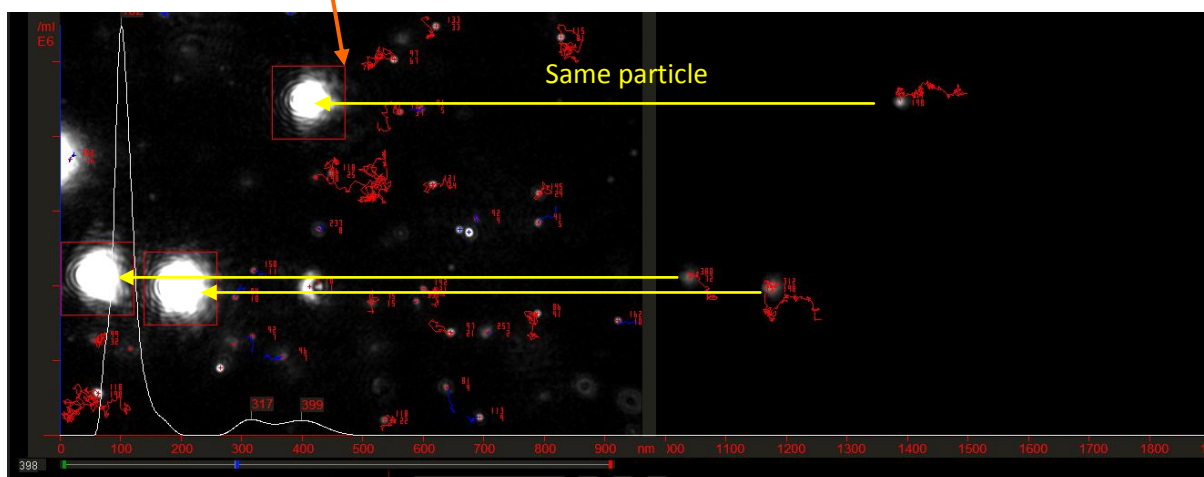
- *left* mouse button to adjust the grey slider bars applicable to Channel A
- *right* mouse button to adjust the **blue** slider bars applicable to Channel B
- *left* and *right* buttons on the automatic **Detection Threshold** check box will activate this function for Channels A and B respectively. The check box will show (half grey and/or blue) which channel is activated.



The Min Expected Particle size should be set based on the smallest particle present in Channel A.


Analysis

During analysis, NTA analyses both screens simultaneously but those particles which have been identified, centred and tracked in Channel B (whose settings are usually optimised for large particles) are excluded from analysis in Channel A (whose settings are adjusted to be optimal for the smaller particles in the sample). Those which are excluded in Channel A (typically over-exposed) are masked by an exclusion area depicted by a red square.



When EDR is activated, the requirement for two screens (showing the two interleaved videos, Channel A and Channel B) means that the 2D Intensity vs. Size Plot is **re-located** to lower left of the NTA 2.0 screen.

Live Capture/display - When this option is selected, NTA 2.0 captures and analyses the output of the camera directly, generating particle size distribution data in near real-time without having to capture the camera output to disc/hard drive first. This allows the user to collect results (but not raw video) over greatly extended time periods (e.g. many hours of continuous analysis).

 *Note, It is recommended that, for best results, NTA 2.0 is restarted before using Live Display.*

To activate “Live Display”, press the **red** button




Click **BASIC/CAM** to change the camera settings and screen shot

Once the live display function is running, the **grey** frame capture indicator line on the **Progress Bar** moves to show the number of the frame that has been captured in the RAM buffer. The **red** indicator line shows the frame currently being analysed. Typically, processing will be slower than capture leading to an increasing falling behind between the two activities in the RAM buffer. When the capture position




indicator overtakes the processing position indicator, the camera capture is momentarily interrupted and the two re-synchronise. This leads to a small jump in the displayed video. This does not affect the accuracy of results obtained.


During live processing the **Reset** button immediately above the Live capture button acts to erase all data and results captured up to that instant and starts a new analysis. Pressing the **Stop** button resynchronises the processing with the current capture frame.


 *Note, Because no raw data is captured to file or disc under this option, it must be remembered that re-analysis of a video is not possible and that the only information retained is that of the Particle size Distribution profile of the sample at the time of analysis (*.his).*

Live display gives the user the following benefits;

- In general use, the user is offered an immediate and direct generation of particle size distribution allowing a rapid identification of sample characteristics and/or confirmation that capture and analysis settings are appropriate for the sample under analysis.
- The user can run very extended analyses allowing very much improved particle size distribution statistics for low concentration samples in which the numbers of particles are very limited and the results therefore not very reproducible over normal experimental durations.
- Similarly, extended analyses addressing many thousands of individual particles allows features within a particle size distribution profile such as very minor peaks or shoulders on peaks to be confirmed as being 'real' and not merely statistical artefacts associated with limited data.

 *Under normal analysis (i.e. not the Live analysis described here) the maximum number of tracks that can be stored is 50,000 (**Completed Tracks**). When operating in Live mode, this 50,000 tracks limit is retained and when this limit is reached or exceeded, the current database of track histories is erased and a new database is begun. However, the particle size distribution graph (the 'result') is not erased, it being the accumulation of results from all particles seen.*

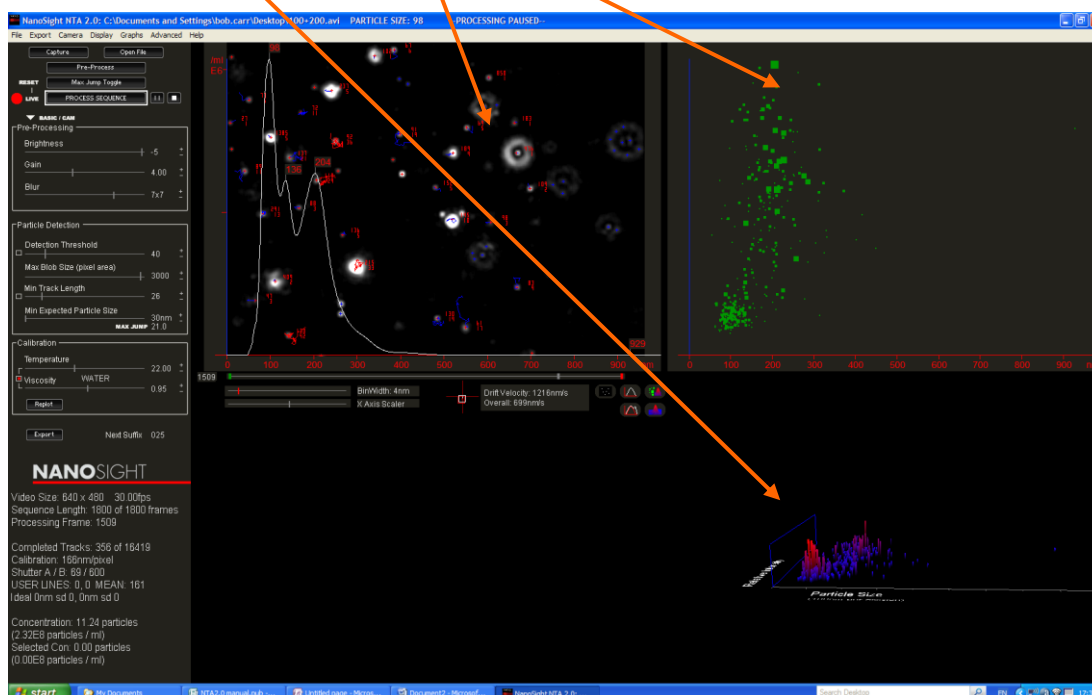
 *But because only the distribution profile data is retained, re-analysis of the data using different parameters such as track length restriction, temperature/viscosity changes and exclusions on the intensity scatter graph cannot be employed. Attempts to apply such settings will only affect those results which reside in the current particle database in RAM and cannot be applied to the whole history of particles. This is also the case for the datas created by the File Export facility.*

 *When creating a report or exporting data this should be performed whilst the **Live** is processing, i.e. Do not stop processing first.*

Display Options

On viewing the opening screen, two areas to the right of the main image screen are seen;

- Main graph/analysis screen
- 2D Intensity vs. Size screen
- 3D Plot Screen



Main Graph/Analysis screen

Display options for this screen are discussed from page 31.

Adjusting X axis

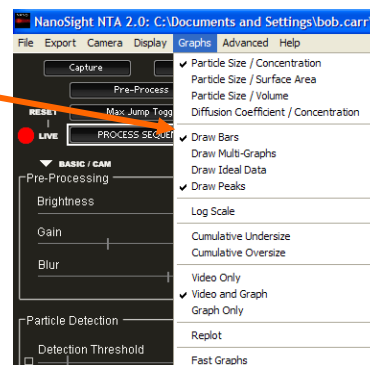
- The scale of the x-axis can be shrunk or expanded from its default range of 0– 1000nm (approx) by use of the x-axis adjust slider bar to the axis.
- The x-axis can also be moved without changing the scale by click and dragging on any of the numbers in the x-axis label.
- This will adjust the x-axis on all three screens.

Adjusting Bin Width

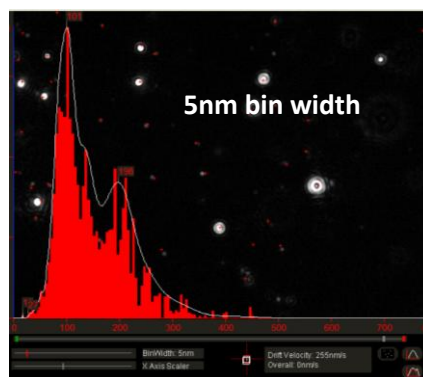
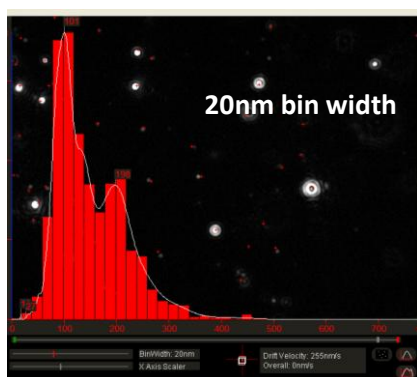
When in histogram mode (see 'Draw Bars' under the 'Graphs' pull-down menu), the output of the size Distribution

Profile can be displayed as bars of a histogram instead of the rolling average curve which is the default display option.

By using the Bin Width slider, the size of the bins in which the data is plotted can be adjusted. Thus, the default setting of a bin width of 20nm (left below) can be reduced to a bin width of, for instance, 5nm (right below), enabling a higher resolution plot to be displayed.



Because of the possible sensitivity of the displayed mode size to bin widths which are sometimes set too

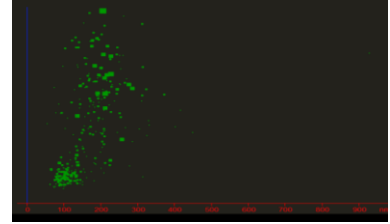


wide to be suitable for a given data set, the default display mode for

NTA 2.0 is that of a smoothed, rolling average curve (the white line). The degree to which the line is smoothed by adjacent bins can be adjusted by the user.

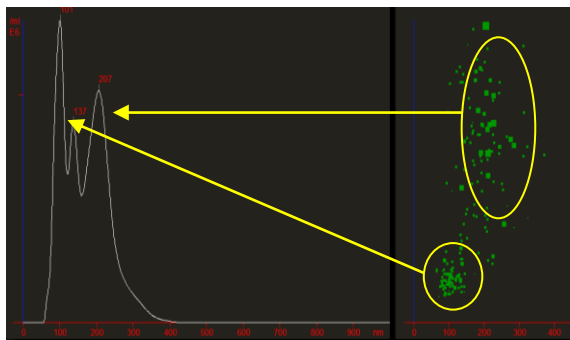
2D Intensity vs. Plot Screen

During the tracking and Brownian motion analysis of a particular particle, NTA 2.0 also measures the peak intensity (i.e. sum of all pixel values within a particle image or blob) exhibited by the particle during the time it is visible and being tracked. This allows the relative light scattering intensity of the particle to be plotted against the estimate of the particle size. These two independent but simultaneous measurements allow increased differentiation of particles from each other. For instance, it is possible through NTA 2.0 to differentiate between particles of similar size but which have a different refractive index. Similarly, two particles which scatter similar amounts of light which have different diffusion coefficients (e.g. diameters) can also be discriminated.

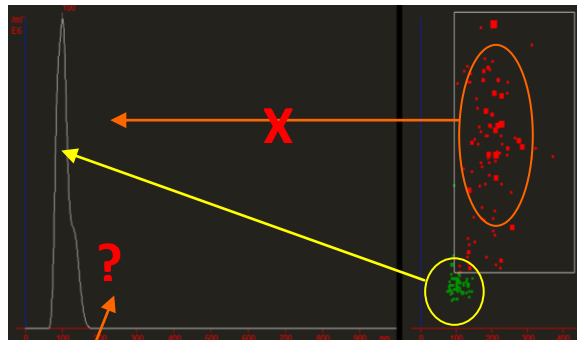


It is possible to graphically isolate one part of a population from another on the 2D plot by *left-clicking* and dragging the cursor (only from top left to bottom right) to highlight a particular part of the population.

As can be seen below, when highlighted, the particles are shown red and their contribution to the main particle size distribution plot on the left is removed.



Highlighted part of the population is removed from



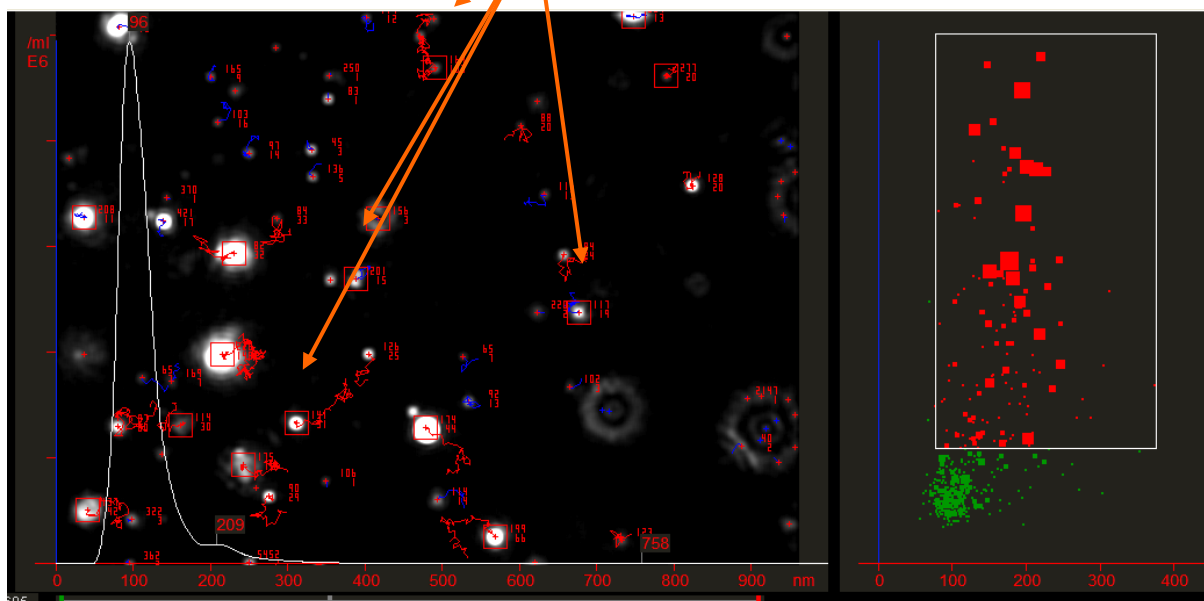
main particle size distribution plot.

Visualising highlighted particles during analysis

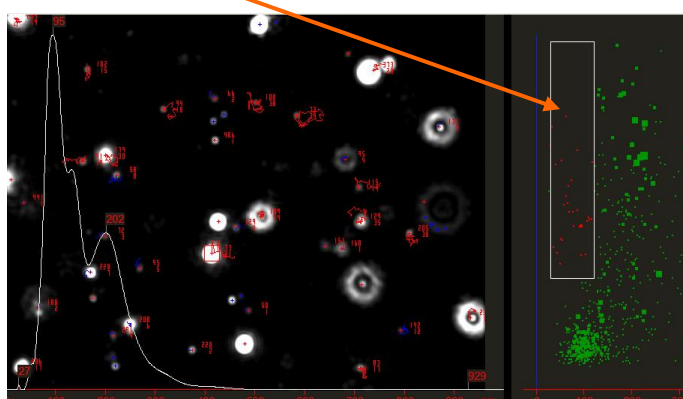
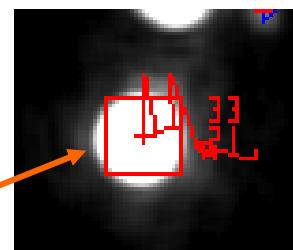
It is possible, while the sample is being analysed, to see which of the particles in the main screen are contributing the **highlighted** region.



Those particles which fall in the highlighted region can be identified in the left-hand main image and plot screen as particles surrounded by a small red square.



This capability proves useful in identifying sources of noise in the results which can sometimes appear as apparently small particles of high relative light scattering intensity. This occasionally occurs when an inappropriate 'max jump distance' or 'minimum expected size' setting is selected. In this case, large particles may be incorrectly tracked and trajectories may, in large particles, jump from the true centre to the edge of the particle blob and back again. If this occurs, the particle intensity remains high but the program interprets this additional false movement as a much more rapidly moving, and therefore much smaller, particle. This results in the apparent emergence of a high intensity, low size population in the region shown below.



*The emergence of significant numbers of particles in this region must be treated with caution and investigated further (e.g. by trying different 'min expected particle size' settings. Additional **Blur** or use of Extended Dynamic Range may help minimise such problems.*

However, it should be remembered that it is possible that the population does contain small, highly scattering particles (e.g. metal particles) as well as lower refractive index (e.g. polymeric) particles of a similar size.

Track length indication in 2D plot

The sizes of the green squares shown in the 2D Intensity vs. Size plot reflect the length of time over which a particle has been tracked; the longer the particle trajectory, the larger the square. This is useful for confirming that larger particles, on average, track for longer than smaller particles and that anomalies to this general principle should be investigated (as discussed above).

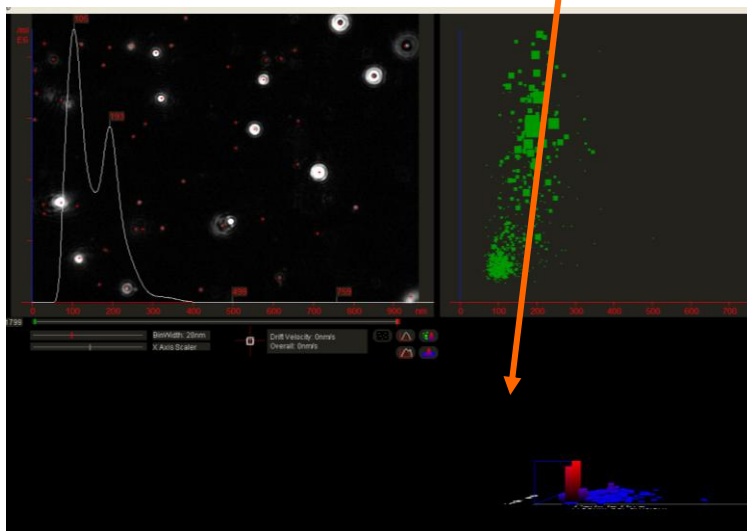
To switch off this feature, simply **right-click** on the **2D plot** and the track length-adjusted related square size will change to simple dots showing merely the presence of a particle without reflecting its track length. *Right click* again to return.

3D Plot

The 3D plot (shown lower right below) shows a plot of particle **Size** (x-axis in nm) vs. **Intensity** (yaxis in arbitrary units) vs. particle **Concentration** (z-axis in relative proportions—not enumerated).

This plot can be manipulated in a number of ways;

- It can be rotated in three dimensions
- It can rotate automatically
- The plot can be expanded or reduced in size
- The resolution of the plot can be adjusted in x and y (i.e. In Size and Intensity)



Rotation of the 3D plot in three dimensions

By clicking the left mouse button anywhere on the 3D plot and dragging (holding the left-hand mouse button down while moving the mouse), the plot can be rotated in 3 dimensions.

Rotating automatically

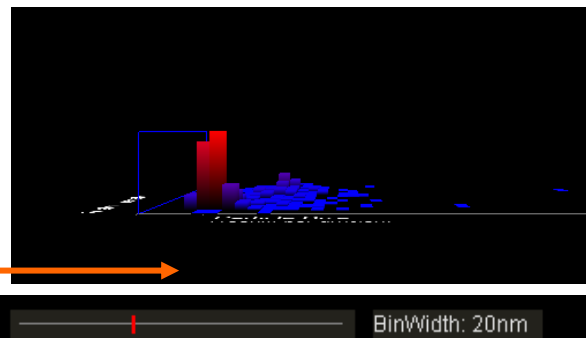
By pressing **Ctrl + 5**, the 3D plot rotates automatically and continues to rotate until the mouse is right-clicked on the plot.



Changing Resolution of the 3D plot

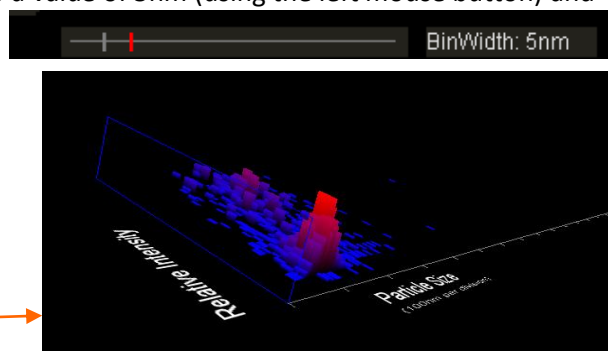
The resolution (i.e. Bin Width) of the plot (in both size and intensity) can be changed by using the **Bin Width** slider bar.

NTA 2.0 defaults to a Bin Width of 20nm and a plot of aspect as shown right.



- The resolution with which the size (x-axis) is plotted can be adjusted by *left clicking* on the **Bin Size** slider bar (grey line) and moving to the required resolution.
- The resolution with which the Intensity (yaxis) is plotted can be adjusted by *right clicking* on the **Bin Size** slider bar (**red** line) and moving to the required resolution.

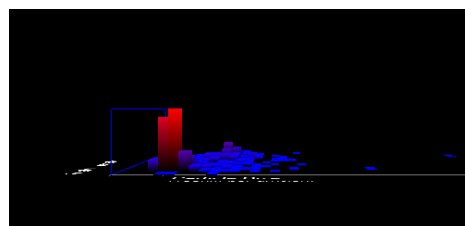
For example, changing the size bin width (grey line) to a value of 5nm (using the left mouse button) and changing the intensity bin width (red line) to a value of 10 (using the right mouse button) gives a plot as shown below. The value displayed for the bin width is always that of the Size.



Note the plot shown right has been rotated as described earlier for clarity.

Expanding or reducing the size of the plot

- Right clicking on the 3D plot and dragging from size to side adjusts the plot size (i.e. Zooms in and out)
- *Double left clicking* on the 3D plot acts to expand the plot to fill the right-hand side of the computer screen to improve clarity. *Double left clicking again* returns the image to its default size.

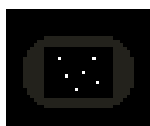
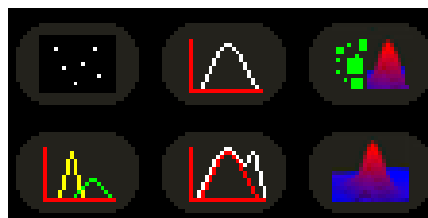




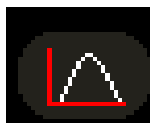
Plot and Display Icons

Clicking on one of the cluster of plotting icons seen in the centre of the NTA 2.0 screen allow the user to:

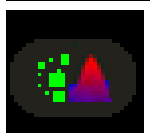
- Change the main window display
- Switch between showing the Particle Size Distribution plot or the video Image (or both overlaid)
- Smooth the 3D plot
- Fit mathematically generated model curves to the experimental Particle Size Distribution plot obtained
- Show the degree to which the model fits the experimental data, also showing residuals to the fit.



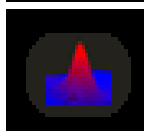
Video Image Display This switches off the graph plotting leaving only the video Image display. To draw graphs again, press the Graph Plot icon.



Graph Plot This switches off the video image display leaving only the graph plotting, display. To show the video image display again, press the video image display icon.



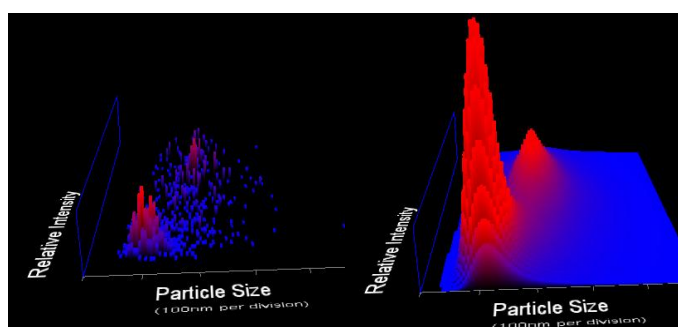
Main Display Option Toggling this icon allows the user a choice of which display to show in the main screen (i.e. video image/particle size plot, 2D plot or 3D plot)



3D Plot smoothing This icon allows the user to smooth and auto-rescale the 3D plot. The degree of resolution employed in smoothing will be determined by the x-axis and y-axis widths selected. For instance, the effect on smoothing a 3D plot can be seen below right.



- Note: very small Bin Width values (e.g. <3) may involve significant computer processing and slow replotting times.
- Simultaneously clicking left and right buttons on the main plotting screen will define a limit to the range over which the smoothed curve is fitted.





- When smoothed, it is possible to **adjust the vertical axis** using a right mouse button click and drag up and down (as well as the right button click and drag side-to-side zoom facility and the left button click and drag rotate facility).

Model Curve Fitting

The white, particle size distribution plot drawn during the analysis of a sample comprises the smoothed experimental data obtained directly from analysis of the trajectories of the particles tracked.

This data is based on an estimate of any given particle's size derived from the average distance the particle has moved during the time it was being tracked. For small particles which move rapidly under

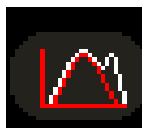
Brownian motion, the length of time the particle is present in the laser-illuminated scattering volume is likely to be restricted. The number of steps taken by the particle during this short time is similarly limited. Thus, any estimate of the average step length taken by a particle, resulting from a finite number of steps, results in a certain degree of unavoidable but statistically predictable broadening of the measured particle size distribution.

This broadening effect can be mathematically modelled and subtracted from the experimental data to allow more accurate particle size distribution profiles to be obtained for monodisperse or bimodal samples of small (e.g. <200nm diameter) size.

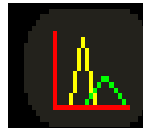
NTA 2.0 allows the user to fit a single, model log-normal plot to the experimental data. The peak of the model curve is centred on the mode of the experimental data obtained and the width of the model distribution is calculated from the standard deviation of the experimental particle size distribution plot



NTA 2.0 then recalculates a "finite track-length adjusted" (FTLA) plot which compensates for the predicted profile broadening. This is shown by the yellow line. Those parts of the original (white) experimental plot which are cannot be fitted to the model log-normal profile are shown as green residuals.



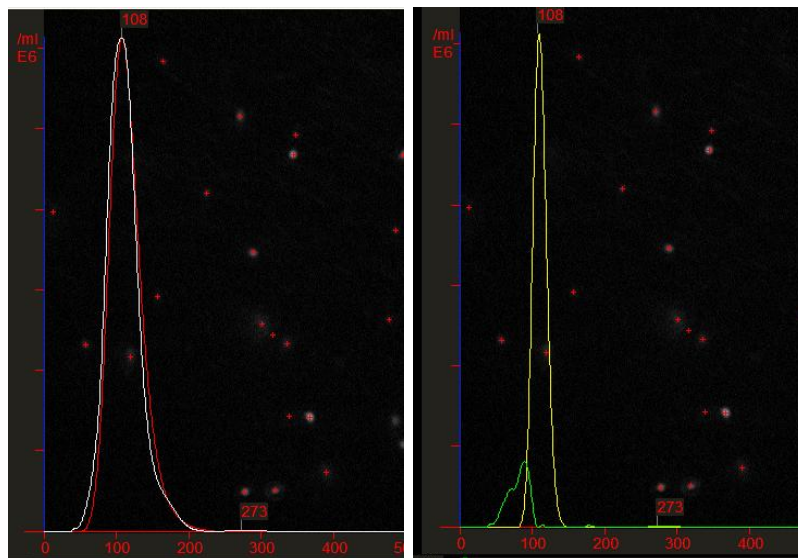
By pressing the **Model Fitting** icon once, a single log-normal model plot (red) is overlaid on the experimental profile (white). The icon beeps once and flashes on and off while the model is being calculated.



This **FTLA + Residuals** plot (the icon appears only when the Model Fitting is completed) can be switched on and off by toggling the icon.

Monomodal Model Fitting

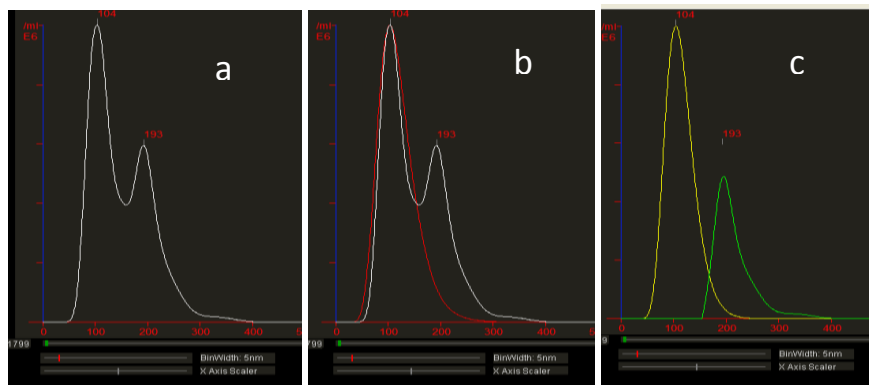
An example of the operation of these two functions can be seen here. In this case, a monodisperse sample of purified virus particles (experimental plot shown in left as white line) was fitted by **model fitting** a single lognormal profile (red line on left). A very close fit to a model log normal profile was confirmed on re-plotting the **FTLA + Residuals** plot (right) in which the true narrow profile (**yellow**) of the sample can be seen and the purity of the sample indicated by the low amount of residuals (**green** lines—representing data which did not fit to the model log-normal distribution).



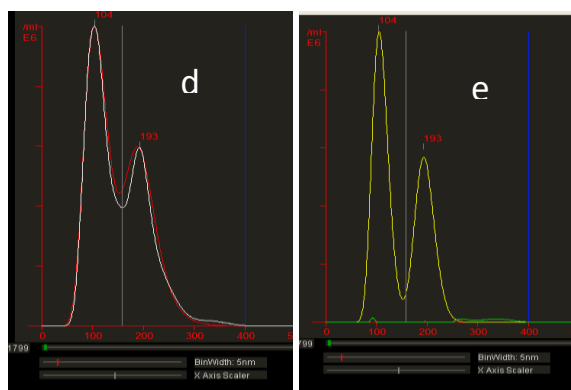
Bimodal Model fitting

If a particle size distribution profile is obtained which appears clearly bi-modal, the NTA 2.0 can be configured to apply a bimodal model to the profile as follows. After the analysis is complete, the user can move the cursor to the main particle size distribution plot screen and place a pair of marker (user) lines each side of the second (right hand side) peak to be fitted. This is done by left clicking at a point between the two peaks (a white line will appear) and right clicking at a point to the right of the second region or peak to which the secondary log-normal curve is to be fitted (**blue** line will appear).

In this example, a mixture of 100nm and 200nm particles has been analysed and a monomodal fit attempted.



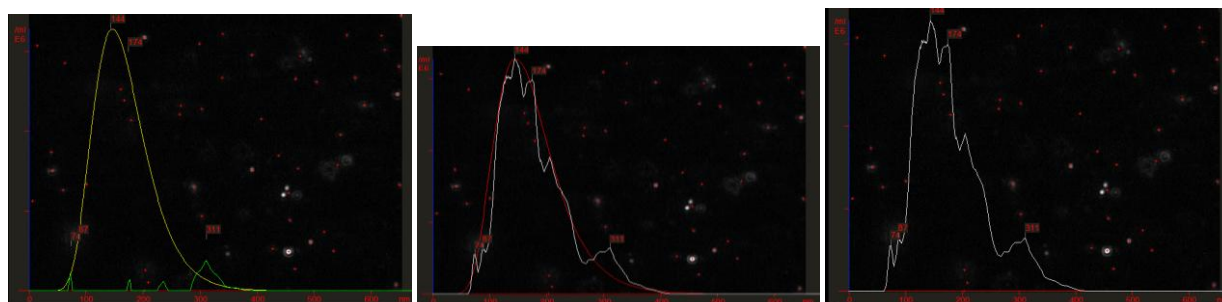
The first plot (a) of the experimental data is fitted with a single lognormal model curve (b) centred on the mode of the largest peak and on converting to the yellow FTLA plot (c) it can be seen the second peak remains as a residual.



By placing the white (left click) and **blue** (right click) markers to the left and right respectively of the second peak and pressing the Model Fitting icon, a bimodal fit will be made. The icon will beep twice (indicating a bimodal fitting step) and flash while the model is being calculated. When fitted, the red model curve will appear (d) and on clicking the FTLA icon, the corrected 'true' bimodal will be shown. Any residuals (very few in this case) will be shown again as green residual curves (e). It should be noted that the width of the two FTLA peaks is narrower reflecting the extent to which NTA 2.0 can successfully correct for limited particle tracking statistics.

Polydisperse sample types

In this final example of a polydisperse, truly log-normally distributed sample, it can be seen that attempting to fit a log-normal model is successful and that the FTLA plot does not differ significantly from the model



Log-normal plot. This is because of the polydisperse nature of the sample and the fact that the standard deviation of the sample is so wide that the range of multiple particle sizes present do not exist as, and cannot be resolved into, separate peaks.

i *Fitting of mono-modal and bimodal curves and extrapolation of these curves to FTLA plots is only successfully applied, and should be restricted, to samples which are actually very monodisperse or which are clearly bimodal, such as those of calibration quality materials or mixtures thereof.*

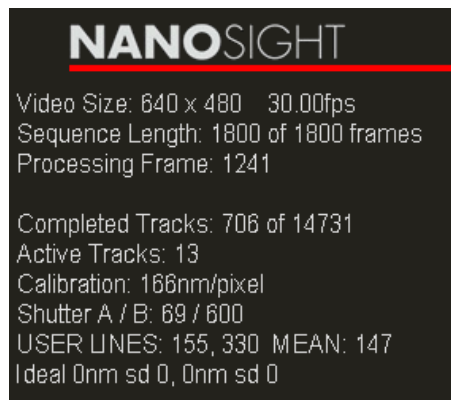


Sample Tracking Statistics area

The area display in the bottom left of the main screen contains information about various parameters associated with the progress and display of the analysis as it is proceeding. The location of this area may be different under different screen resolutions.

Video Size This shows the current video size (in pixels) and the current frame rate.

Sequence Length Shows the length of the video in numbers of frames (divide this value by 30 for sequence length in seconds when running at 30fps)



Processing frame Shows the frame number being processed at any given instant.

Completed Tracks Shows the number of tracks that have been successfully analysed and that will contribute to the Particle Size Distribution profile being plotted. This value will change significantly if Min Track Length is changed.

Active Tracks Shows the number of trajectories being tracked at that instant.

Calibration Shows the factory set-calibration. This will change if the x10 Magnification is selected under the Advanced menu.

Shutter Shows the current shutter speed values set for Channel A and Channel B.

USER LINES and MEAN

The user can, by *left* and *right clicking* on the main plotting screen, add one or two 'user lines' (*left* is grey, right is blue). Their position, in terms of the value on the x-axis being displayed, is shown here.

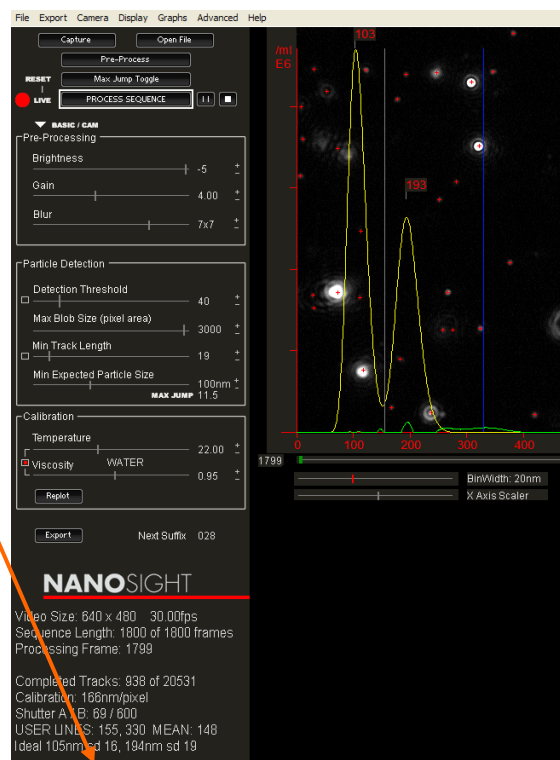
The MEAN value is the Mean of the entire distribution shown.



Ideal

When the Model Curve fitting option is selected and the FTLA Plot is selected (see page 38), the exact peak position and width (the Standard Deviation) of the peak (or both peaks if a bi-modal fit is selected) is shown here.

USER LINES: 155, 330 MEAN: 148
Ideal 105nm sd 16, 194nm sd 19

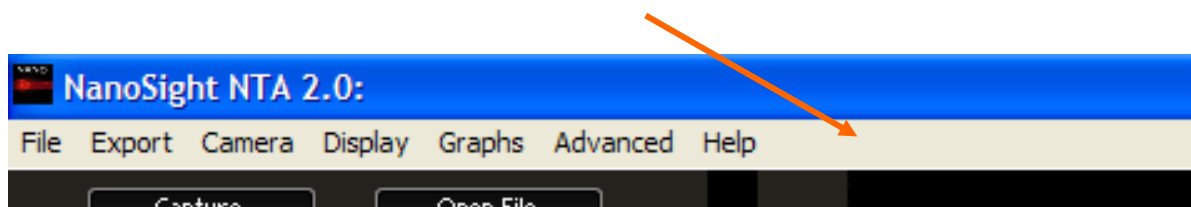


Concentration

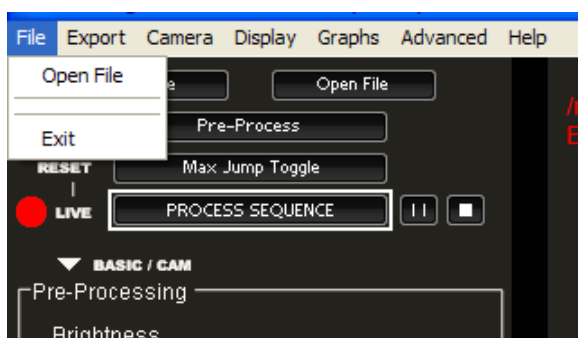
For information about the Concentration information area immediately below the above, please go to page 53.

Menu Bar and Pull-down menus

NanoSight's NTA 2.0 program is a sophisticated and multi-functional program offering the user many options which can be accessed via the Menu Bar and which will now be discussed.

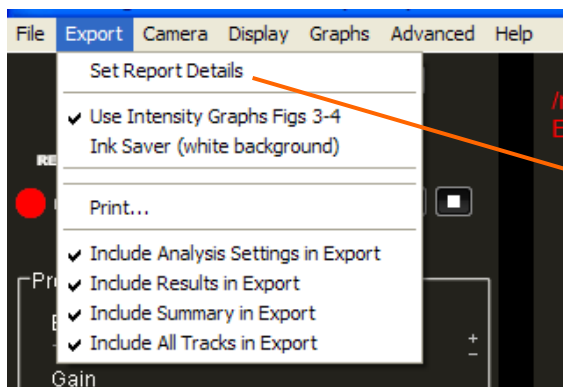
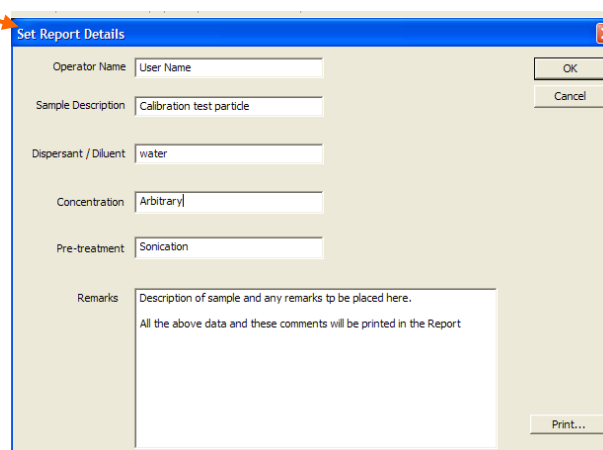


File Menu



Open File Serves the same function as Open file on the Main Screen and allows a previously saved video to be loaded into NTA 2.0

Exit Exits NTA 2.0

Export Menu

Set Report Details

Allows the user to input relevant data, sample description and remarks as required. All information input here will be printed in the Final Report

**Use Intensity Graphs Figs 3-4**

This tells the program to use the 2D and 3D plots shown on the Main Screen as figures 3 and 4 of the four figures allowed in the Report.

Un-checking this option allows the user to select their own Figs for inclusion in the Report.

Ink Saver (white background)

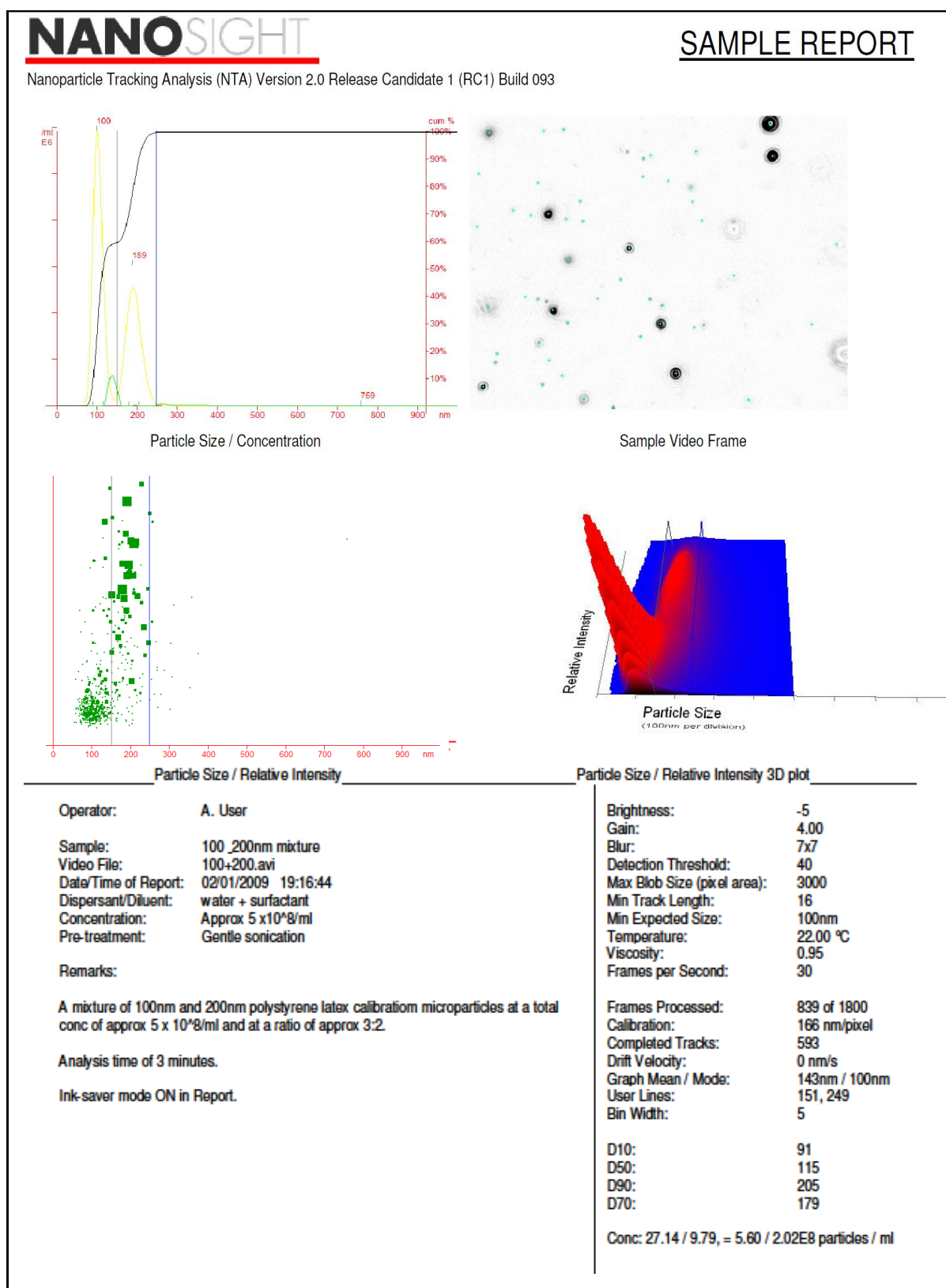
This option allows the user to print the normally black background figures from NTA 2.0 in “negative” (white background) thus saving on printer ink when printing Reports.

Print

This takes the user to a conventional Printer option page in which, depending on Printer types loaded, the report can be printed to .pdf, Notebook, or hardcopy. A bitmap file can be formed from the screen within the area that the image is normally displayed. This is created by pressing F12 button on the keyboard.



An typical example of a printed NTA 2.0 Sample Analysis Report:





Exported Files

Analysis file

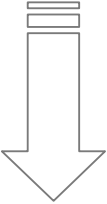
The following comma separated values (.csv) file is generated via the Export File facility within the program and is named "xxxx-001-analysis.csv". It is saved to the same location in which the video file (xxxx.avi) is saved (user-selectable) and has the same name as the video. Note that repeated analyses of the same video will be saved separately with an auto-incrementing number to distinguish the different analyses, e.g. *usertest-001-analysis.csv*, *usertest-002-analysis.csv*, etc.. These files can be loaded to/by Excel for further manipulation.

```
[Settings]
Pulldown = 3
Gain = 4
Blur = 2
DetectThresh = 39
MaxBlob = 250
MinTrackLength = 20
MaxParticleJump = 10
Temperature = 19.0
Viscosity = 1.0227
BinWidth = 10
```

Parameters set for processing video (also printed out in report)

Results file

The following comma separated value (.csv) file is generated via the Export File facility within the program and is named "xxxx-001-result.csv". It is saved to the same location as the video file (xxxx.avi) is saved (user-selectable) and has the same name (xxxx...) as the video. Note that repeated multiple analyses of the same video will be saved separately with an auto-incrementing number to distinguish the different analyses, e.g. *usertest-001-result.csv*, *usertest-002-result.csv*, etc.. These files can be loaded to/by Excel for further manipulation.

Size (nm)	Count
60	1
63	1
64	1
66	3
67	1
69	1
	
218	1
223	1
235	1
238	1
256	2
268	1
282	1
303	1
382	1
848	1

Values for each bin containing a non-zero value, i.e. empty bins are not displayed.

Note that the raw data stored in this table at a 1nm resolution while the particle size distribution graph displayed on the screen or in the report will depend on the bin width selected by the user.



Summary File

The following comma separated values (.csv) file is generated via the Export File facility within the program and is named “xxxx-001-summary.csv”. It is saved to the same location in which the video file (xxxx.avi) is saved (user-selectable) and has the same name (e.g. xxxx as the video. Note that repeated analyses of the same video will be saved separately with an auto-incrementing number to distinguish the different analyses, e.g. *usertest-001-summary.csv*, *usertest-002-summary.csv*, etc.. These files can be loaded to/by Excel for further manipulation.



[ResultsSummary]

Operator=
SampleDescription=
Diluent=
Concentration=
PreTreatment=
Comments=
TotalFrames=1800
ProcessedFrames=1799
CompletedTracks=776
OverallDrift=228.489557
X-Drift=55.969406
Y-Drift=-221.528560
Calibration=166
Mode=98
Mean=112
UserLine=0

Sample description as entered by the user in the Report page

Total no of frames in video

Number of frames used in analysis

Number of particles sized

Drift automatically compensated for unless option deactivated

Fixed on factory set-up

Value (size in nm) of highest bin [weighted by values of adjacent bins]

Sum of particle size values for all particles ÷ number pf particles

PercentileUndersize	size
1	67
2	72
3	73
4	74
5	76
96	174
97	184
98	191
99	233
100	382

Percentile undersize values at 1% resolution

BinCentre	Count
5	0
15	0
25	0
35	0
45	0
55	0
65	8
75	38
85	87
95	161
105	151
1975	0
1985	0
1995	0

Values at centre of each bin (including zero values) at user selected bin width (in this case, 10nm)



All-tracks File

The following comma separated values (.csv) file is generated via the Export File facility within the program and is named “xxxx-001-alltracks.csv”. It is saved to the same location in which the video file (xxxx.avi) is saved (user-selectable) and has the same name as the video. Note that repeat analyses of the same video will be saved separately with an auto-incrementing number to distinguish the different analyses, e.g. *usertest-001-alltracks.csv*, *usertest-002-alltracks.csv*, etc.. These files can be loaded to/by Excel for further manipulation.

Particle ID	Track Step	X1	Y1	X2	Y2	Diffusion Co	Particle Size
1	2	100.4584	3.868786	95.32211	3.192722	361.9166	117.0273
1	3	95.32211	3.192722	98.14208	2.710382	361.9166	117.0273
2	2	160.4091	2.396104	164.0134	2.3125	172.6027	232.7189
2	3	164.0134	2.3125	165.9162	2.021739	172.6027	232.7189
4	2	99.38546	16.35463	101.3246	22.68264	517.314	78.79213
4	3	101.3246	22.68264	103.5821	23.76014	517.314	78.79213
7	2	450.9534	100.4378	455.8448	99.74518	453.3191	92.52557
7	3	455.8448	99.74518	458.1433	101.3496	453.3191	92.52557
7	4	458.1433	101.3496	454.4592	101.8439	453.3191	92.52557
7	5	454.4592	101.8439	454.0251	100.6831	453.3191	92.52557
7	6	454.0251	100.6831	454.7009	105.0745	453.3191	92.52557
7	7	454.7009	105.0745	457.2028	104.3766	453.3191	92.52557
7	8	457.2028	104.3766	457.1436	107.8561	453.3191	92.52557
7	9	457.1436	107.8561	456.2762	104.8696	453.3191	92.52557
7	10	456.2762	104.8696	456.6437	103.0476	453.3191	92.52557

Identity given to each particle

Consecutive steps taken by particle

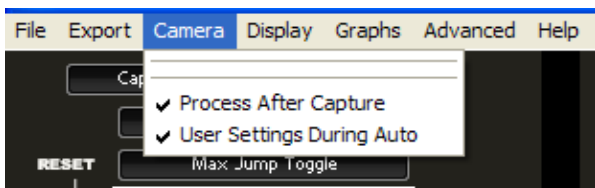
Estimated diffusion coefficient at end of track

Estimated particle size at end of track

x and y position at current frame

x and y position at next frame

Camera Menu



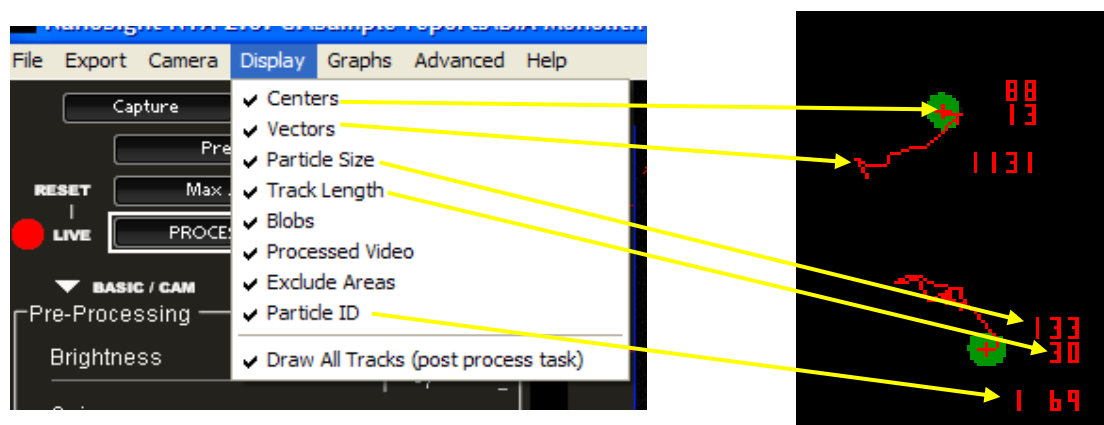
Process after Capture

Automatically opens a capture file for analysis once it has finished recording

User Settings During Auto

Uses currently loaded pre-processing settings when setting Auto-capture.

Display Menu



When checked/ticked, the following are displayed....

Centres The red cross denoting the centre of a blob found by a centre of mass algorithm

Vectors The particle trajectory history—the ‘track’.

Particle Size The estimated particle diameter in nanometres at that instant/frame.

Track Length The number of steps the particle has taken so far (and on which size is based)

Blobs The computer generated particle image on which the mass centring algorithm operates

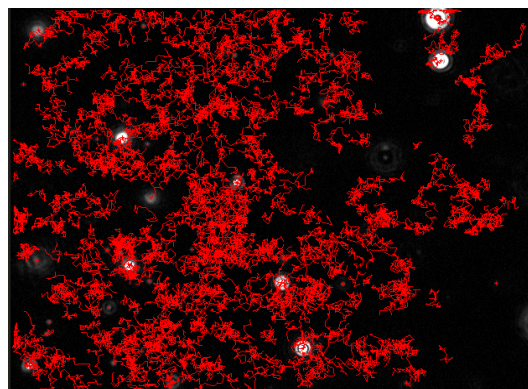
Processed Video The video display

Excluded areas The masked regions (if any selected) used to exclude sources of noise.

Particle ID The Identification number allocated to each particle when centred and tracked.

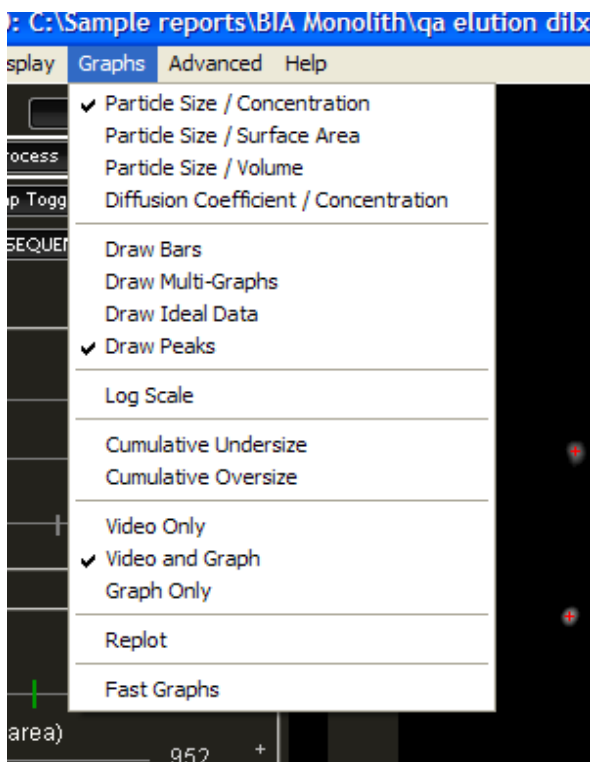
Draw All Tracks An option to show all the tracks analysed and which contributed to the final Particle Size Distribution profile.

Note that particles selected by **Highlighting** on the 2D Plot (see page 33) will not be included in the Draw All Tracks option.



Graphs Menu

This menu offers the user a choice of graphs display options.



Particle Size / Concentration

Under this (default) option, the Particle Size Distribution plot is of particle diameter in nanometres on the x-axis plotted against particle concentration (particles x 10⁶/ml) on the y-axis.

Particle size

The particle is assumed to be a sphere.

! NTA 2.0 measures the Diffusion Coefficient (D_t) of a particle (the mean squared displacement) and, via the Stokes-Einstein equation, assumes a spherical particle and ascribes a diameter to that particle's D_t . This sphere equivalent model is, in practice, valid for the vast

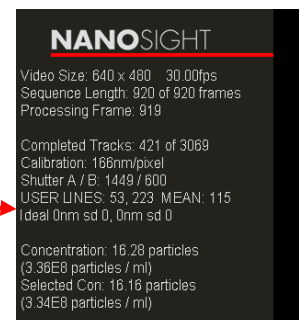
majority of nanoparticle types encountered in a wide range of scientific disciplines and industrial applications. Only when particle asymmetry exceeds an aspect ratio >3 does the Stokes-Einstein approximation becomes inappropriate.

For particle types which are known to be highly asymmetric, care should be exercised when using particle 'diameter' as a measure.



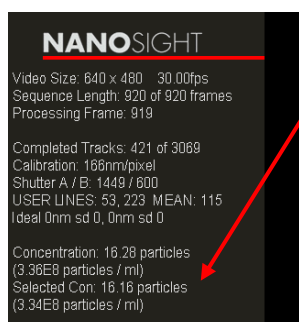
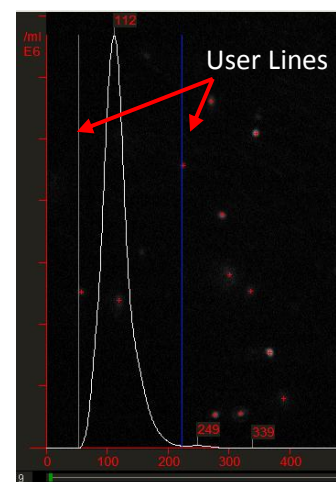
Concentration Measurement

Estimation of the concentration of the particles is based on the assumed scattering volume calculated from the dimensions of the field of view (at a given magnification) and the depth of the laser beam. By counting the number of particles tracked at any instant the average concentration per scattering volume can be estimated. This is shown in the lower left side of the main screen.



The average number of particles per millilitre of sample is then simply extrapolated from the assumed scattering volume.

The user can select a Region of Interest (RoI) within the distribution by *left* clicking on the distribution plot to generate a white user line defining the start of the RoI and the *right* clicking to the right of the RoI to generate a second **blue** user line.



All particles within the white and blue user lines (the RoI) will then be counted separately and shown as the Selected Concentration.

The user RoI can be removed by *left* and *right* clicking exactly on the y-axis.



Limitations to Estimate of Particle Concentration.

There are a number of considerations that must be taken into account when estimating particle concentration as described above:

Initial Sample Concentration.

The scattering volume in which particles are visualised for tracking and analysis is very small and, for a statistically significant number of particles to be analysed, the sample should contain particles in a concentration which exceeds 10^7 /ml. Samples which contain significantly lower will contain statistically insufficient numbers of particles to be tracked and the statistical accuracy of the particle size distribution and particularly of the particle concentration estimated will be affected.

Samples which contain particles at concentrations exceeding 10^{10} /ml may also not be analysed correctly. At these higher concentrations, particles become so close to each other that, for larger particles, it may not be possible to resolve them from each other, resulting in loss of data. Similarly, for small particles which are in close proximity to each other, even though they may be successfully identified as discrete entities, their Brownian motion trajectories may very quickly cross over each other. Clearly, given the random nature of such motion, NTA 2.0 will not be able to determine which trajectory belongs to which particle and accordingly the program is designed to cease tracking both particles until they have moved a sufficient distance from each other (the Maximum Jump Distance set during Pre-processing described earlier.)

- For optimum determination of particle concentration, the sample should be diluted to a concentration between 10^8 and 10^9 particles per millilitre.

Sensitivity to particle size/type.

Given the Gaussian nature of the illuminating laser beam, the 'edges' of the beam exhibit a gradual reduction in intensity with radius. Accordingly, larger or higher refractive index particles may remain visible at greater distances from the beam centre than would smaller or lower refractive index particles. Thus, the effective scattering volume may vary with particle type. This may significantly affect the accuracy of particle concentration determination.

Exclusion areas.

Placing Exclusion zone masks on an image (page 19) obviously reduces the field of view but NTA automatically compensates for this and the Concentration shown is re-calculated accordingly

Highlighting

Highlighting sub-populations of a Particle Size Distribution profile on the 2D plot acts to remove such particles from the main profile and automatically reduces the value of the concentration estimation



accordingly. This allows the user to estimate the concentration of particular sub-populations within a sample.

Pre-Processing and Analysis settings.

NTA 2.0 allows the user to adjust camera capture, pre-process and analysis parameter settings to suit a specific sample. Clearly, changing these settings significantly may change the numbers and types of particles seen. This may subsequently affect estimation of particle concentration considerably.



For higher accuracy particle concentration estimations for a given sample it is strongly recommended that the user:

- calibrates the NanoSight system to the sample under investigation by use of known concentrations of that sample type,
- retains all capture, pre-processing and analysis settings between such samples to ensure reproducibility
- dilutes the sample to the optimum concentration range of 10^8 and 10^9 particles per millilitre
- Use a track length of 5




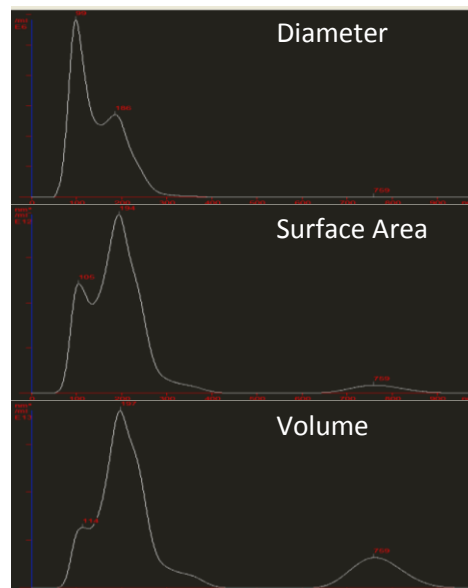
Particle Size / Surface Area

This option plots particle Surface Area in nm^2 (as a simple extension of the estimated sphere diameter).

Particle Size / Volume

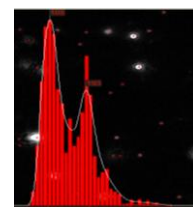
This option plots the particle volume in nm^3 based on the estimated sphere diameter.

 As can be seen on the right, it is sometimes informative to be able to see the relationship between particle diameter and other sphere parameters. For instance, it is often not appreciated that a single 1micron sphere (1000nm diameter) has the same volume as 1,000 particles of 0.1micron (100nm) diameter! Thus,, the presence of even a low number of aggregates can represent a significant proportion of the total 'primary' present in a sample.



Diffusion Coefficient / Concentration

This option allows the data to be displayed as the diffusion coefficient of the particles (in $10^{-9} \text{ cm}^2/\text{second}$). This is the parameter which is measured directly by the NTA 2.0 and is therefore independent of particle shape.

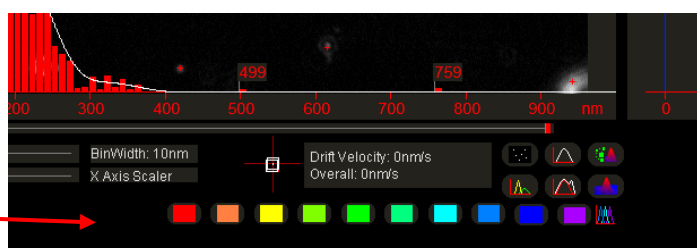


Draw Bars

This option allows the results to be drawn as a histogram, the bin width of which can be selected by the user by adjusting the Bin Width slider bar. The default setting is a bin width of 20nm.

Draw Multi-Graphs

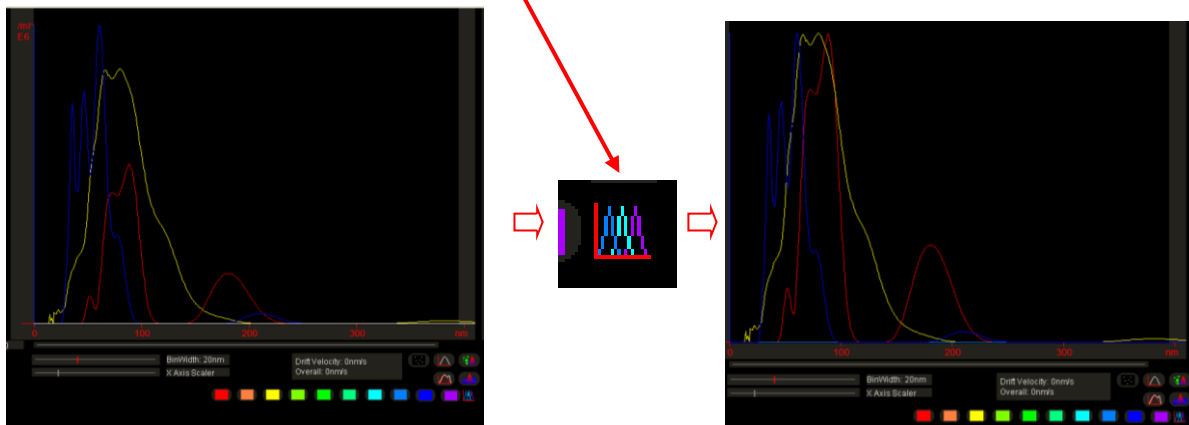
This option allows the user the draw multiple particle distribution plots overlaid on each other. Up to 10 different plots can be overlaid on each other, each of a different colour. The palette of colours is shown here.



This option is designed to be used in conjunction with the **Batch Process** and **Load Multi-graph** options under the **Advanced Menu** (see later). From left to right, (red to blue) the different plot colours are allocated on a sequential basis.

For examples, red is line 1, yellow is line 3, blue is line 9, etc.. An example of three such over-laid plots is shown below left.

The final icon in this series and shown here allows the user to normalise the mode peaks of all plots with respect to each other, as seen below.





i Each line can be added or removed by clicking on the coloured button for that line. To view which line represents which graph it is possible to right click and hold on the coloured button. The file name of that coloured line will then be displayed near the top of the screen.

Draw ideal Data

This option allows the user to display, or switch off, the red, “ideal data” log-normal plot generated by the **Model Curve Fitting** option discussed earlier.

Draw Peaks

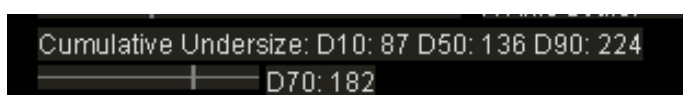
This option allows the user to display (at the top of the peak) the numerical value associated with a peak position depending on what has been selected to be displayed (e.g. Size, surface area, volume, etc..).

Log Scale

This option allows the user to plot the x-axis on a log scale. Note, when the **Draw Bars** option is selected, the histogram bins are also log distributed.

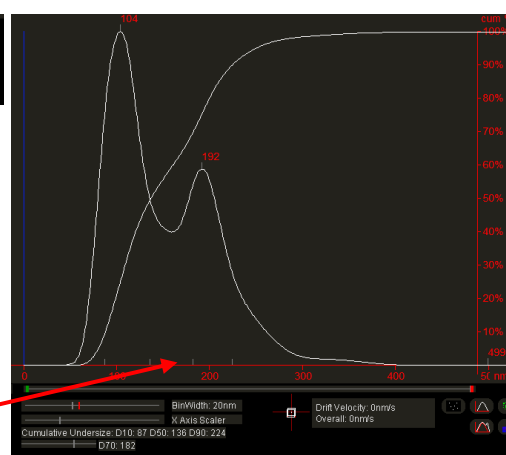
Cumulative Undersize and Cumulative Oversize

This allows a plot of cumulative undersize (or cumulative oversize) data to be displayed. Three default values are generated, D10, D50 and D90.



The D10, for example, is the diameter (in nanometres) of the particle that is the 10th percentile; i.e. 10% of all particles analysed are below (or above) this size.

NTA 2.0 also allows the user to set their own D value by using the slider bar which appears when the Cumulative Undersize/Oversize option is activated.



i Note: the small markers on the x-axis which show the relative positions of the D values.



Video Only, Video and Graph and Graph Only

This allows the user to select one of the display modes described. These options can also be selected by use of the Display icons serves the same functions as the **Video Image Display** and **Graph Plot** icons described earlier.

Replot

Re-plots all plots after changes are made to any analysis settings which modify the results (e.g. Temperature, Viscosity, etc.)

Fast Graphs

Changes the rate at which the particle size distribution plot (smoothed line or bars) is updated and redrawn during analysis from the default value of 3 (redrawn every third frame) to 1 (redrawn every frame). Setting this value to 3 is sometimes useful when running NTA 2.0 on PCs which exhibit restricted processing speed (older models or limited RAM or memory capacity).

Advanced Menu

Advanced	Help
Loop Processing (Demo Mode)	
Batch Capture...	
Batch Process...	
Load Custom Viscosity Table	
Use Custom Viscosity	
Magnification 10X	
✓ Magnification 20X	
Extract Background	
Show Background	
Load Multi-Graph 1	
Load Multi-Graph 2	
Load Multi-Graph 3	
Load Multi-Graph 4	
Load Multi-Graph 5	
Load Multi-Graph 6	
Load Multi-Graph 7	
Load Multi-Graph 8	
Load Multi-Graph 9	
Load Multi-Graph 10	
✓ Auto Drift Correction	
Hide Intensity Graphs	
Interleaved Mode	
Swap Frame Order	
Show Temperature Control Interface	

This menu contains many features that are new to NTA 2.0 and which allow greater flexibility and functionality in the NanoSight technique

Loop Processing (Demo Mode)

Used for demonstration or training purposes only. This option causes the analysis to be automatically repeated.

Batch Capture

Allows the user to automatically capture a series of videos of user-selected duration (**Duration of Each Sequence**), at userselected intervals (**Frequency of Capture**) for a user-selected number of times (**Number of Captured Sequences (1-25)**).

When this option is activated, the videos are automatically saved to the default location of

C:\ \My Documents\NanoVideos\batch.avi.

The user is, however, given the option of saving to a different location or folder and selecting a different file name (**Set File Base Name**).

The files names are auto-incremented with a numerical suffix , for example,

C:\ \Nanovideos\testbatch-001.avi

C:\ \Nanovideos\testbatch-002.avi

C:\ \Nanovideos\testbatch-00n.avi

.....etc



When the necessary information is entered, pressing **Go** starts the automated capture without further under intervention.

Batch Capture

Duration of Each Sequence (seconds)
30

Frequency of Capture (seconds)
900


Number of Captured Sequences (1 - 25)
10

Base Name for Captured Sequences (01 etc will be added during capture)
C:\Documents and Settings\Bob.Carr\My Documents\NanoVideos\batch.avi

Set File Base Name

Batch 0 in progress, Total Batch Time: 0 Time until next: 20

For each cycle the video is captured at the start of the cycle and then written to disc. For long video clips, for example >60 seconds, the software may take up to 10 seconds to transfer the information from RAM to disc after capture. This time may increase for computers whose memory is becoming limited.

 *This facility is useful for monitoring and analysing sample types which are unstable over time. For instance, samples which are aggregating, dissolving or precipitating can be analysed and changes in the sample particle size distribution profiled seen on analysis of these sequential videos. It should be noted, however, that over extended time periods, adhesion of sample onto the walls of the sample chamber, or slow sedimentation of parts of the sample population, may occur.*

Batch Process

This option allows the user to

1. Automatically load and analyse a sequence of videos captured under Batch Capture or
2. Load and analyse a series of different (un-related) videos selected by the user.

Find Sequence

Allows the user to locate and automatically load a series of videos captured under **Batch**

Capture mode. When the first file is located, the program automatically finds all *.avi files with the same base name and loads them sequentially according to the auto-incremented numerical suffix, e.g.

..\testfile-001.avi,

\testfile-002.avi,

etc...

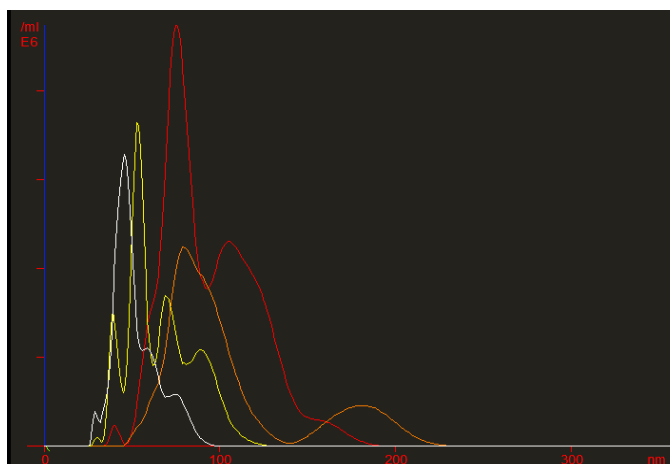
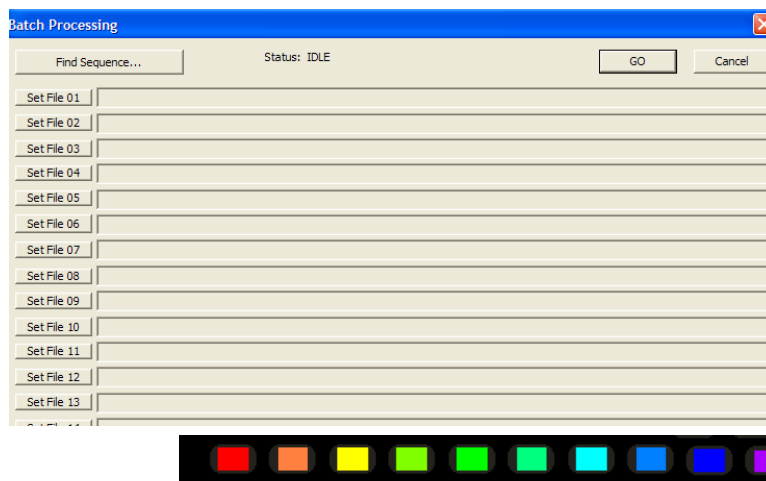
The Size Distribution Plots drawn, following analysis, will be coloured according to the sequence of colours in the Palette described in **Draw Multi-Graphs** command discussed on page 64.

Set File (01)

This option allows the user to select a series of different video files which were not captured under the Batch Capture command.

Accordingly, different video files of different samples taken at different times and saved in different locations can be loaded and analysed sequentially and plotted on the same plot automatically without further user intervention.

The colours allocated to the different plots will be determined by the **Set File** value chosen in combination with the colour palette sequence. The last file analysed will be white.





! *Note: All videos analysed when loaded using the Find Sequence or Set File modes described above will be analysed using the same analysis settings. The settings used will be those loaded with the first video analysed (if it has been analysed previously) or will be those remaining from whatever analysis was last carried out. Accordingly, it may be necessary to re-set the analysis parameters.*

However, for a sample analysed under Batch Capture mode over an extended time period or which has changed its character (e.g. aggregated), the nature of the sample may have changed to such an extent that the optimum analysis settings for these different videos (track length, gain, blur, etc..) may be very different. Similarly, batch analysing very different sample types with the same analysis settings would probably result in poor quality data.

! *If over-plots of different sample particle size distribution profiles are required for comparative purposes, it is advised that the user analyses each of the required videos separately (with the correct analysis settings (or those saved when the video was previously analysed). Over-plotting in this way can only be done by using the **Load Multi-graph** option described on page 64.*

Load Custom Viscosity Table

The accurate determination of particle sphere-equivalent hydrodynamic diameter by the Stokes- Einstein approximation requires knowledge of the viscosity of the solvent at any given temperature. The relationship between viscosity and temperature of a solvent depends on the solvent type. NTA 2.0 defaults to a viscosity-temperature relationship based on that of water. As has been seen earlier, the relationship between the solvent viscosity and temperature can be linked automatically when the red square is checked (see pages 2026 and 63).

It is possible, however, to load a different solvent viscosity-temperature curve if known. NanoSight NTA 2.0 contains a library of viscosity-temperature curves for a variety of common solvents. The Load Custom Viscosity Table function allows the user to load one of these tables.

Viscosity-Temperature curves cannot be supplied for all solvents or mixtures of solvents. If, however, the user has, and wishes to use, such data, NanoSight will custom modify NTA 2.0 to include such data. Please contact NanoSight at support@nanosight.com for customisation of this feature if your solvent type is not included in the look up tables supplied.

Use Custom Viscosity


This option allows the custom Viscosity-Temperature curve selected to be employed in an analysis.



Magnification x20 and Magnification x10.

The accurate determination of particle size by NTA 2.0 requires knowledge of the calibration factor needed to convert movement of a particle as seen by the camera (in camera pixels) to actual distances moved in nanometres. This is usually factory pre-set at a value of 166 for the x20 objective supplied as standard.

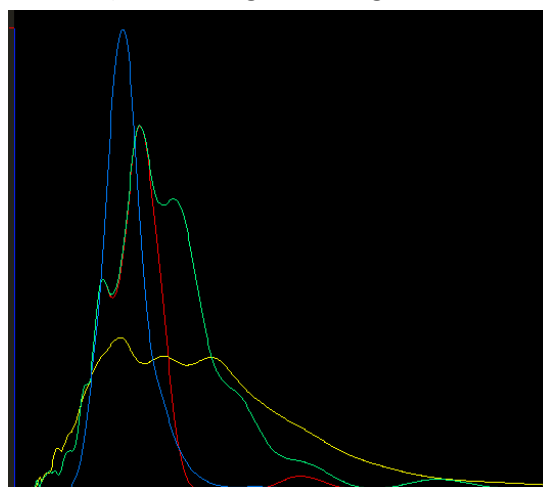
However, it is possible to employ a different magnification microscope objective in the optical train visualising the particles in the laser beam. For samples containing a low concentration of large particles, it is possible to employ a lower powered objective (e.g. x10) to give a wider field of view encompassing more particles. This improves statistical accuracy of data for low numbers of particles. However, the calibration factor required needs to be changed. Switching to the x10 magnification calibration factor by clicking Magnification x10 when using a x10 objective retains the correct calibration of the system ensuring sizing accuracy is maintained.

 *Note, that if the beam no longer fills the field of view when using a lower powered objective it is advised to use the **mask/exclude area** function (page 20), to mask the area of the screen in which the beam is not present, this will help to obtain a more accurate concentration measurement for the sample.*


Load Multi-Graph

When a sample has been analysed, a file (C:\ \uservideofilename.his), containing the histogram data from which the particle size distribution plot is formed, is automatically saved in the same location as the video. (This file is saved automatically and does not require selection in the **Export** menu).

NTA 2.0 allows up to 10 of these *.his histogram files to be loaded and the results over-plotted. Depending on which value Load Multi-Graph is used, the loaded histogram will be plotted as a colour defined by the sequence of the palette described earlier. For instance, whichever *.his file is loaded as Load Multi-Graph 1 will be plotted as a **red** line; any file loaded as Load Multi-Graph 9 will be plotted **blue**.



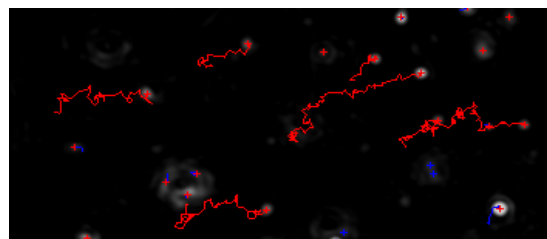
To see which colour of line corresponds to which file, *right click and hold* on the requisite colour on the palette bar. The file name will appear above the plot.

 *Use of this **Load Multi-Graph** option allows Particle Size Distribution plots generated from analysis of different samples to be compared against each other. Because each sample video had been previously analysed with its own optimum pre-processing and analysis settings, the results are all valid (unlike analysing in **Batch Process** mode where a single set of pre-processing and analysis settings are applied to all the videos selected.)*



Auto Drift Correction

Occasionally, particles within the sample are seen to undergo a unidirectional drift due to one of a number of causes including leakage through poor sealing of the O-ring or syringe, bubble formation within the sample or slight heating of absorbing sample types leading to convection. This additional movement on top of the random Brownian motion can be misinterpreted by the program as a faster moving and therefore smaller particle.



Given the random nature of Brownian motion, for a static sample the sum of all the vectors undertaken by the particles should sum to zero. NTA 2.0 measures, for each and every particle tracked, the overall vector of the particle trajectory as well as the average distance moved per frame. If the sum of these vectors is more than zero, this value is interpreted as a collective, unidirectional drift and the value is subtracted from the trajectory of each particle thereby automatically correcting for drift.

The panel below the main window shows the magnitude (in nm's/second) and direction of the average drift measured for the sample (the white square) compared to the instantaneous drift value measured for total particle motion in a specific frame (the red square).



Hide Intensity Graphs

Gives the user the option to switch on or off the 2D and 3D plots.

Extended Dynamic Range

Activates EDR as described on page 16. Selecting this function acts to re-organise the main screen display, re-set camera driver settings and change camera frame rate and capture parameters.

Swap Frame Order

Changes the allocation of Channel A and Channel B in the video playback should these be misallocated.

Show Temperature Control Interface.

Applicable only to the NanoSight LM20 system with temperature control fitted. [See the Temperature Control Manual for details.](#)



Frequently Asked Questions

Fundamental questions

How much light is scattered by a particle?

The particles that we size are generally in the Rayleigh or Rayleigh-Debye-Gans scattering regime.

As an approximation, the Rayleigh regime, which is the simplest, will be briefly discussed. In this case the efficiency of light scattering is given by:

$$\sigma_s = \frac{2\pi^5}{3} \frac{d^6}{\lambda^4} \left(\frac{n^2 - 1}{n^2 + 2} \right)^2$$

Thus the factors affecting the amount of light scattered are:

d is the particle diameter. If you double the particle size you increase the power of light scattered by a factor of 64.

λ is the wavelength of light. If you half the wavelength of light you increase the scattering by a factor of 16.

n is the ratio of the particle refractive index to the solvent refractive index.

For example; for latex particles (ref. ind. 1.59) in water (ref. ind. 1.33), n is 1.195 and the factor $\left(\frac{n^2 - 1}{n^2 + 2} \right)^2$ is 0.0157.

However, for the case of gold (ref. ind. 0.14-3.7i) in water n is 0.1053 + 2.7820i and $\left(\frac{n^2 - 1}{n^2 + 2} \right)^2$ is 2.3, a factor of 147 greater than for the latex particles.

The amount of light scattered is also directly proportional to the intensity of incident illumination.

What is the smallest particle that can be analysed?

The minimum size of particle that is detectable depends on the scattering being sufficiently strong. To understand this question better see 'How much light does a particle scatter?'. As a general guide:

- Low Ri (e.g. biological/polymer) will be detectable down to approximately 30-40nm.
- Higher refractive particles (e.g. ceramics, metal oxides) will be detectable down to approximately 15-25nm.



- Exceptionally high Ri particles (e.g. Ag sol, Au colloid) can be detected at diameters as low as approximately 10nm.

What is the upper size limit of particles that can be sized by your system?

The upper size limit of 1000nm that we suggest is an approximation and dependent on particle type.

The NanoSight NTA technique analyses the movement of a particle undergoing Brownian motion by tracking in time the spot of light it scatters. For large particles:

1. The diffusion of the particle becomes increasingly limited making the accurate determination of its mean displacement progressively less accurate.
2. For dense (metal or metal oxide) particles, sedimentation rates begin to exceed the limited Brownian motion.
3. The spot of light becomes so large and its edges so variable between frames, that identifying its centre becomes increasingly difficult leading to centring errors which are as large as the particle's movement.
4. The numbers of particles that can be viewed at any one time decreases (because of the amount of light they scatter)

These effects get progressively larger as particle size increases so there is no specific limit to our upper size range. This upper limit will depend on the nature of the system. In practice, for low density and low Ri particles such as polystyrene microspheres their buoyancy and weaker scattering allow them to remain Brownian movers with easily identifiable centres and the upper size limit is 600- 800nm or above.

For dense, high Ri particles such as gold, their rapid sedimentation rate and excessive scattering restricts the upper size at which they can be analysed to approx <200nm.

How long is a sample stable for? Will the nanoparticles aggregate?

The science behind dispersion chemistry is not simple and we do not claim that we can stop your particles aggregating. We often use a little ultrasound but usually look to the customer to provide a protocol to disperse and stabilize the system. We also refer to ISO 14887 Sample Preparation – Dispersing procedures for powders and liquids.

However if the sample is at suitable viewing concentration then the injection and time to the start of the measurement can be less than 30 seconds and a measurement can take as little as 10 seconds.

Therefore our system can be used to monitor aggregation if the process is sufficiently slow (e.g. takes longer than 5 minutes).

Note also that if a sample settles to a significant degree over an hour or two, this strongly indicates that it contains particles in the mm range and which are therefore not true colloidal nanoparticles and therefore may be at the extreme of our analytical capability.



The particles are only being imaged in two dimensions, but they must move in three dimensions. Is this a problem?

No, Stokes-Einstein theory which is used to calculate the particles size from the particle motion can take into account whether two or three dimensions are tracked (see equations below).

$$\overline{(x, y)^2} = \frac{2kTt}{3\pi a\eta} \quad \overline{(x, y, z)^2} = \frac{kTt}{\pi a\eta}$$



Sample measurement

How accurately can particle size be determined?

For samples in which an adequate number of monodisperse particles are present in the sample (typically $>10^9/\text{ml}$), the determination of particles size can be as accurate as that of equivalent dynamic light scattering techniques such as Photon Correlation Spectroscopy. For samples in which solvent viscosity and temperature is correctly entered, the diameter of a well prepared, monodisperse, calibration polystyrene microsphere can, for instance, be accurately measured to within 5 percent of nominal. Increasing analysis time (e.g. 1 to 2 minutes) will increase accuracy of measurement.

For polydisperse samples, the NanoSight system exhibits significant advantages over PCS in that the size distribution profile obtained is based on the separate but simultaneous analysis of each and every particle seen during the analysis period. This is in stark contrast to the data obtained by PCS in which a large ensemble of particles are measured and from which only an intensity weighted (z-average) is obtained.

What is the best particle concentration to use?

The NanoSight system relies on the measurement of each and every particle in the field of view over a period of time. The trajectory described by each particle is tracked for as long as it is visible or until it crosses with that of an adjacent particle. By determining the average step distance moved by any particle, and estimate of Dt and therefore sphere equivalent size can be determined.

Statistically, low numbers of particles measured for too short a time period will not generate data which is as good as that obtained by looking at higher numbers of particles for longer. Adequate statistics can be obtained from samples in which 50+ particles are visible at any given instant in the field of view. This equates to approx 10^7 particles / ml as an acceptable minimum. Of course, for low concentration samples, statistical accuracy can be improved by extending analysis time from, for instance, 10 or 20 seconds to 1-2 minutes.

Upper concentration ranges are defined by the acceptable proximity of particles to each other in the field of view. The trajectories described by small, fast moving particles very soon cross those of their nearest neighbour at which point tracking is terminated. This necessarily restricts the number of steps over which such particles can be tracked, reducing the accuracy with which the necessary average step length and therefore size can be determined. Therefore a maximum concentration of approximately $10^{9-10}/\text{ml}$ of these smaller particles is approached.

Are there enough particles in my analysis?



For there to be enough particles in the analysis the only criterion that can reliably be used is that as more data is added to the histogram, the shape of the histogram does not change significantly. This is a function of the concentration of particles, the time of analysis, the tracking success of particles, the bin width and the polydispersity of the sample. As a guideline we would recommend that your first frame has greater than 10 particles (to keep the experimental time required to a reasonable length) and less than 100 (otherwise there is a significant risk of particle tracks being discarded due to ambiguity about their origin).

As a rough guideline a 60s movie clip of monodisperse 100nm particles at a reasonable concentration will have sufficient number of tracks for a measurement. Another guideline is that the number of completed tracks should be greater than 500.

How well can different sizes be resolved from each other in a mixture?

Under ideal conditions (two very monodisperse populations of similar type and of similar number density), we have achieved resolutions of less than a factor of 1.25. Should either of these populations exhibit any significant breadth of distribution, their diffusion coefficients become too similar to differentiate easily and the size ratios at which they can be resolved will also increase accordingly.

In practice, for all but the most well prepared of calibration particles, resolution of mixtures of particles within a 1.5 diameter ratio can rarely be achieved with a high degree of confidence. This, however, represents a significant improvement on the resolutions routinely achievable with PCS which, in practice cannot resolve systems by greater than a factor of 4.

How reproducible is the result?

Given the statistical nature of analysis of a population of particles, reproducibility is a function of the numbers of particles measured over a given period of analysis. For optimum concentrations of monodisperse samples containing no contaminating aggregates, results reproducible to within approx 2-3% can be achieved in a comparatively short time (e.g. 30seconds). Clearly, for low concentrations of particles exhibiting a range of particle sizes, extended analysis periods will be required to obtain reproducibility of results. Note that analysis times need to be increased for larger particle diameters to achieve similar reproducibility.

Can I use different solvents in the sample chamber?

Yes. Whilst the system is typically optimised for water as the solvent we get reasonable results from other solvents. As an indication solvents with a refractive index up to a value of approximately 1.45 can be analysed. For solvents with a higher refractive index please consult NanoSight. Water is 1.33 and a water-



optimised LM unit will work up to about Ri of 1.4. Above this, a unit optimised from 1.40 should work up to 1.52, but best way is to try this.

How long can I leave a sample in the cell?

Providing that the system that you are looking at is suitably stable, the water will not evaporate over a period of many days. Note however, that some particle types will 'plate out' on the internal surfaces of the sample chamber (through, for instance, electrostatic interactions) and may increasingly contribute to noise within the system. Also, on re-analysis, there may be a significant reduction in particle number.

Why do my particles twinkle and appear to diffuse asymmetrically?

Whilst currently we cannot put quantitatively answer this, our current understanding is that this is due to the particles being significantly aspherical (e.g. oblate or prolate)

How does a larger particle (such as an advanced agglomerate or contaminant) affect the measurement?

Unlike other light scattering techniques we are not strongly affected by large contaminants. This is because we visualise individual particles. Whilst an unusually large particle will scatter far more than the primary particles, its scattered light will be confined to and tracked only in a discrete region of the image.

How do I calibrate my system?

Our method of particle tracking is absolute as it is based on dynamic measurements in the time domain. The only parameters that need to be known are the solvent temperature and the viscosity of the solvent at that temperature. Therefore there is no calibration required other than ensuring the field of view of the camera is correct. In all systems we supply this is factory set and not user adjustable.

How long does the sample temperature take to equilibrate?

The sample volume, at approx 500 μl , is smaller than is typically used in other light scattering measurements. Therefore, providing the solvent (or the already diluted sample) is not at a very different temperature to ambient, the time taken for the liquid temperature to equalise with that of the metal housing is relatively small. 30s should be adequate time for temperature equilibration.



Common User Issues

I want to put my movies in a presentation but they are very large. Can I compress these?

Yes, the easiest way we have found to do this is using the Windows Movie Maker software that comes with, for example, Windows XP. The settings we use are all the default ones. Note, video clips cannot be re-analysed following compression.

Is it possible I could have blocked the inlet outlet ports with particles?

This is very unlikely; it would take particles of over 100 microns to block the inlet ports.

I am still seeing a significant flow of particles, but have waited longer than 30s. Why is this?

There are four possibilities why this may be so and these are listed below in increasing likelihood:

- Sample leakage. When setting up, it is imperative that the optical flat surface is correctly seated in the viewing unit, the sealing 'O' ring is correctly seated in the top plate and that the top plate has been brought into contact with the optical flat surface horizontally. Refer to the manual for a description of these processes.
- The beam has become incorrectly aligned and light is being absorbed by the optical flat surface. If this is the case check that the flat was seated correctly in the LM12 body.
- The particles are very large and very absorbing causing heating in the cell
- The solvent you are using is very absorbing or has a low specific heat capacity, which is causing convective currents.

My new version of NTA2.0 does not open. Why could this be?

The new version of NTA requires a software licence in addition to the dongle. For more details about this, see the installation instructions for the software.



Specification questions

What are the dimensions of the sample chamber?

Cell volume: Approximately 0.25 ml; Cell depth: 0.5mm

What are the dimensions of the sample viewed?

Approximately 100 x 80 x 20 μ l.

How long is the lifetime of laser source in LM12 laser viewing unit?

10,000 hours specified by manufacturer, however the laser is very sensitive to electrical interference, to mechanical impacts and to excessive moisture. Therefore this component should be taken care of.

Is the system safe?

Yes. The system is a Class I Laser product if used according to the manufacturer's instructions as it has a fail-safe mechanism.

What is the unit made of?

The parts of the LM10 unit in contact with the sample are made of glass, stainless steel and the plastic of the inlet/outlet ports. The seal is Viton.

Do I need an anti-vibration table for the system?

Possibly; any solid and heavy surface that is suitable for conventional microscopy will suffice, though extended analyses will be affected by significant and continuous vibration. If the image on the screen is seen to move systematically as someone walks past the area/bench on which the microscope is located, a vibration isolation table might be appropriate.



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User Notes