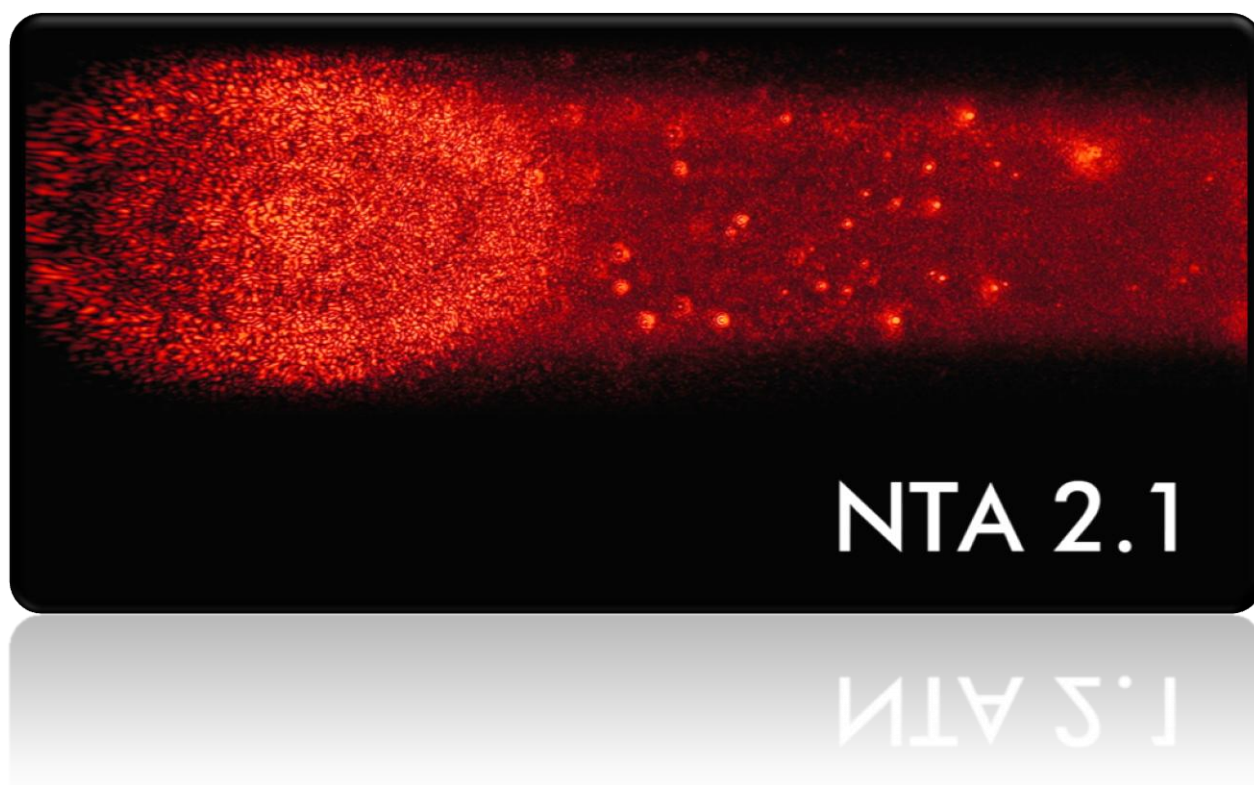


NanoSight NTA 2.1 Analytical Software



Operating Manual

Version 1.0

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Introduction

Basic principles behind the NanoSight NTA Program.

NanoSight 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. 30 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 to 60 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 2.1 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 software selects 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. 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.



System Requirements

The software is designed for optimal use with a system operating on Windows 7 or Windows XP Professional (32 bit).

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 1333MHz DDR2 RAM
- 250 GB, 7200rpm SATA hard drive
- 1680 x 1050 Display
- IEEE 1394 port (Firewire) + 2 free USB ports

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.

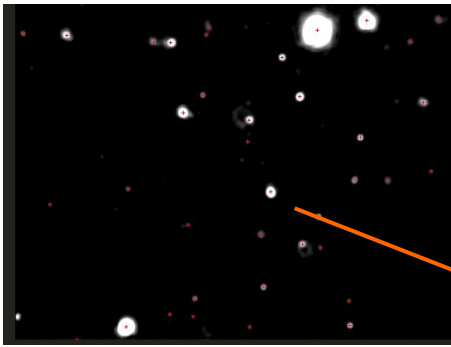
Software protection

For capture of videos the NTA 2.1 software 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 relevant installation document.

For users who wish to carry out 'off-line' analyses, on a different computer, of videos previously captured on the NanoSight instrument it is possible to install the software onto the required computer. NanoSight allows the user to install three additional copies of the NTA 2.1 analysis program on such other computers without charge. Contact NanoSight (at support@nanosight.com) for details on how to enable these free copies of the program.

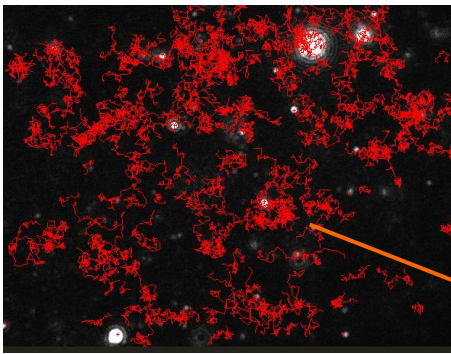
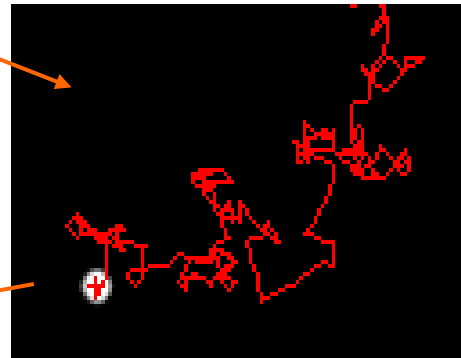
For quotes for further copies of the NTA program, please contact NanoSight.

Principle of Operation of NTA 2.1 at-a-glance



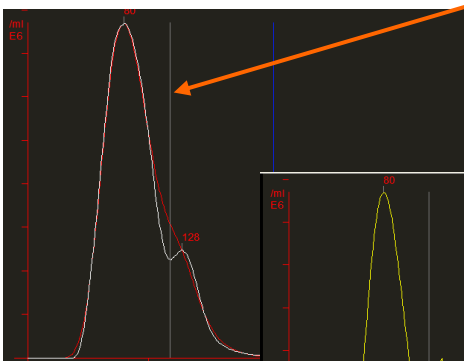
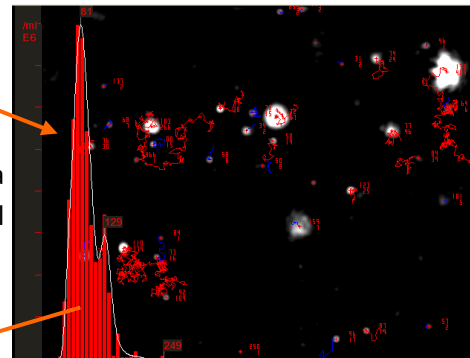
NTA 2.1 captures a video of particles moving under Brownian motion.

NTA 2.1 automatically locates and follows the centre of each and every particle and measures the average distance it moves per frame

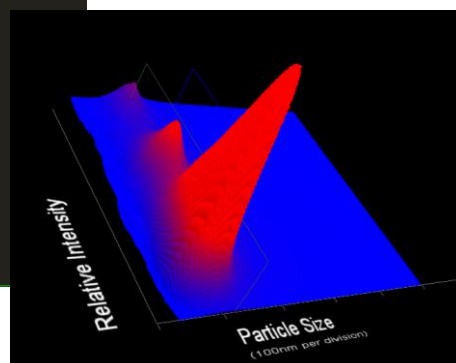
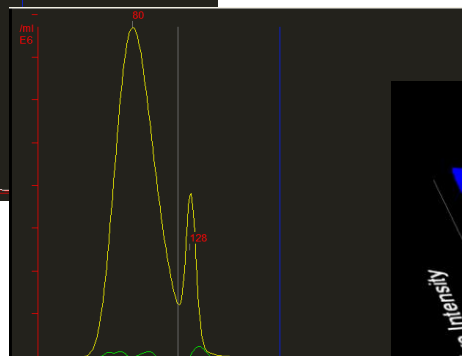


This is done simultaneously for all particles until hundreds or thousands of particles have been tracked

NTA 2.1 converts the distances moved into a particle size and plots accumulated results in real time as a particle size distribution profile.

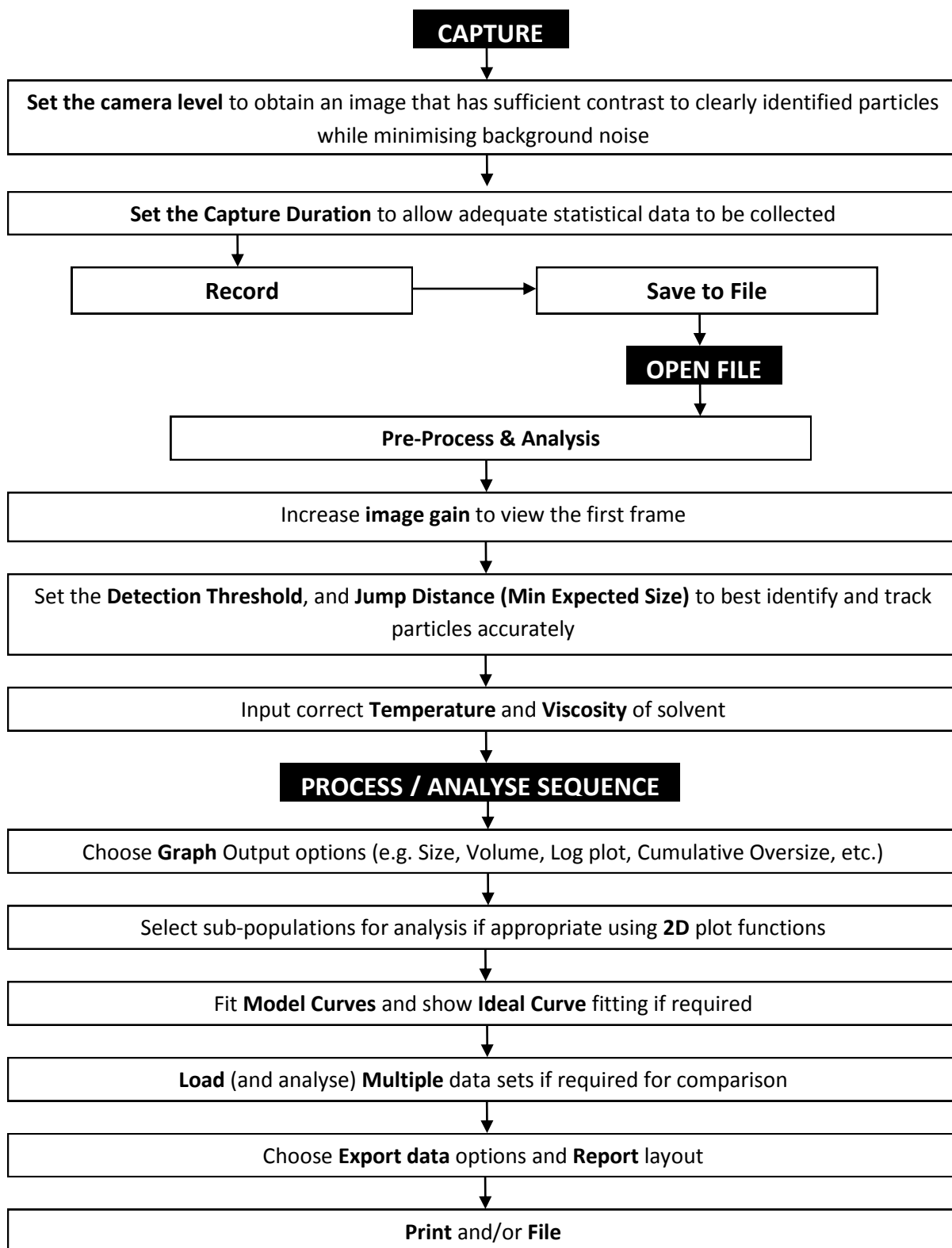


NTA 2.1 analyses the raw data, fits model distributions or displays different particle parameters (size vs relative intensity vs number) against each other. Concentration is also measured.



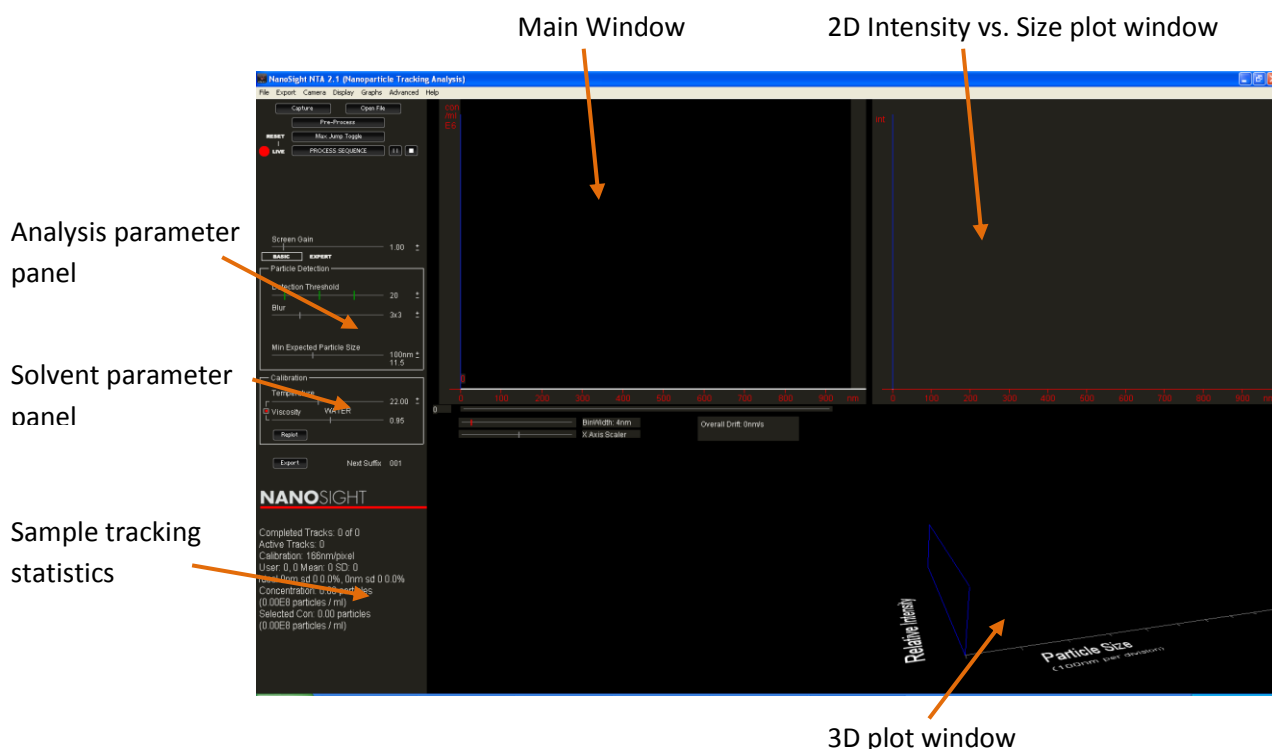
NTA measurement process flowchart

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



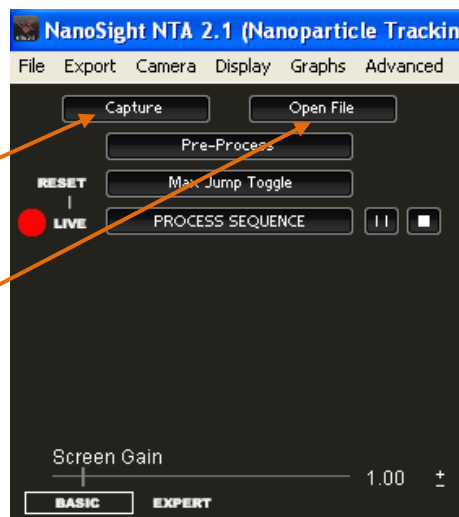
NTA 2.1 Opening Screen

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



When first opening the program, 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 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)

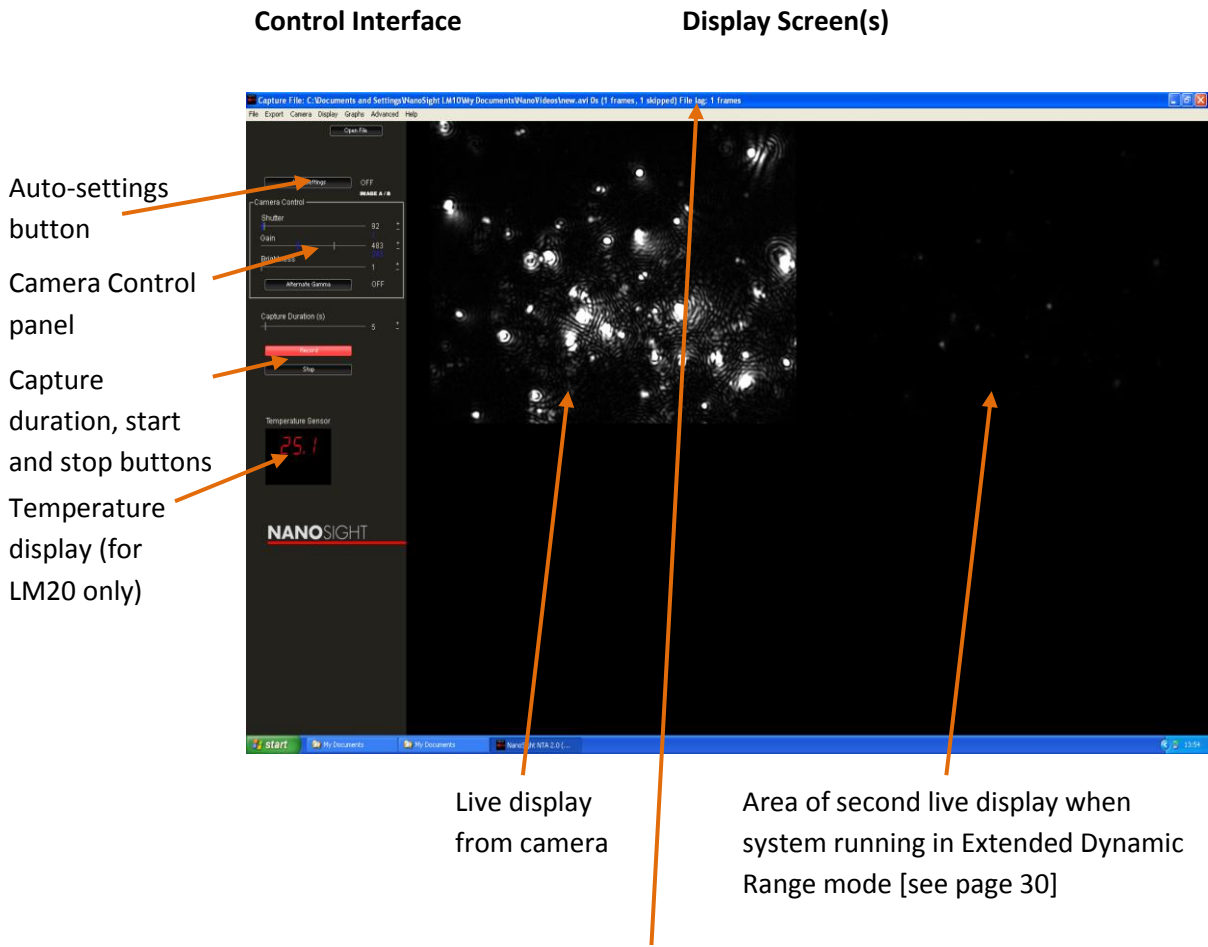
***NTA display on non standard resolutions.** Dependent on screen resolution, the NTA 2.1 Interface presents a slightly different layout to the standard 1680 X 1250 pixels. The differences are such as when displayed on a 1280 X 1024 screen are summarised as follows:

- Under Extended Dynamic Range (see page 30) 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 2.1 Capture Screen

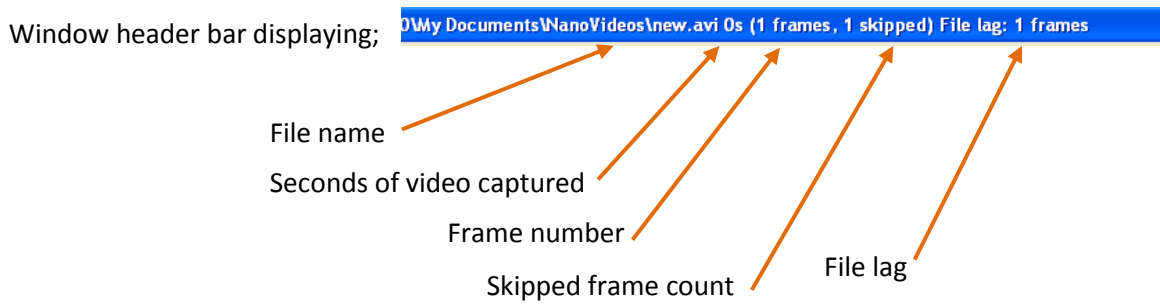
The Capture Screen illustrated below is broken into two different functional areas;

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.



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 30]



Window header bar displaying;
 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.

Start-up and Capture

Start-Up

The software comes pre-installed on NanoSight-supplied computers and can be run either from the desktop icon (NTA 2.1) or via the start menu (under All Programs > NanoSight NTA 2.1). Depending on which instrument you are using it may be necessary to first run the NTA Temperature Comms program or the NTA Stage Comms program – please refer to the hardware manual for details.

Pre-capture

Before entering the capture screen it may be necessary to adjust some menu settings depending on your hardware configuration.


1. In the **camera** menu select the appropriate camera. Two choices are available; CCD (AVT Marlin, standard camera) and EMCCD (Andor Luca, High Sensitivity camera).
2. When using the CCD camera, there is also an option to use Extended Dynamic Range (EDR) mode. This option can be found in the **camera** menu, see page 40 for more details.
3. The objective magnification (x10 or x20) should be selected in the **advanced** menu to ensure that the correct calibration information is associated with the captured video.

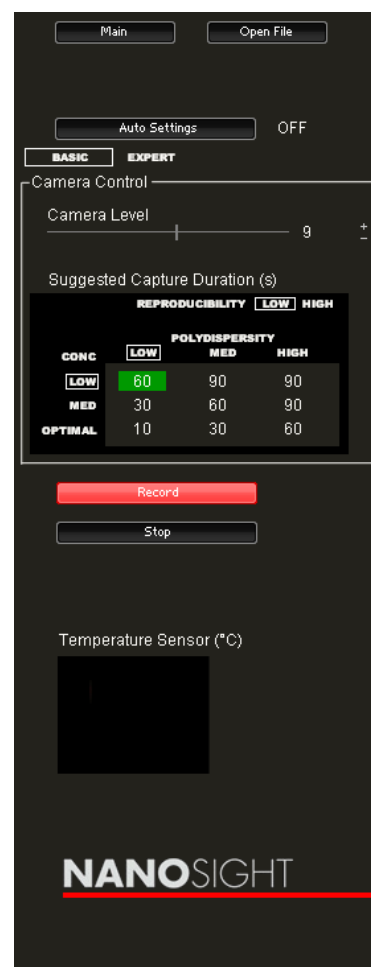
Video Capture

The video capture interface comprises the camera control interface and the live display.

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.*





Camera Control

The camera control interface is responsible for setting the camera controls manually. There are two options available, basic and advanced. Basic mode should be sufficient for most samples and we would recommend only using advanced mode if you require more precision in the camera settings or recording duration. The aim of using these settings is 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).

Warning triangles Warning triangles appear when the capture screen is either too dim (displaying a ‘Dark!’ warning) or has too much saturation (display a ‘Bright!’ warning) –these should be used as a guide only.

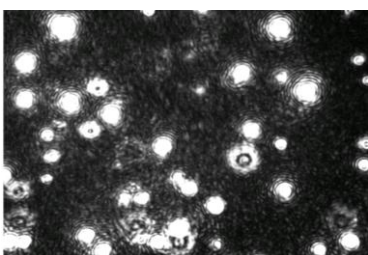
i *Saturated pixels appear as red blobs on the capture screen. High saturation of particles can lead to tracking errors and hence should be kept to a minimum; however, in almost all samples it will be necessary to have some saturation to allow the dimmest particles to be visible. If the sample is very 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 [14](#).*

Basic mode – camera level Determines the brightness of the image (using a combination of shutter and gain used in advanced mode)

Advanced mode - 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.

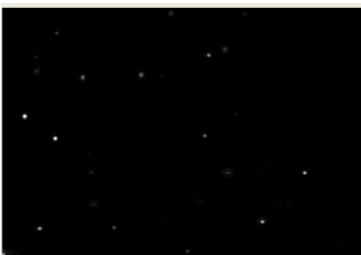
i *If the shutter setting bar turns red, the camera is being over exposed and the shutter setting will need to be reduced until the red warning bar disappears.*

Advanced mode - 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.



Example of overexposed image

i *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

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. In basic mode, suggested capture durations are selected from a table based on approximate concentration, polydispersity and repeatability required.




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 ($>10^9$ 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 [25](#))*

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, if there is no temperature readout available, the user is prompted to input the temperature of the sample at the time of analysis. When using the LM10, this can be obtained from use of the Temperature Probe that is supplied with the instrument (see relevant manual).

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

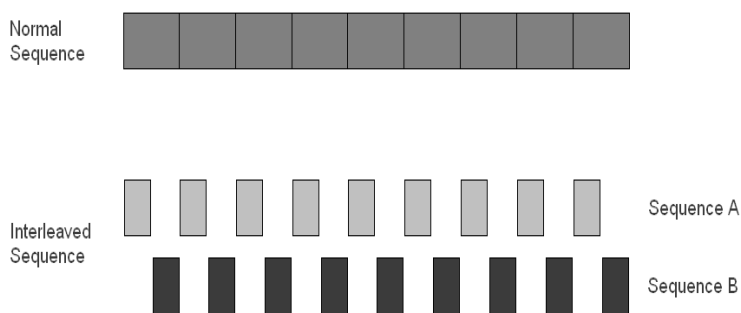
 *This default save location can be changed by modifying the configuration settings in the nano.ini file*

Extended Dynamic Range (EDR) capture

! Due to the high processing and disk access demands on the computer when running this NTA 2.1 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 2.1 running in standard mode. This is described in the previous section Video Capture.

The Extended Dynamic Range (EDR) option described here offers additional functionality to the standard operating mode of NTA 2.1. 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:



Upper line; normal, single channel, 30fps video output. The lower graphic represents an interleaved mode output at 60fps when EDR is selected.

Sequence A (Channel A) might be set at high gain to detect small particles,

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:

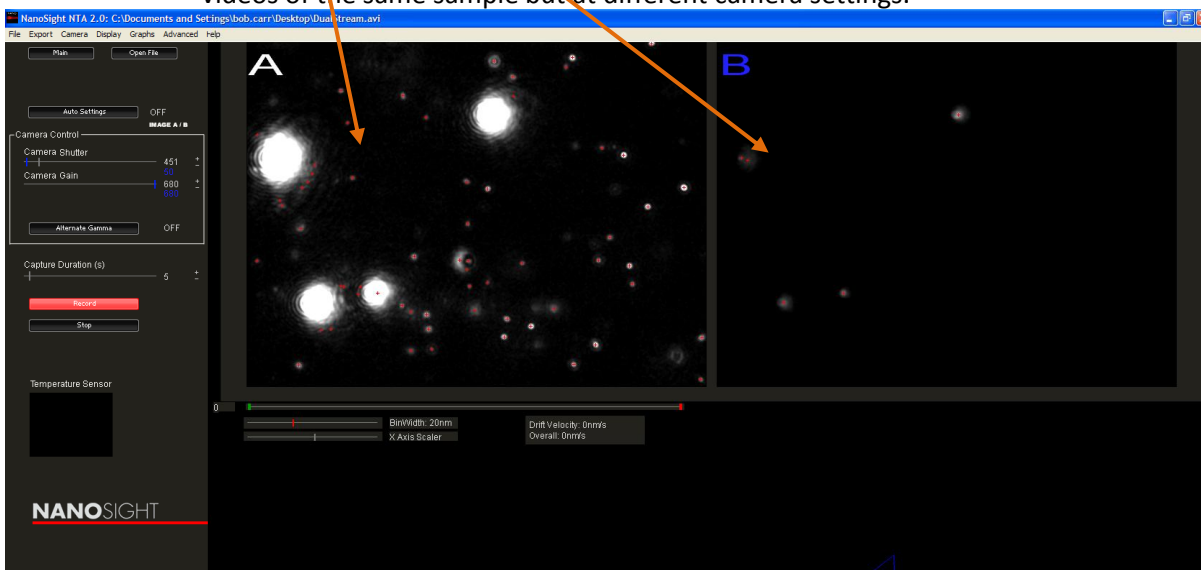
1. If in NTA already, exit the program and restart NTA 2.1 from the start menu or the NTA icon on the desktop.
2. Click on **Extended Dynamic Range** in the Pull down "**Advanced**" menu.
3. Select Capture mode by pressing the Capture button.
4. The EDR Capture screen will then be displayed.

! Note:

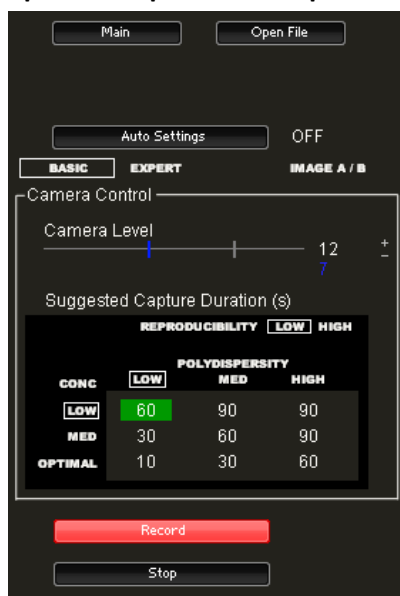
- Once the camera is initialised in 60fps EDR mode, the program must be restarted to enable 30fps normal mode.
- Auto Capture Settings mode is disabled in EDR Mode

EDR Capture Screen

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



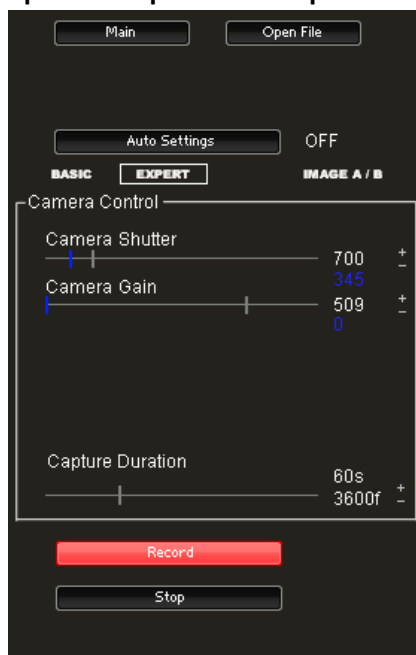
Optimise exposure for capture – Basic mode



In basic mode the camera levels are always at a fixed distance apart. The camera level should be increased such that the smallest (dimmiest) of the particles present can be seen in the “A” Capture window. This should allow the larger, brighter particles to be tracked successfully in the “B” capture window.


If more flexibility is required in the settings – for example, the particles in the “B” capture window are still too bright – advanced mode can be used to independently change settings for each channel.

Optimise exposure for capture – Expert mode



“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

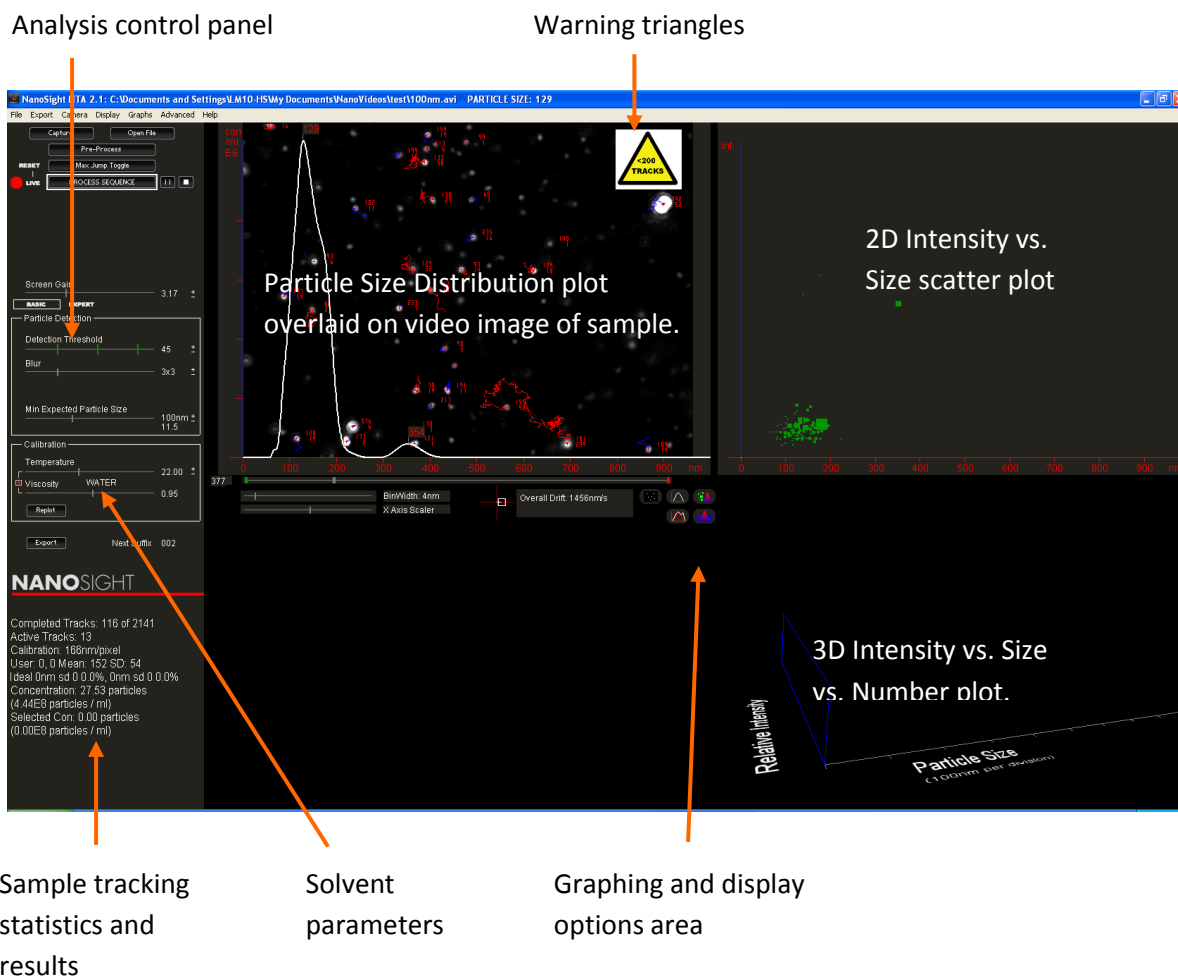
- In EDR mode the maximum shutter value is 700
- Shutter settings **must** either be **both above 180** or **both below 180** to avoid a camera configuration conflict.

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

! NTA display on lower resolution screens.

- NTA 2.1 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



Analysis control panel

Warning triangles

Particle Size Distribution plot overlaid on video image of sample.

2D Intensity vs. Size scatter plot

3D Intensity vs. Size vs. Number plot.

Sample tracking statistics and results

Solvent parameters

Graphing and display options area

- Analysis control panel**
 Allows adjustment of the image and the parameters for particle detection and tracking. 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. Settings can be modified during processing and remaining frames will be processed using these modified settings.
- Solvent parameter—Temperature and Viscosity Input**
 Allows input of solvent temperature and viscosity – allows re-plot of a particle size distribution when temperature and viscosity and changed
- Sample tracking statistics and results**
 Selected results from tracking and analysis
- Graphing and display options**
 Allows different graph and display options to be selected

- **Warning triangles**

Displays the following warnings –



The <5 objects warning may be seen in **pre-process** when there are less than 5 centres found on screen – this indicates that the concentration of the sample is very low, or the detection threshold setting is too high.



The >200 objects warning may be seen in **pre-process** when there are more than 200 centres found of screen – this indicates that the concentration of the sample is very high, or the detection threshold setting is too low, detecting a significant amount of noise. Adjust the sample concentration if necessary; use blur, extract background or increase the detection threshold to remove noise.



The <100 tracks warning indicates that there are less than 100 completed tracks at the current point during the analysis. This is a very low amount of statistics to be included in the results except for very monodisperse samples.



The <200 tracks warning indicates that there are less than 200 completed tracks at the current point during the analysis. This is a low amount of statistics to be included in the results.



The high min expected warning indicates that the selected min expected particle size may be too low for the current analysis. This is shown when a significant peak is found below the current min expected particle size.

- **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

Image Pre-Processing


Introduction

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. There are two modes available, 'basic' and 'expert'; the use of basic mode is recommended under most circumstances.

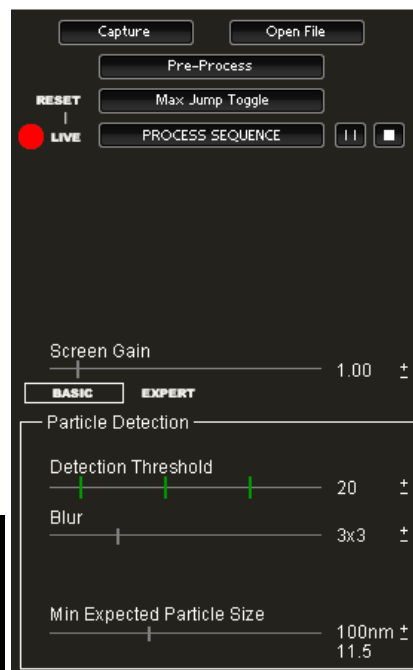
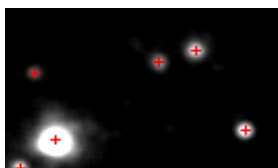
Basic mode

In basic mode the background is automatically removed from the video – if using a video captured with an older software version there may be no background file present, in this case you will need to use 'make background' from the **advanced** menu.

Screen Gain Adjusts the brightness of the image displayed on screen with no effect on detection – use this setting to help visualise the dimmest particles when setting the detection threshold

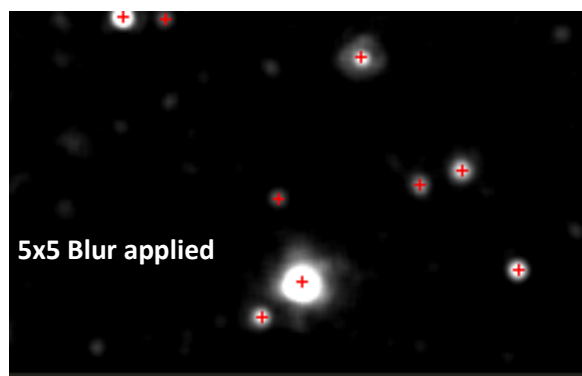
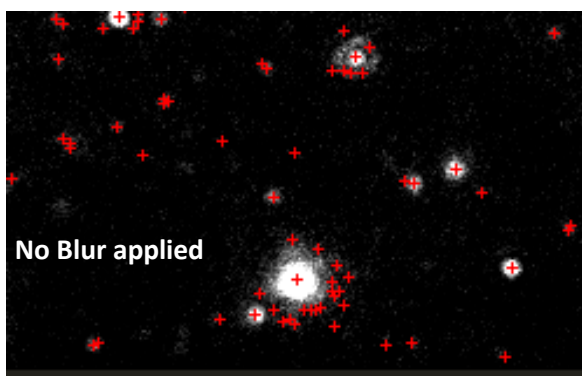
 The 'Screen Gain' bar goes up to a maximum of 10.00, however this limit can be exceeded by using the + and – buttons or using CTRL+Page Up and CTRL+Page Down.


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.



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 below, 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.

Min Expected Particle Size

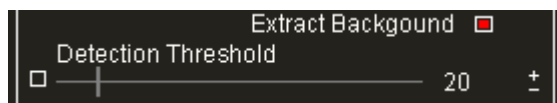
The distance a particle moves between frames (i.e. within 30ms) will depend on its size (as well as the solvent viscosity and temperature). The value the user selects here determines the maximum distance (pixels) from the particle’s position in a given frame that the software will search for a particle 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. 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. Max jump toggle can be used to assess the suitability of the max jump selected for unknown samples, see page 22

 *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.*

Expert Mode

In addition to the settings described in basic mode, expert mode has the follow settings available;

Extract Background This can be toggled on and off using the red box



Single detection threshold Un-selecting the red box on the left of the detection threshold slider reduces the detection threshold

Min Track


Length Because NTA 2.1 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 Min Track Length 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

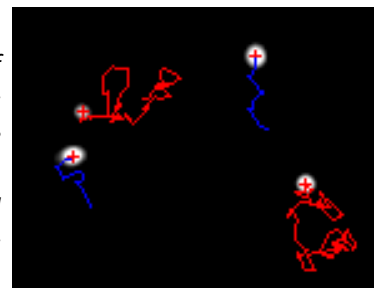
in the particle size distribution plot. Entering too large a value will bias the results to larger, slower moving particles in a mixture of particle sizes, or, for a more monodisperse sample, simply reduce the proportion of included tracks.

 *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.

i 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.



Additional pre-process settings

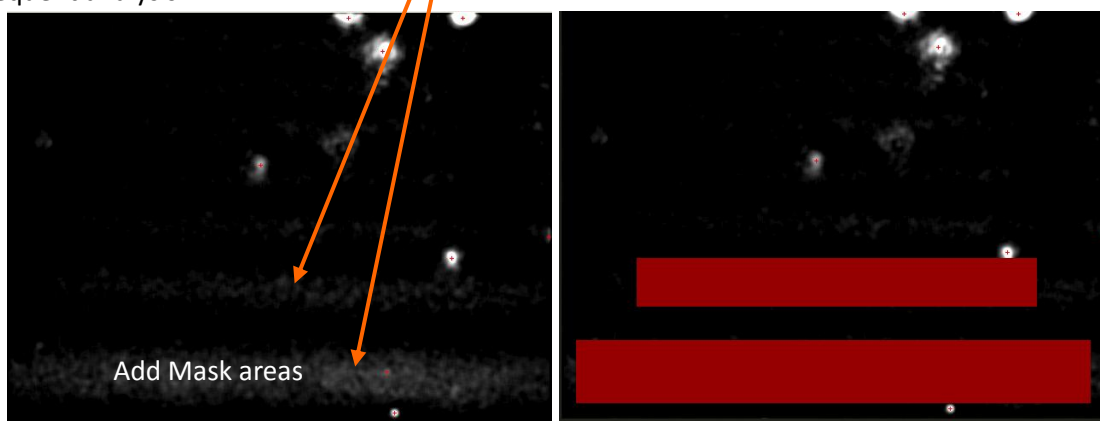
Masking/excluding an area from analysis: Occasionally, during video capture, an area of stray light or flare which is not associated with particles appears. This might arise from contaminant adhesion onto one of the optical surfaces, bubble formation in the sample, etc.. This area of static stray light 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

i 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.

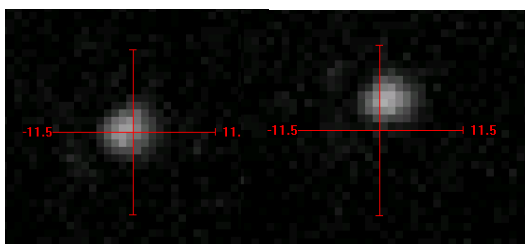
Min expected particle size The distance a particle moves between frames (i.e. within 30ms) will depend on its size (as well as the solvent viscosity and temperature). The value the user selects here determines the maximum distance (pixels) from the particle's position in a given frame that the software 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


In order to assess the suitability of the ‘max jump’ determined by the minimum expected particle size, 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).

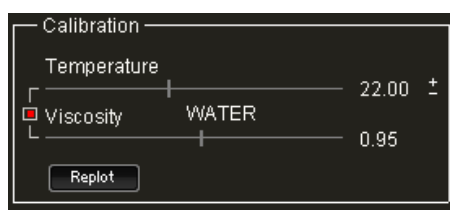
This movement is overlaid with a crosshair showing the extent of the maximum particle distance selected (Note; the centre of the crosshair is zero). The size of the crosshair alters as the user changes the ‘Min Expected Particle Size’ slider. Using the slider, the user can adjust this distance such that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

Calibration

Temperature & viscosity


The temperature and viscosity of the solvent needs to be set before processing and analysis begins. 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.

 *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.*

Analysis

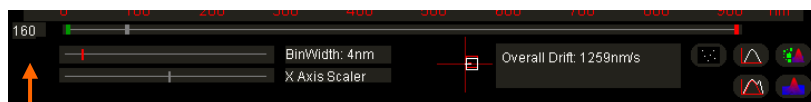


Process Sequence Starts the analysis of the Captured video sequence based on the Pre-processing settings previously selected.

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 'Ctrl' + 'right arrow' keys 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

In pre-processing, 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 (which can be moved to any frame by *left clicking* on the progress 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 14.

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

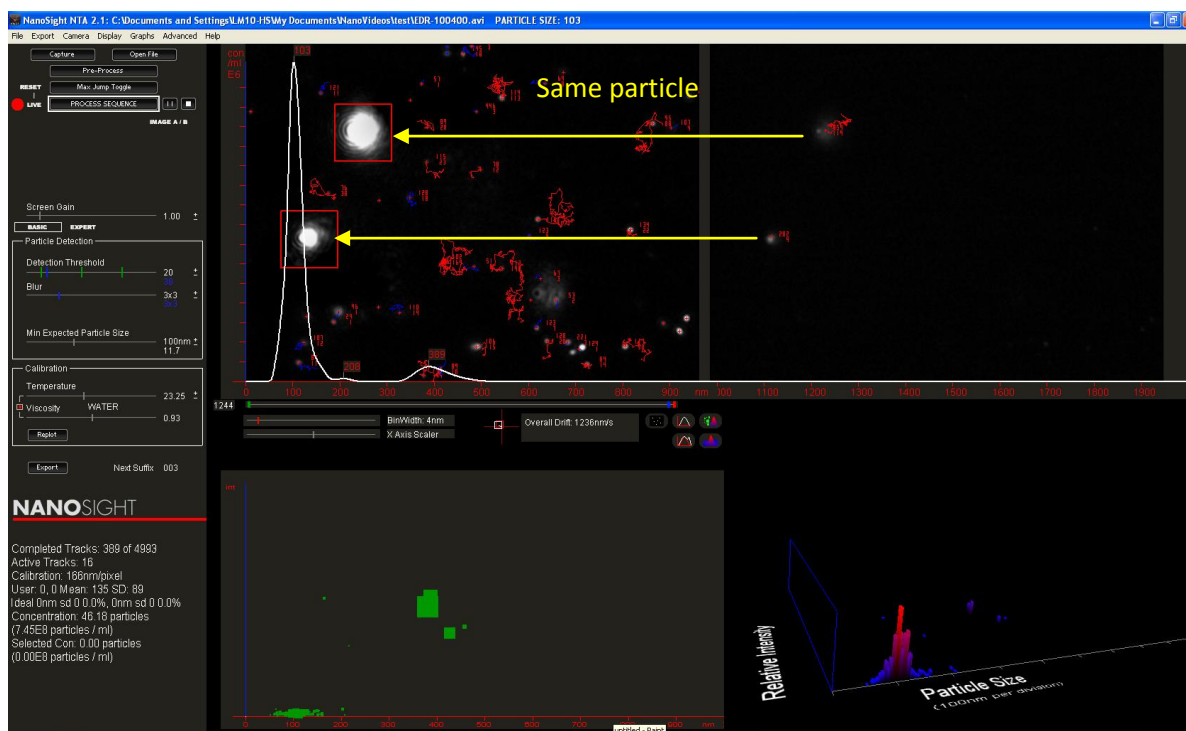
Detection threshold and **Blur** are all adjusted as usual but using the

- *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 **Detection Threshold** check box in expert mode will activate this function for Channels A and B respectively. The check box will show (half grey and/or blue) which channel is activated.

Changes to Screen Gain, Minimum Track Length and Min Expected Particle size apply to both Channel A and Channel B. 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 2.1 screen.



Live Capture/display


When this option is selected, NTA 2.1 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).

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




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 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 data created by the File Export facility.*

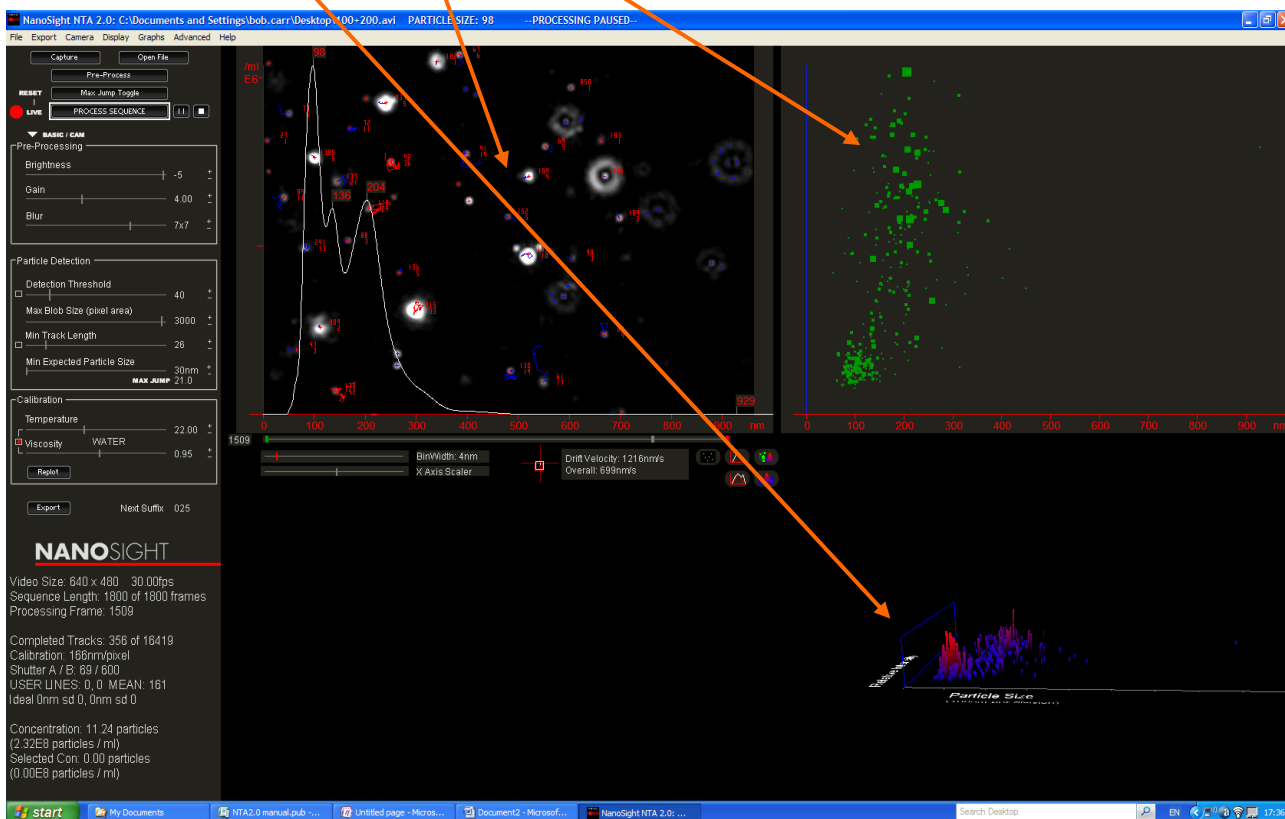


! When creating a report or exporting data this should be performed whilst the **Live** is processing, i.e. Do not stop processing first.

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

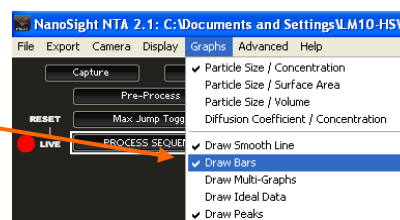
Adjusting X axis

- 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.

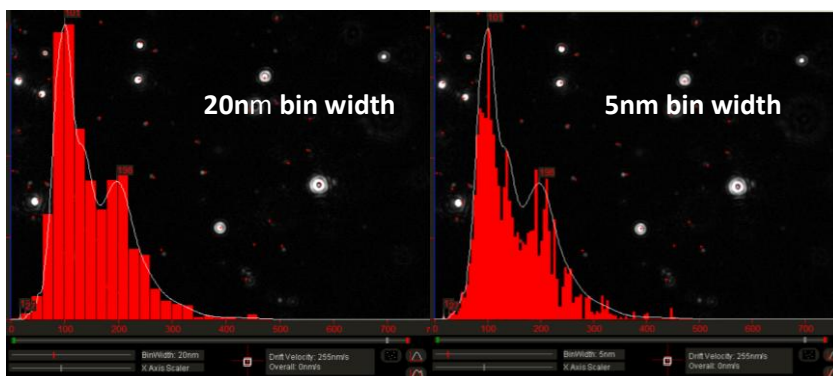
Adjusting Bin Width

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.



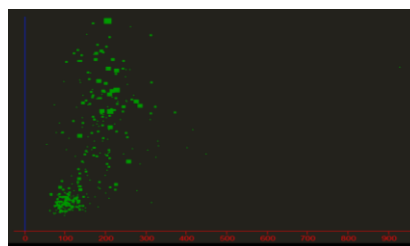
By using the Bin Width slider, the size of the bins in which the data is plotted can be adjusted. Thus, the default setting of a bin width of 20nm (left below) can be reduced to a bin width of, for instance, 5nm (right below), enabling a higher resolution plot to be displayed.



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 2.1 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.

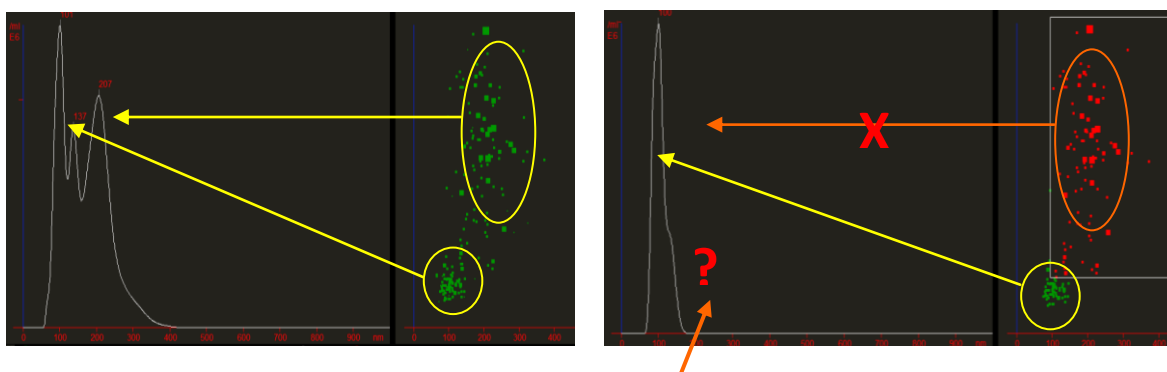
2D Intensity vs. Plot Screen

During the tracking and Brownian motion analysis of a particular particle, NTA 2.1 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 2.1 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.

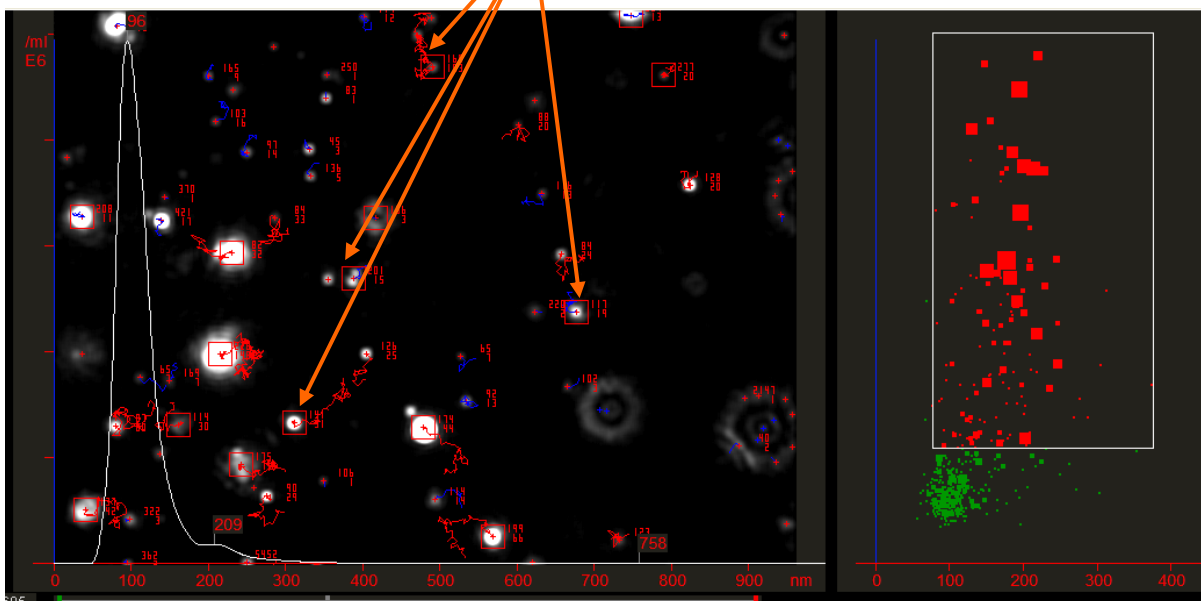



Highlighted part of the population is removed from main particle size distribution plot.

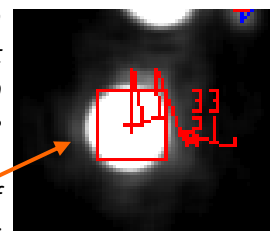
Visualising highlighted particles during analysis

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

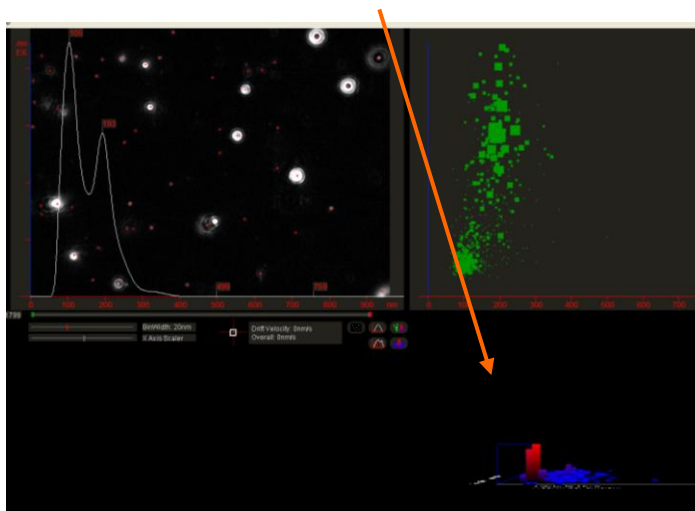
The sizes of the green squares shown in the 2D Intensity vs. Size plot reflect the length of time over which a particle has been tracked; the longer the particle trajectory, the larger the square. This is useful for confirming that larger particles, on average, track for longer than smaller particles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

3D Plot

The 3D plot (shown lower right below) shows a plot of particle **Size** (x-axis in nm) vs. **Intensity** (y axis 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.

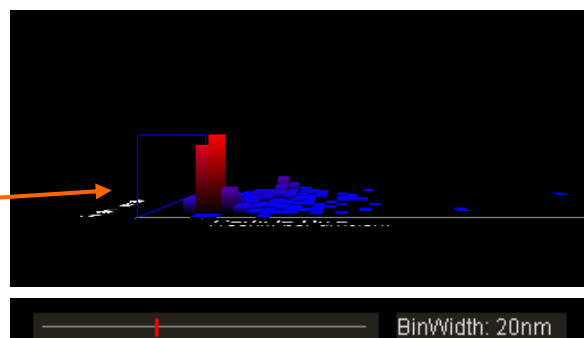
Rotating automatically

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.

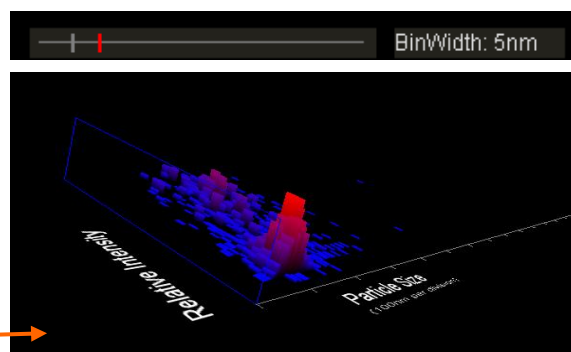
NTA 2.1 defaults to a Bin Width of 20nm and a plot of aspect as shown right.



- 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 (y axis) is plotted can be adjusted by *right clicking* on the **Bin Size** slider bar (red line) and moving to the required resolution.

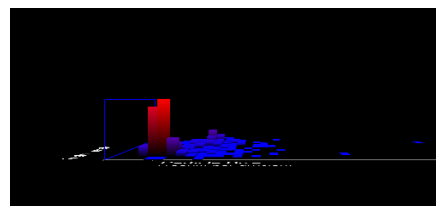
For example, changing the size bin width (grey line) to a value of 5nm (using the left mouse button) and changing the intensity bin width (red line) to a value of 10 (using the right mouse button) gives a plot as shown below. The value displayed for the bin width is always that of the Size.

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



Expanding or reducing the size of the plot

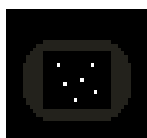
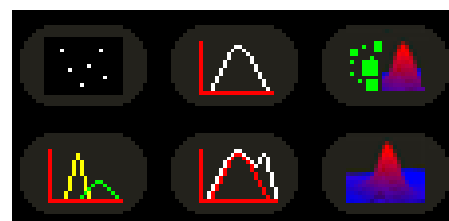
- Right clicking on the 3D plot and dragging from side to side adjusts the plot size (i.e. Zooms in and out)
- *Double left clicking* on the 3D plot acts to expand the plot to fill the right-hand side of the computer screen to improve clarity. *Double left clicking again* returns the image to its default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



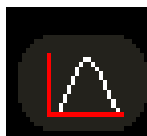
Plot and Display Options

Clicking on one of the cluster of plotting icons seen in the centre of the NTA 2.1 screen allow the user to:

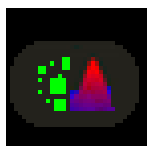
- Change the main window display
- Switch between showing the Particle Size Distribution plot or the video Image (or both overlaid)
- Smooth the 3D plot
- Fit mathematically generated model curves to the experimental Particle Size Distribution plot obtained
- Show the degree to which the model fits the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



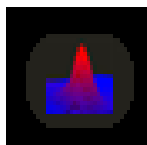
Video Image Display This switches off the graph plotting leaving only the video Image display. To draw graphs again, press the Graph Plot icon.



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.



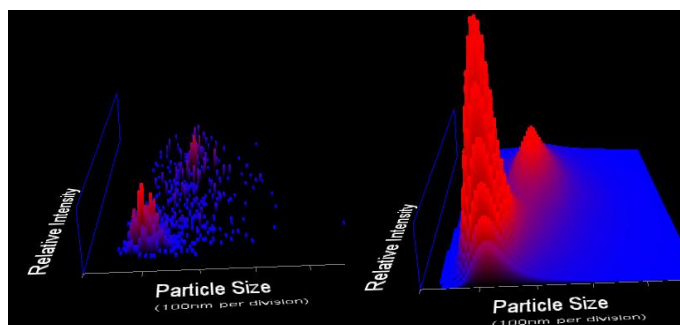
Main Display Option Toggling this icon allows the user a choice of which display to show in the main screen (i.e. video image/particle size plot, 2D plot or 3D plot)



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

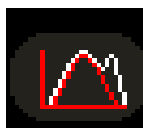
Brownian motion, the length of time the particle is present in the laser-illuminated scattering volume is likely to be restricted. The number of steps taken by the particle during this short time is similarly limited. Thus, any estimate of the average step length taken by a particle, resulting from a 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.

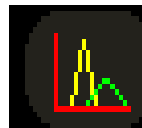
NTA 2.1 allows the user to fit a single, model log-normal plot to the experimental data. The peak of the model curve is centred on the mode of the experimental data obtained and the width of the model distribution is calculated from the standard deviation of the experimental particle size distribution plot



NTA 2.1 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.



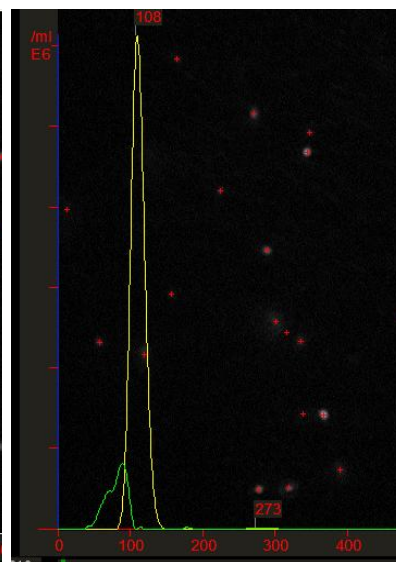
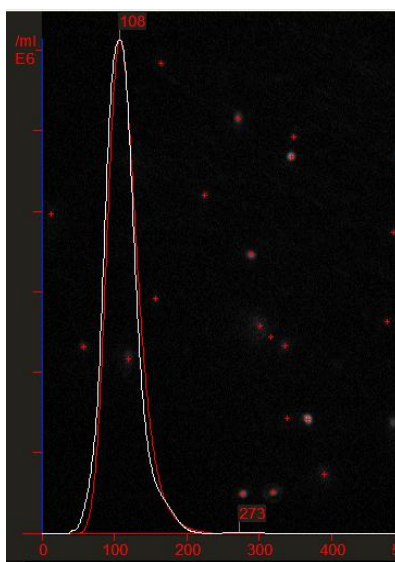
By pressing the **Model Fitting** icon once, a single log-normal model plot (red) is overlaid on the experimental profile (white). The icon beeps once and flashes on and off while the model is being calculated.



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



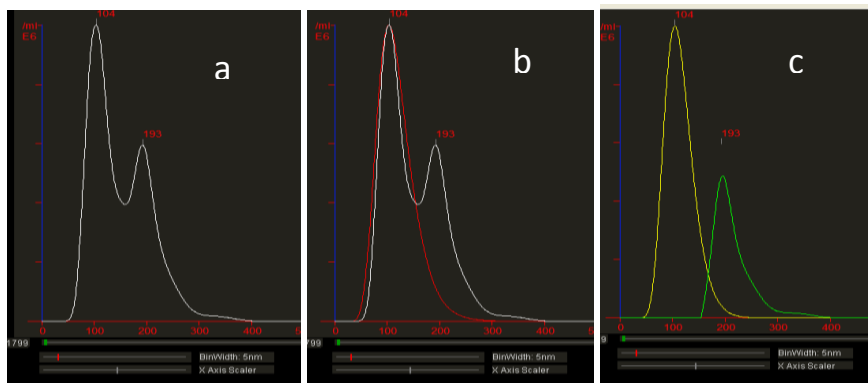
the model log-normal distribution).

Bimodal Model fitting

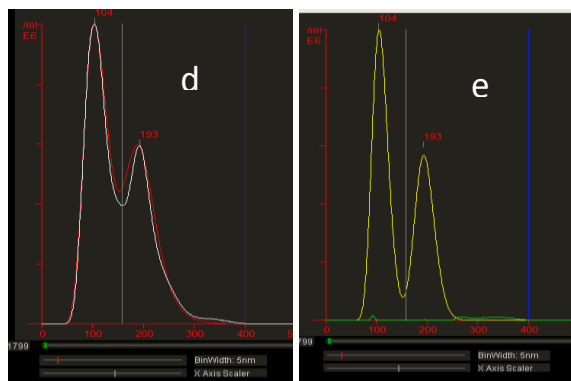
If a particle size distribution profile is obtained which appears clearly bi-modal, the NTA 2.1 can be configured to apply a bimodal model to the profile as follows. After the analysis is complete, the user can move the cursor to the main particle size distribution plot screen and place a pair of marker (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.

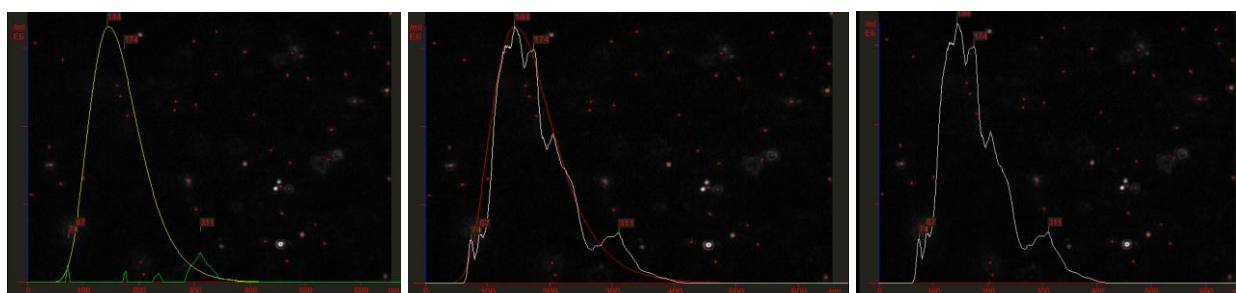


By placing the white (left click) and blue (right click) markers to the left and right respectively of the second peak and pressing the Model Fitting icon, a bimodal fit will be made. The icon will beep twice (indicating a bimodal fitting step) and flash while the model is being calculated. When fitted, 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 2.1 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.

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.

User (User lines), Mean and SD

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.

Mean displays the average size of the particle size distribution shown on screen.

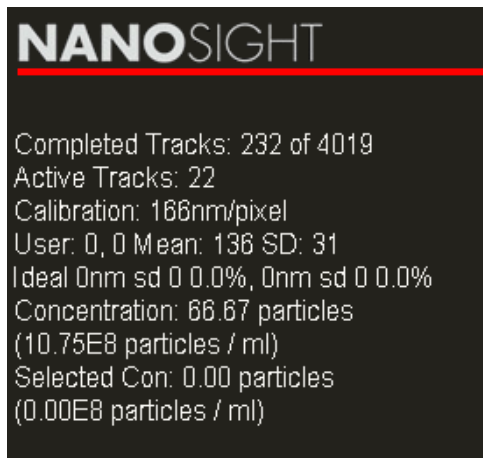
SD displays the standard deviation of the particle size distribution shown on screen.

Ideal

When the Model Curve fitting option is selected and the FTLA Plot is selected (see page [33](#)), the exact peak position, width (the Standard Deviation) and relative proportion of the peak (or both peaks if a bi-modal fit is selected) is shown here.

Concentration

For information about the Concentration information area immediately below the above, please go to page [41](#).

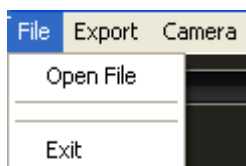


Menu Bar and Pull-down menus

NanoSight's NTA 2.1 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.



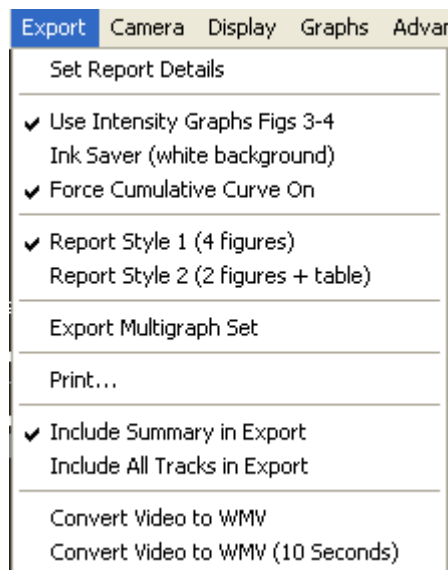
File Menu



Open File Serves the same function as Open file on the Main Screen and allows a previously saved video to be loaded into NTA 2.1

Exit Exits NTA 2.1

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 on 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 2.1 in "negative" (white background) thus saving on printer ink when printing Reports.

Report Style 1/Report Style 2 NTA has the option of two different report styles to export. These are shown on pages 37 and 38.

Export Multigraph Set This exports a *.csv file containing bin data of the current multi-graphs displayed on screen

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.

Include Summary/All Tracks in Export These options allow you to select which files are exported automatically when a video has completed processing. For more information on these files see page 39.

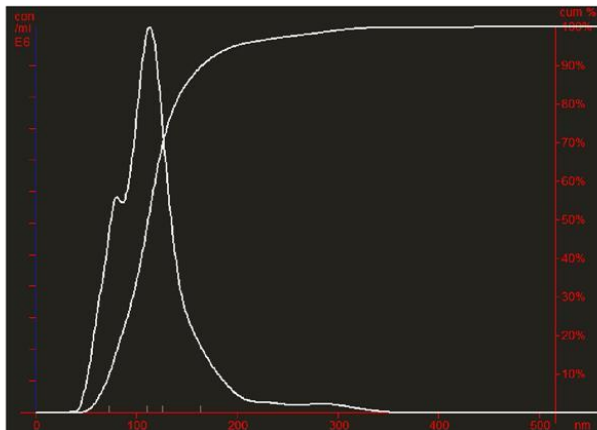
Convert Video to WMV This option converts the currently loaded video to a WMV file. Although this file cannot be processed using the software, it is ideal to use in presentations or sending via email (e.g. for support purposes) as its file size is significantly smaller.

A typical example of a printed NTA 2.1 Sample Analysis Report (Style 1):

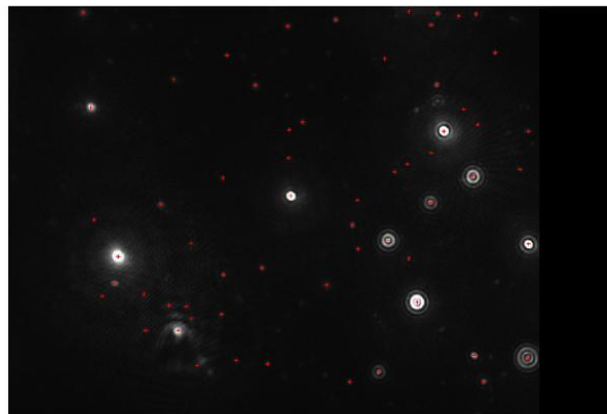
NANOSIGHT

Nanoparticle Tracking Analysis (NTA) Version 2.1 Build 0325

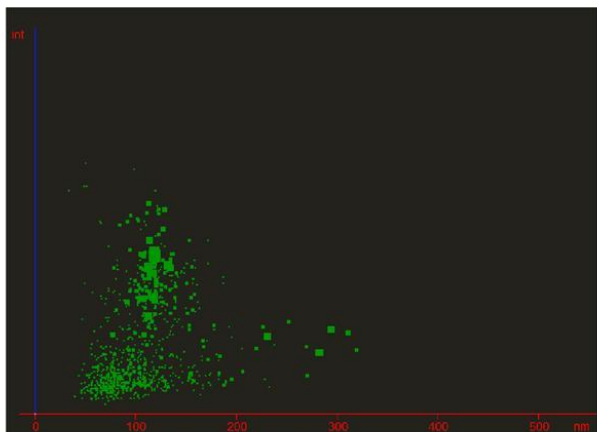
SAMPLE REPORT



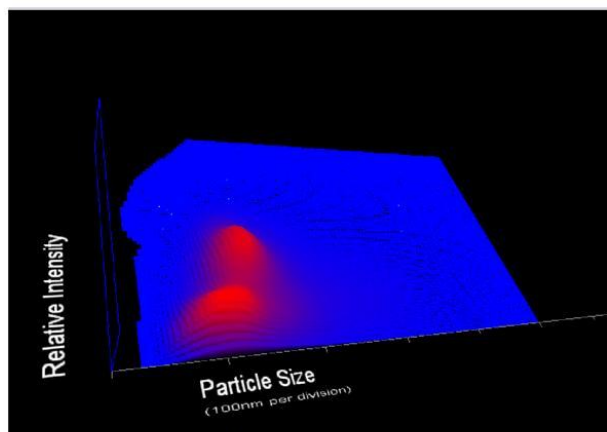
Particle Size / Concentration



Sample Video Frame



Particle Size / Relative Intensity



Particle Size / Relative Intensity 3D plot

Operator:

Sample: Polymer + Biological Mix
Video File: Mix.avi analysis no. 003
Date/Time of Report: 08/10/2010 15:30:42
Dispersant/Diluent:
Concentration:
Pre-treatment:

Remarks:

RESULTS:

Distribution Data: Mean: 118nm Mode: 113nm SD: 43nm
Cummulative Data: D10: 73nm, D50: 111nm, D90: 164nm, D70: 126nm
User Lines: 0nm, 0nm
Total Concentration: 50.29 particles / frame, 8.11E8 particles / ml
Selected Concentration: 0.00 particles / frame, 0.00E8 particles / ml
Fitted Curve 1: Mode: 0nm, SD: 0, Fitted Share: 0.0%
Fitted Curve 2: Mode: 0nm, SD: 0, Fitted Share: 0.0%
Completed Tracks: 1217
Drift Velocity: 891 nm/s

ANALYSIS SETTINGS:

Frames Processed: 1779 of 1780
Frames per Second: 29.61
Calibration: 166 nm/pixel
Brightness: -0
Gain: 1.11
Blur: 5x5
Detection Threshold: Auto
Max Blob Size (pixel area): 3000
Min Track Length: Auto
Min Expected Size: 100nm
Temperature: 24.70 °C
Viscosity: 0.90 cP

A typical example of a printed NTA 2.1 Sample Analysis Report (Style 2):

NANOSIGHT

Nanoparticle Tracking Analysis (NTA) Version 2.1 Build 0325

Sample:

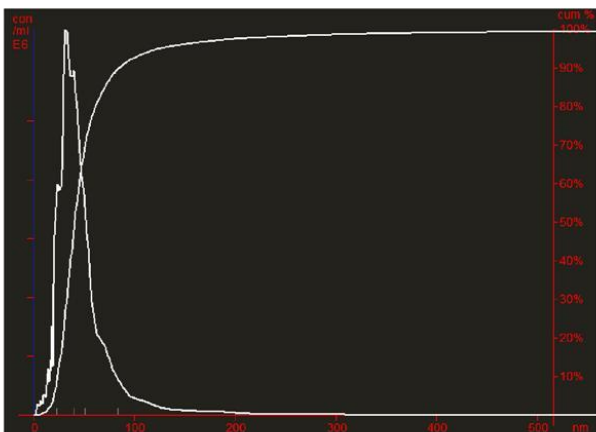
Video File: #1 4-25000 andor.avi; Analysis no. 002

Date/Time of Capture: 19 March 2009 11:57

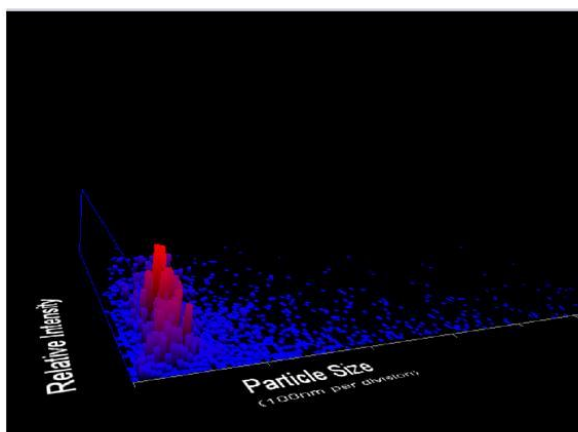
Operator:

Comments:

SAMPLE REPORT



Particle Size / Concentration



Particle Size / Relative Intensity 3D plot

Bin Centre (nm)	Concentration E6 particles / ml	Percentile Undersize
10	8.247	0.71%
30	98.164	21.97%
50	76.224	68.09%
70	23.880	84.73%
90	9.726	91.26%
110	4.826	93.89%
130	2.509	95.38%
150	1.716	96.21%
170	1.371	96.85%
190	1.139	97.39%
210	0.684	97.77%
230	0.502	98.00%
250	0.508	98.22%
270	0.495	98.43%
290	0.404	98.63%
310	0.297	98.77%
330	0.258	98.89%
350	0.247	99.00%
370	0.212	99.10%
390	0.164	99.18%
410	0.132	99.24%
430	0.117	99.29%
450	0.104	99.34%
470	0.092	99.38%
490	0.085	99.42%
510	0.084	99.45%

Bin Centre (nm)	Concentration E6 particles / ml	Percentile Undersize
530	0.086	99.49%
550	0.086	99.53%
570	0.082	99.56%
590	0.076	99.60%
610	0.070	99.63%
630	0.064	99.65%
650	0.061	99.68%
670	0.057	99.71%
690	0.052	99.73%
710	0.045	99.75%
730	0.037	99.77%
750	0.030	99.78%
770	0.023	99.79%
790	0.019	99.80%
810	0.016	99.81%
830	0.014	99.82%
850	0.013	99.82%
870	0.013	99.83%
890	0.013	99.83%
910	0.013	99.84%
930	0.013	99.84%
950	0.013	99.85%
970	0.013	99.85%
990	0.013	99.86%
1000-2000	0.322	100.00%

Results

Mean: 56 nm
Mode: 32 nm
SD: 77 nm
D10: 23 nm
D50: 40 nm
D90: 84 nm
User Lines: 0, 0 nm
Concentration: 2.33 x 10⁸ particles/ml

Measurement Conditions

Temperature: 22.20 °C
 Viscosity: 0.95 cP
 Frames Per Second: 31.30
 Measurement Time: 59 of 59 seconds
 Drift Velocity: 299 nm/s

Analysis Conditions

Brightness: -0
 Gain: 1.11
 Blur: 5x5
 Detection Threshold: Auto
 Max Blob Size (pixel area): 3000
 Min Track Length: 1
 Min Expected Size: 30 nm



Exported Files

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. xxxx-001-summary.csv, xxxx-002-summary.csv, etc... These files can be loaded to/by Microsoft Office Excel for further manipulation.

[Results Summary] Contains information entered by the user using Set Report Details, video information and analysis statistics.

[Settings] contains the analysis settings used to process the video in NTA.

The summary file also includes;

- 1nm resolution bin data for size against concentration, surface area and volume; with the addition of FTLA plots if applicable
- Percentile undersize values at 1% resolution

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. xxxx-001-alltracks.csv, xxxx-002-alltracks.csv, etc... These files can be loaded to/by Excel for further manipulation.

Particle ID gives each track a unique ID, corresponding to the ID shown in NTA (when switched on in Display)

Used in plot states whether the track was used in the resulting particle size distribution

Start Frame and **End Frame** give the frames where the particle track began and ended respectively

Track step gives the current step corresponding the X and Y positions

X1, Y1, X2 and **X2** give the X and Y coordinates of the positions at the beginning at end of the current step

Diffusion Co gives the estimated diffusion coefficient for the particle

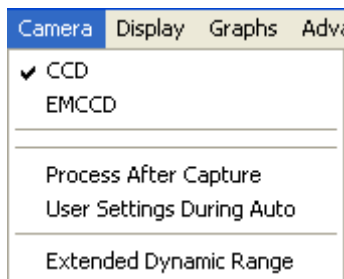
Particle Size gives the estimated hydrodynamic diameter for the particle

Mean Intensity gives the average intensity of the imaged particle

NanoLog.csv

This file is created by NTA 2.1 on first opening and is updated each time a new analysis is run. For each analysis it records the date, time, file name, results and analysis settings.

Camera Menu



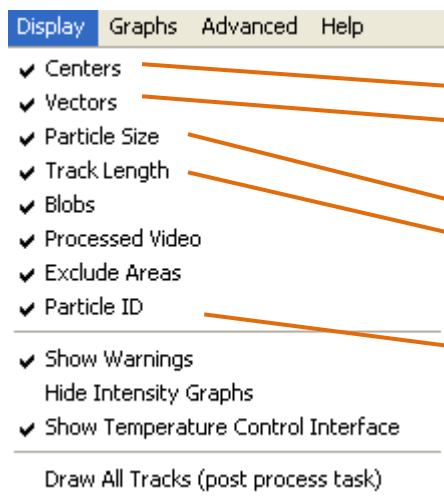
CCD/EMCCD Select either the CCD (AVT Marlin) or EMCCD (Andor Luca HS) for capture

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

Extended Dynamic Range Enables the interleaved camera mode when using the CCD camera

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

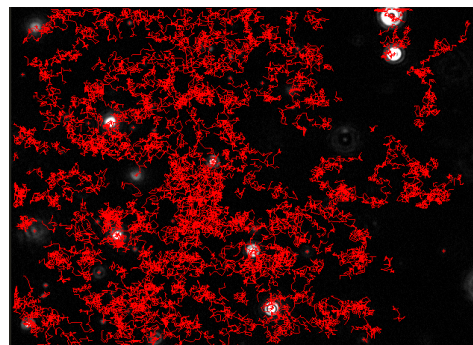
Show Warnings Warning triangles in capture and process

Hide Intensity Graphs Hides the 2D and 3D Intensity graphs

Show Temperature Control Interface For temperature control enabled hardware only – shows the temperature control interface in capture

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 29) will not be included in the Draw All Tracks option.



Graphs	Advanced	Help
<input checked="" type="checkbox"/> Particle Size / Concentration		
Particle Size / Surface Area		
Particle Size / Volume		
Diffusion Coefficient / Concentration		
<hr/>		
<input checked="" type="checkbox"/> Draw Smooth Line		
Draw Bars		
Draw Multi-Graphs		
Draw Ideal Data		
<input checked="" type="checkbox"/> Draw Peaks		
<hr/>		
Log Scale		
<hr/>		
Cumulative Undersize		
Cumulative Oversize		
<hr/>		
Load Multi-Graph Set		
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		
<hr/>		
F1 - F10 Merge By Sum		
<input checked="" type="checkbox"/> F1 - F10 Merge By Mean		

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 2.1 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 become inappropriate.*

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

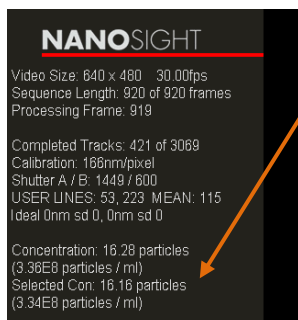
Concentration

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.

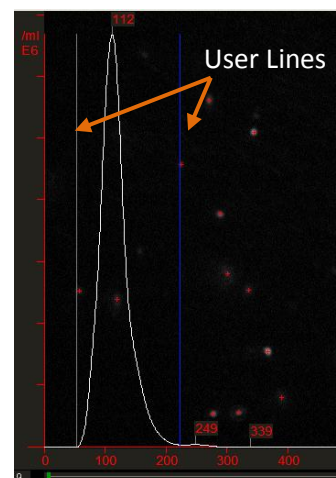
NANOSIGHT	
Video Size: 640 x 480	30.00fps
Sequence Length: 920 of 920 frames	
Processing Frame: 919	
<hr/>	
Completed Tracks: 421 of 3069	
Calibration: 166nm/pixel	
Shutter A / B: 1449 / 600	
USER LINES: 63, 223	MEAN: 115
Ideal 0nm sd 0, 0nm sd 0	
<hr/>	
Concentration: 16.28 particles	
(3.36E8 particles / ml)	
Selected Con: 16.16 particles	
(3.34E8 particles / ml)	

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 5×10^9 /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 2.1 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 21) 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 2.1 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.

i 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

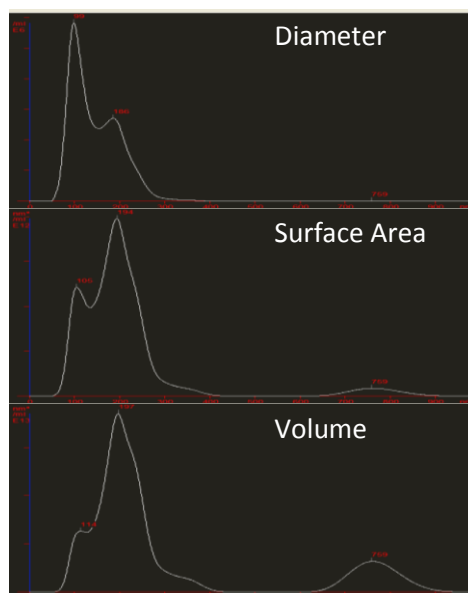
Particle Size / Surface Area

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.

i 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

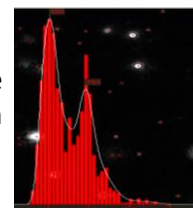
This option allows the data to be displayed as the diffusion coefficient of the particles (in $10^{-9} \text{cm}^2/\text{second}$). This is the parameter which is measured directly by the NTA 2.1 and is therefore independent of particle shape.

Draw Smooth line

This option allows the results to be as a smoothed line, this is the default setting.

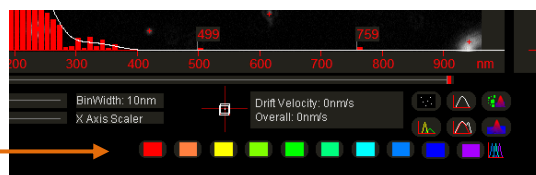
Draw Bars

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.



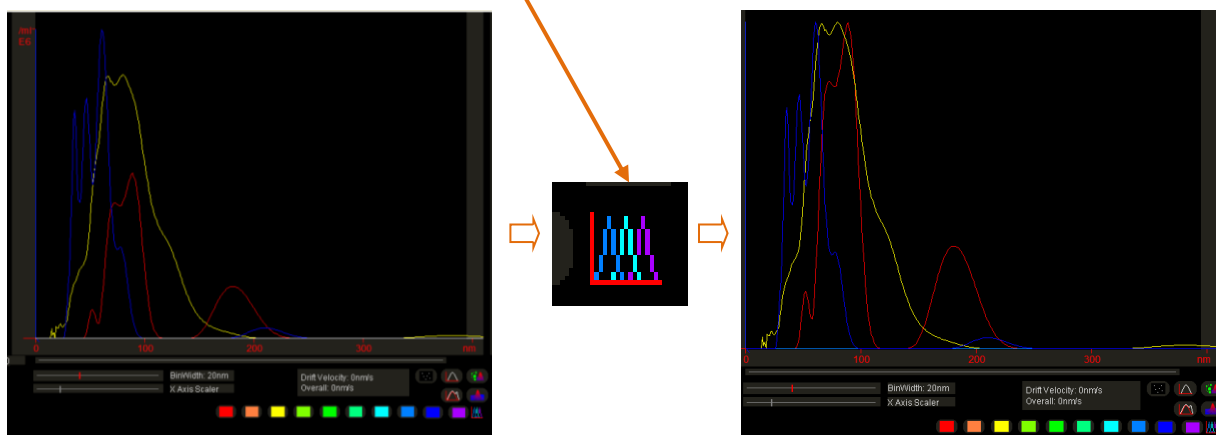
Draw Multi-Graphs


This option allows the user to 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 example, 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.



 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

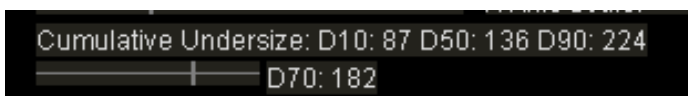
This option allows the user to display (at the top of the peak) the numerical value associated with a peak position depending on what has been selected to be displayed (e.g. Size, surface area, volume, etc..).

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.

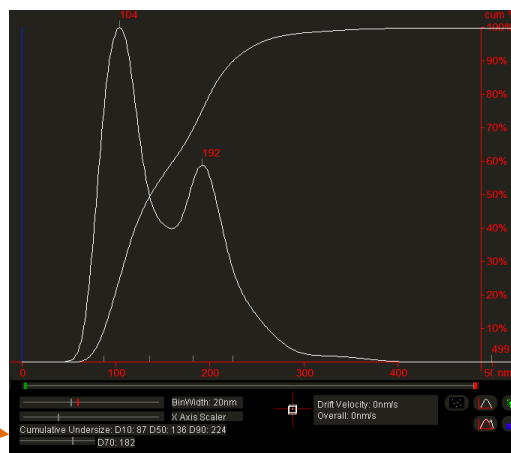
Cumulative Undersize and Cumulative Oversize

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 2.1 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.

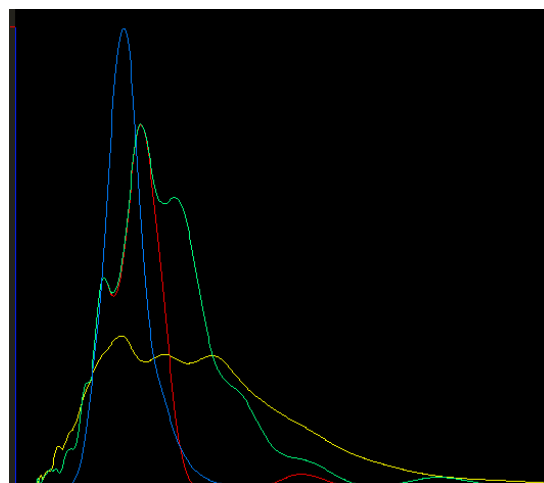


 *Note: the small markers on the x-axis which show the relative positions of the D values.*

Load Multi-Graph

When a sample has been analysed, a file (C:\ \filename.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 2.1 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**.



The colours on the palette bar appear as the his files are loaded in; *left click* toggles the graphs on and off, if data is present but the graph has been switched off the box will appear grey. 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 (and sample concentration) will appear above the plot. The graph button on the right of the palette bar toggles height normalisation on or off; this can be useful for comparing samples with differing concentrations.

If the multi-graphs are named with number suffixes in the format 01,02, ect... they can be loaded in as a set using **Load Multi-Graph Set** by selecting one .his file from the sequence.

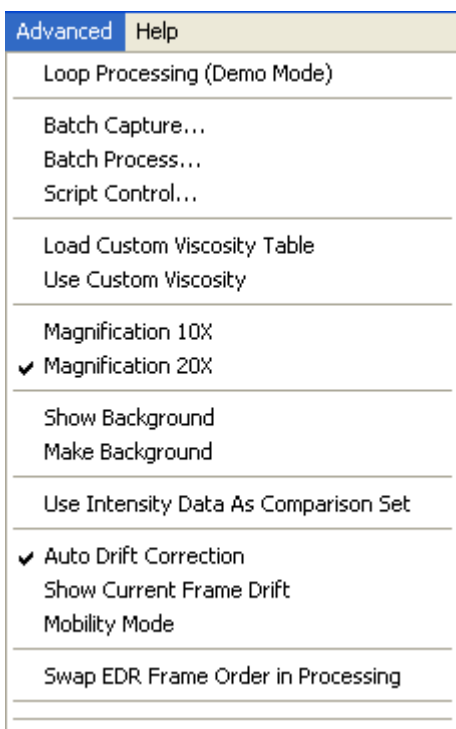
The multi-graphs can be exported into a single file using the **Export Multi-Graph set** function in the **Export** menu.

F1-F10 Merge by Sum/Mean

Any files loaded into NTA as a Multi-Graph can be merged with the current graph (white line) using the 'F' function keys. The option in the menu allows the graphs to be merged by summing or averaging the data. A white box is present around the colour of the colour palette for any graph included in the merge.

Advanced Menu

This menu contains many features that are new to NTA 2.1 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 video**), at user-selected intervals (**Period between video start points**) for a user-selected number of times (**Number of captured videos (1-25)**).

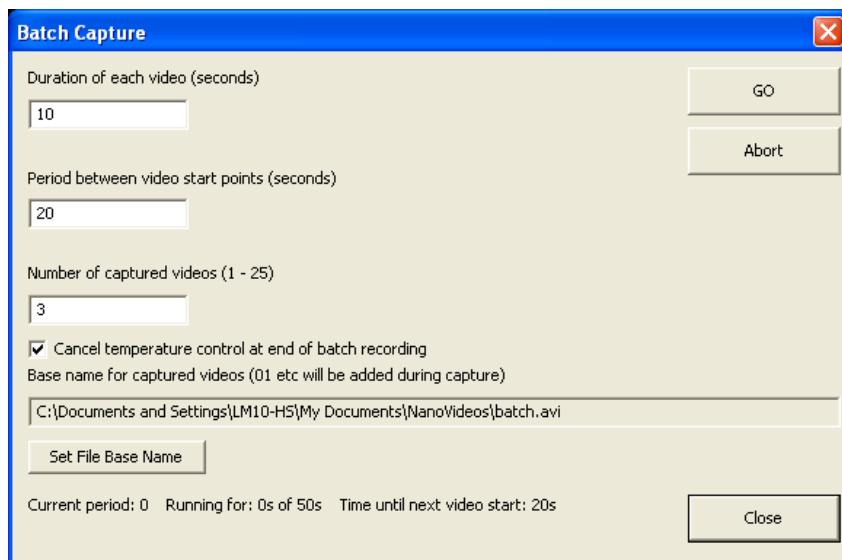
By default, if you are using temperature control, this will be deactivated when the batch capture is complete. This can be prevented by un-ticking the appropriate box.

! We do not recommend that the instrument is left unattended while temperature control is activated.

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\batch01.avi
C:\...\Nanovideos\batch02.avi
etc

When the necessary information is entered, pressing **Go** starts the automated capture without further user intervention. Pressing **Abort** during batch capture stop recording of the current video and prevents further videos from being recorded; however, already captured videos are retained.

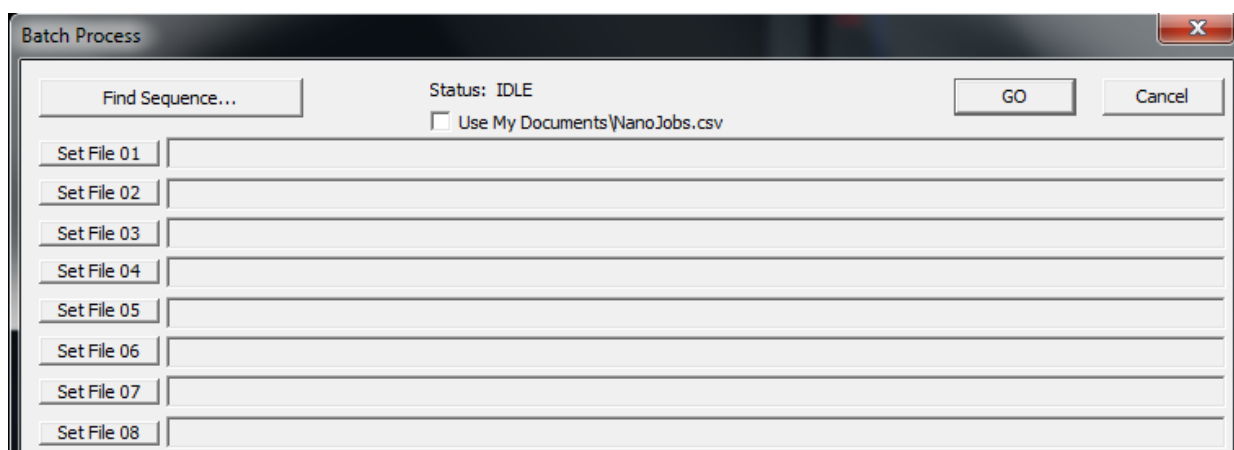
For each cycle the video is captured at the start of the cycle and then written to disc. For long video clips, for example >60 seconds, the software may take up to 10 seconds to transfer the information from RAM to disc after capture. This time may increase for computers whose memory is becoming limited.

 *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
2. Load and analyse a series of videos specified by NanoJobs.csv
3. Load and analyse a series of different (un-related) videos selected by the user

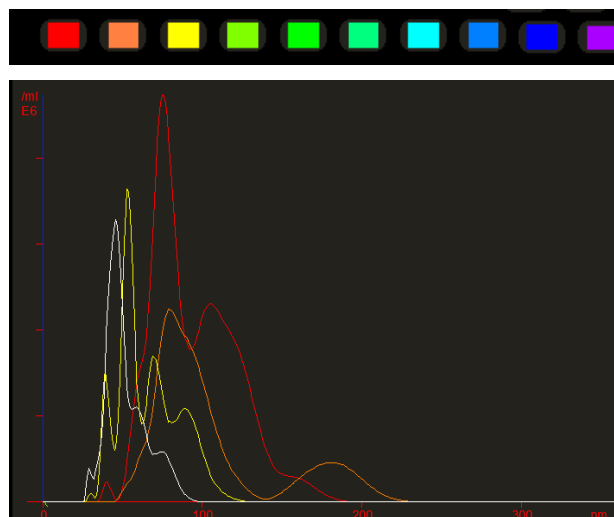


Find Sequence

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 [44](#).



Use NanoJobs.csv

Allows the user to specify files and analysis settings using the file 'NanoJobs.csv' located in my documents. Ticking the box will load the specified videos into the Batch process window and clicking GO will process the videos in sequence with the specified analysis settings.

If this file cannot be found in My Documents an example file (with column headers) will be created when 'GO' is pressed.



Set File (xx)

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.

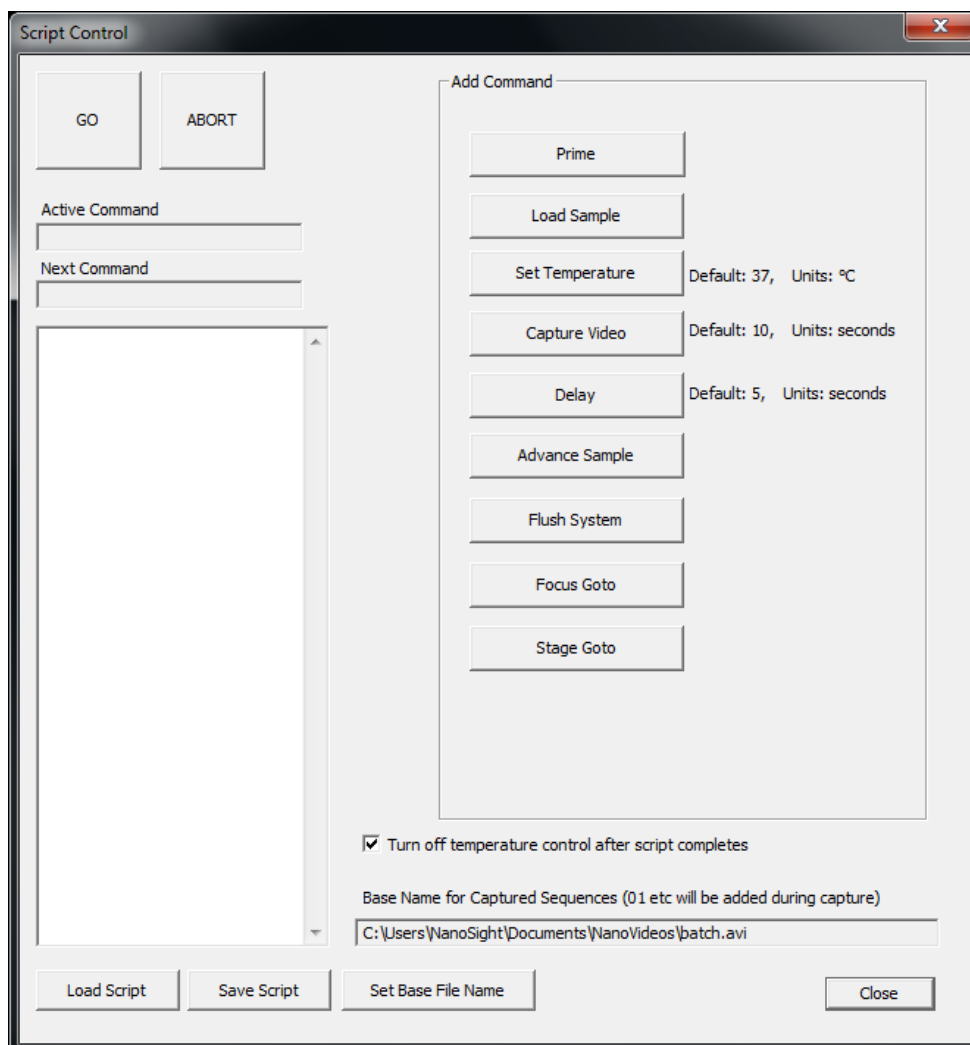
! *Note: All videos analysed when loaded using the Find Sequence or Set File modes described above will be analysed using the same analysis settings. The settings used will be those loaded with the first video analysed (if it has been analysed previously) or will be those remaining from whatever analysis 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.*

Script Control

This opens a control window which allows the user to programme in a sequence of commands for use in creating customised experimental procedures.



Commands

Clicking the command options on the right hand side will add them to the command window on the left, where the default values, e.g. for time duration or focus position, can be altered.

Prime (NS500 only): Run the sample pump only to load sample as far as the Y-connector

Load Sample (NS500 only): Run sample and diluent pumps to load sample into the viewing chamber

Set Temperature (LM14 and NS500): Activate the temperature control and set to the required temperature in °C.

Capture Video: Record a video of the required length in seconds. Videos captured in script mode are saved sequentially using a base file name, which can be set by clicking on the “Set Base File Name” button.

Delay: Pause the programme for a set time in seconds

Advance Sample (NS500 only): Run the “Advance” pump function to introduce fresh sample into the viewing chamber



Flush System (NS500 only): Run the “Flush” pump function to clean the viewing chamber and fluidics

Focus Goto (NS500 only): Move the focus to a specific position

Stage Goto (NS500 only): Move the viewing chamber stage to a specific position

Running Scripts

Programme scripts can be saved to the computer and re-loaded using the “Save Script” and “Load Script” buttons. Once a script has been entered or loaded from the computer, click the “GO” button to start running the programme. The script can be stopped at any time by clicking the “ABORT” button.

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 2.1 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.

It is possible, however, to load a different solvent viscosity-temperature curve if known. NanoSight NTA 2.1 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 2.1 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 2.1 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 [21](#)), 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.*

Show background

Displays the background file in the processing video.

Make background

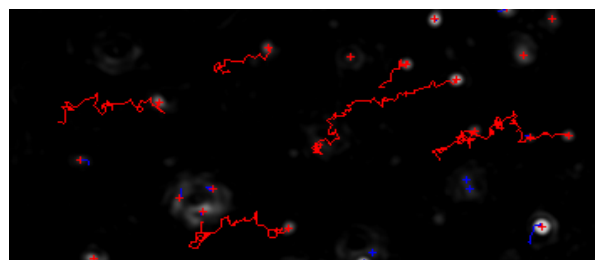
Creates a background file from the currently loaded video – this may be necessary for videos captured using a previous version of the software.

Use Intensity Data as Comparison Set

This setting loads in the current intensity-size scatter plot into the software (in blue) to allow comparison with the scatter plot from a subsequent analysis.

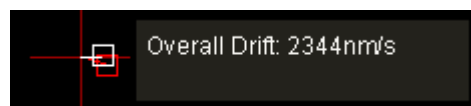
Auto Drift Correction

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 2.1 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).



Show Current Frame Drift

Shows the current frame drift in the drift velocity window

Mobility mode

Mobility mode allow individual drift velocities to be plotted against particle size on the 2D scatter plot window and particle sizes are corrected for drift on an individual basis. This may help compensate for drift when drift is changing over time or different particles are drifting at different speeds.

Swap EDR Frame Order in Processing

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



Sample Preparation and Dispersion

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).

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.

1. 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.

2. 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.

3. 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 de-gassed prior to use.

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.

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 $<2 \times 10^9$ 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 around 10-100 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 around $100\mu\text{m}$ is observed.

Dilution

The sample must be diluted to a number concentration of between 10^8 and 10^9 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.

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.

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.

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.

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.

Dispersion and Deagglomeration

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:

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;

- deglomering 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.



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:

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

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 increasing 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 $\sim 10^9$ /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 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 10^8 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 /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 μ l, 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, NTA 2.1 software can compress videos to wmv files using the 'Convert video to WMV' found in the export menu. Please note that these files cannot be processed using the software.

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 NTA2.1 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 5 ul.

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