Direct visualization, sizing and concentration measurement of fluorescently labeled nanoparticles using NTA

NANO**SIGHT RANGE**

Visualize and Measure Nanoparticle Size and Concentration

PARTICLE SIZE

PARTICLE CONCENTRATION

Introduction

Nanoparticle Tracking Analysis (NTA) technology from Malvern can be used to analyze the motion and concentration of particles from the light they scatter. In addition, with appropriate experimental design, NTA may also be used to detect fluorescence which is emitted naturally by particles or as a result of fluorescence labeling or tagging.

This technical note discusses the process of fluorescence detection in NTA and the considerations necessary for a researcher hoping to exploit fluorescence in their research.

Purpose of research

Fluorescence labeling can be utilized in any situation where it is necessary to distinguish a particular subset of particles within a complex background. Some examples where fluorescence labeling may be useful include drug delivery nanoparticles, small biological particles such as extracellular vesicles (exosomes and microvesicles) and virus particles or virus like particles (VLPs). The type of labeling used will be dependent on the type of particle to be labeled and the experimental objectives.

Loading

In the case of drug delivery nanoparticles, it is possible to fluorescently tag or load these particles and distinguish them within a complex medium, such as mucin. The mucin itself is highly complex, containing particles that would be seen in large numbers under light scatter mode, making it impossible to see or track the small drug delivery nanoparticles. By fluorescently tagging or loading them, it is possible to insert a specially selected filter and allow only the longer wavelength emission from the fluorescently labeled particles to be imaged and measured.

Antibody labeling

Particle labeling can be very specific, for example using antibody labeling to specifically target a known marker on a particle of interest within a mixed sample. This type of

labeling is often useful when studying small biological particles such as exosomes or microvesicles.

Other labeling

Labeling may be less specific, i.e. targeting all lipids, proteins, or sugars within a sample, using a dye that has an affinity for one of these. Sometimes the dye is only 'switched on' when it becomes attached to the molecule of interest, as, for example, some lipid membrane dyes.

Choosing an appropriate fluorophore for use with NTA

Criteria for choosing an appropriate fluorophore:

- The excitation maximum of the fluorophore should be close to the wavelength of the laser (excitation source) fitted to the instrument
- The emission maximum of the fluorophore should be longer than the wavelength cut-off point of the filter fitted to the instrument (see Table 1)
- Fluorophores with a large Stokes shift (wavelength between the excitation and emission maxima of the fluorophore) will give better results
- Bright and photostable fluorescence emission

Table 1. Available laser wavelengths and the standard filters.

Available Laser Wavelengths (nm)	Standard Filter Supplied (nm)
Violet 405	430 long pass
Blue 488	500 long pass
Green 532	565 long pass
Red 642	650 long pass

Photobleaching, and how to address the problem

Photobleaching occurs when a fluorophore permanently loses the ability to fluoresce due to photon-induced chemical damage or covalent modification. Photobleaching is not well characterized, but can result in a dramatic loss in the fluorescence emission intensity, and a subsequent reduction in the ability to perform accurate sample analysis with the NanoSight system.

Using a synchronization cable and syringe pump can significantly reduce the problem of photobleaching during sample analysis, and further increase the accuracy of particle concentration measuements and sizing when operating in fluorescence mode.

The synchronization cable, which is fitted to high sensitivity systems, is used to pulse the laser in synchrony with the camera shutter, thus limiting the exposure time of the fluorophore to the illumination source, thereby slowing down the process of photobleaching.

The syringe pump further improves the accuracy of fluorescence measurements by enabling a constant supply of fresh sample to be flow through the sample chamber at a speed faster than the bleach rate of the fluorophore. Malvern recommends the use of the syringe pump for all fluorescence measurements.

Table 2. Shows selected fluorophores that have been used successfully with NanoSight (top) and some which have been less successful due to their fast bleach or dim fluorescence emission (bottom).

Fluorophore	Excitation Max (nm)	Emission Max (nm)	Brightness (1-5)	Overall	Sample Type
Alexa 488	495	519	5	+++	Exosomes, Microvesicles
Spyro Red	550	630	5	+++	General Protein Dye
Rhodamine-PE	560	590	5	+++	Lipids
EGFP	488	509	4	+++	Exosomes, Microvesicles
DiO	484	501	3	++	Lipids
Alexa 546	556	573	3	++	Silica
Alexa 647	650	668	3	++	Exosomes, Microvesicles, Viruses
FITC	490	525	2	+	Exosomes

Fluorophore	Excitation Max (nm)	Emission Max (nm)	Brightness (1-5)	Overall	Sample Type
ICG	790	800	1	-	$Gd0_2$ + Polymer
DiR	750/650	790	1	-	$Gd0_2$ + Polymer
V450	404	488	0	-	Microvesicles
Pacific Blue	401	452	0	-	Exosomes, Liposomes

Some considerations when labeling

To achieve optimum signal to noise ratio, try varying the concentration of fluorescent label to sample particles:

- If using primary and secondary antibodies rather than directly labeling, you will need to try varying the ratios of these too.

Adding large quantities of fluorescent label will not necessarily result in better labeling:

- Unbound label in free solution becomes a significant problem, raising the background intensity and drowning out the signal from labeled particles.
- Sometimes too much fluorescent label can result in quenching of the fluorescent signal.

It is better to label particles at a high concentration and then dilute after labeling (immediately prior to analysis):

- This will enable dilution of the sample down to the optimal concentration range for NTA and may also help to dilute out any unbound fluorescent label that is left in the sample.
- It also increases the likelihood (rate) of interaction between fluorophore and target.
- Try overnight incubation of the sample with fluorescent label.

- A thirty minute staining protocol that works for flow cytometry will not necessarily work for NTA.

Sample preparation

Prior to carrying out a fluorescence measurement it is necessary to dilute the sample to an optimum concentration for NTA. The dilution factor is best worked out by preparing a serial dilution of your sample, for example: 10x, 100x, 1000x, 10,000x. This should first be checked in scatter mode, by loading the most dilute sample into the chamber and then gradually loading in more concentrated sample until you reach the optimum concentration for analysis.

Once an optimum dilution for measurement in scatter mode has been achieved, the sample can be checked in fluorescence mode. If no signal is seen, continue to inject sample at a higher concentration until fluorescent particles become visible. It is not uncommon to run fluorescence measurements at a much higher concentration than scatter measurements.

Preparing to make a fluorescence measurement

Prior to sample analysis:

- For an LM10 system - Check that the synchronization cable is connected from the camera to either the laser (LM12 unit), or to the back of the control box (LM14 unit).
- Check that the appropriate camera settings are selected under the camera tab in the software (sCMOS trigger or EMCCD).

If using the syringe pump, open the syringe pump control, connect the syringe pump and flow the sample at the recommended speed for the instrument (Table 3). All measurements using the syringe pump should be carried out in scatter and fluorescence mode at the same flow speed for comparison of results.

Table 3. Recommended syringe pump flow speeds for each of the NanoSight instruments.

Laser Module Top Plate Style	Recommended Flow Speed
LM10-T14 (LM14)	50-80
LM10 (LM12)	20-50
NS300 "O" ring (metal)	50-80
NS300 flow-cell	20-50
NS500	20-50

Step by step: how to make a fluorescence measurement

1. Identify correct position under scatter mode and then insert the fluorescence filter:

LM10 instrument - insert silver lever on right hand side of microscope optical head to put the fluorescence filter in (Image 1). The filter will be on the right hand side. Where two filters have been fitted, one filter will be on the left hand side, below the filter which switches between the microscope oculars and the camera. Note, there will be a colored dot next to the lever indicating that a filter has been fitted.

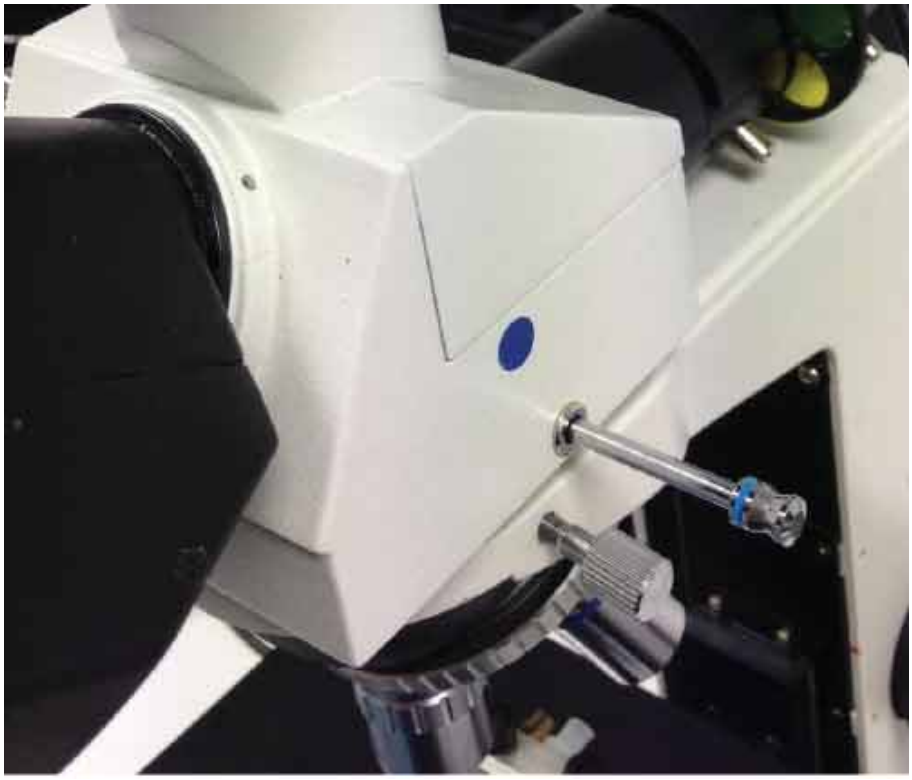

Image 1. LM10 filter position. The fluorescence filter is fitted on the right hand side of the microscope optical head (see red arrow). The colored dot indicates that the filter has been fitted. To insert the fluorescence filter, the silver lever must be pushed in.

NS300 instrument - under the camera menu item, select the relevant filter position for your system (default is filter 2).

NS500 instrument - first check the position of the fluorescence filter in the filter holder, it will be indicated by a colored dot. Either push in or pull out the filter holder until it clicks into position - the direction of movement will depend on the position of the filter in the holder (Image 2).

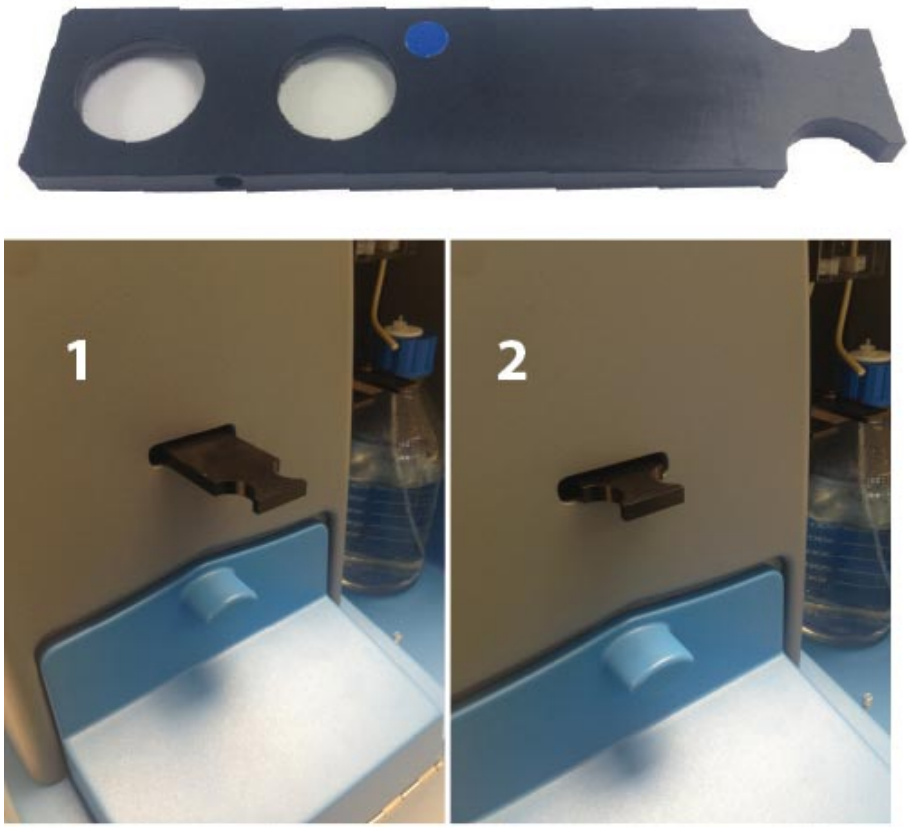

Image 2. (Top) The NS500 filter holder. The colored dot shows the position of the fluorescence filter. (Bottom) Position 1 - the clear glass filter is inserted - this position should be used for light scatter mode. Position 2 - the fluorescence filter is inserted - this position should be used for fluorescence mode. These positions are correct if the fluorescence filter is fitted as shown in the top image.

2. Increase camera level until all particles can be seen - usually to maximum setting.

3. Refocus to see particles:

- LM10 instrument - manually turn fine focus ~1/4 of a turn clockwise
- NS300 and NS500 instruments - click on fluorescence button in the NTA control panel

4. Further adjust the camera level - it is often necessary when working with fluorescent samples that are very dim relative to the image background to use the greyscale histogram beneath the main NTA capture screen, to further increase the brightness of the particles relative to the background (see Image 3)

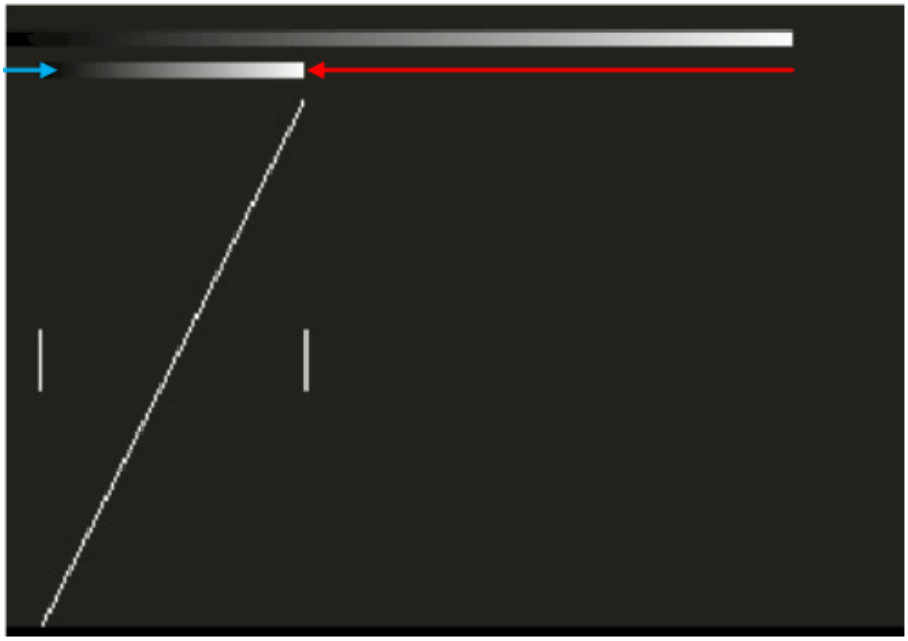

Image 3. Greyscale histogram. The optimal threshold range is achieved by setting the minimum value to a level which allows the dimmest particles to still be visualized: move the grey cursor (blue arrow - left side) into position with the left-hand mouse button. Move the maximum to a level at which the largest particles do not contain many saturated pixels: move the other grey cursor (red arrow - right side) into its maximum position using the right-hand mouse button.

Important controls

Misinterpretation of results is common with fluorescence measurements. To prevent the incorrect interpretation of results it is necessary to run several controls, depending on the labeling method used. All preparation steps should be checked in both scatter and fluorescence mode to enable the user to best understand the results (see Table 4).

Table 4. Directly labeled particles (top) and indirectly labeled particles (bottom). *water, PBS, media etc **should be at same final concentration as in the labeled sample ***If using Qdots, due to their size (~15 nm - 20 nm) it is possible that they will be seen in both light scatter and fluorescence mode, even when not attached to particles. Due to their small size in comparison to sample particles (lower detection limit for NTA), they will not be tracked easily, but may be seen as high background.

Sample	Scatter Mode	Fluorescence Mode
Sample diluent* alone	Check for particles & filter if necessary/possible	Check for fluorescence either as individual particles or a background haze brighter than water alone
Unlabeled sample in diluent	Check particle size and concentration for comparison with labeled sample data	Check that sample does not fluoresce, and that there is no bleed of scatter through the filter (possible with very high intensity particles)
Fluorescent probe in diluent**	Check that there are no large particles/aggregates seen & filter if necessary/possible	Check fluorescence is seen as a flickering background haze, but no large particles seen that could be mistaken for labeled sample particles
Labeled sample in diluent	Check particle size and concentration for comparison with unlabeled sample data and labeled sample fluorescence data	Check particle size and concentration for comparison with scatter

Sample	Scatter Mode	Fluorescence Mode
Sample diluent* alone	Check for particles & filter if necessary/possible	Check for fluorescence either as individual particles or a background haze brighter than water alone
Unlabeled sample in diluent	Check particle size and concentration for comparison with labeled sample data	Check that sample does not fluoresce, and that there is no bleed of scatter through the filter (possible with very high intensity particles)
Primary antibody in diluent**	Check that there are no large particles/aggregates seen & filter if necessary/possible	Check no fluorescence is seen
Secondary antibody in diluent**	Check that there are no large particles/aggregates seen & filter if necessary/possible	Check fluorescence is seen as a flickering background haze, but no large particles that could be mistaken for labeled sample particles***
Primary + secondary Ab in diluent**	Check that there are no large particles/aggregates seen & filter if necessary/possible	Check fluorescence is seen as a flickering background haze, but no large particles that could be mistaken for labeled sample particles***
Sample + primary Ab***	Check particle size and concentration - should be the same as sample alone	Check no fluorescence is seen
Labeled sample in diluent	Check particle size and concentration for comparison with unlabeled sample data and labeled sample fluorescence data	Check particle size and concentration for comparison with scatter

Troubleshooting

If a fluorescent signal is seen in diluent (this can be seen with serum in some laser wavelengths), or any other part of the sample preparation where you would not expect to see fluorescence:

- It may be possible to use a custom band-pass filter that will cut off the unwanted fluorescent emission and only allow through the emission from the fluorescent label.
- Alternatively use another laser wavelength which does not cause auto-fluorescence in the sample preparation, and change the fluorophore to suit.

If large particles are seen in the fluorescent probe or secondary antibody when the fluorescence filter is inserted:

- Filter the fluorescent probe/antibody solution with 20 nm filter prior to labeling.

If a high fluorescence background is seen in a labeled sample preparation, but no labeled particles are seen:

- Vary the concentration of fluorescent probe to sample until signal to noise ratio is reduced and particles can be seen - labeling may have been successful but particles are not visible against high background of unbound fluorophore.

Summary

- Choose a method of labeling that is suited to the particles being studied.
- Choose an appropriate fluorophore for use with NTA:
    - It is very important to match the excitation and emission characteristics of the fluorophore to the NanoSight instrument that is being used.
    - Bright and photostable fluorophores work best.
- Use the synchronization cable and syringe pump to address the problem of fluorophore photobleaching.
- Optimize the labeling procedure until the best signal to noise ratio is achieved:
    - Vary the ratio of fluorescent probe/antibody to sample.
    - Try incubating the sample with fluorescent probe for longer.
    - Label the sample at high concentration and dilute after incubation.
- Dilute the sample to an optimum concentration for NTA in scatter mode before trying to analyze in fluorescence mode.
    - If necessary, increase the concentration of sample for fluorescence analysis.
- Increase the camera level in fluorescence mode and adjust focus to see fluorescent particles clearly.
- If using the syringe pump, carry out both scatter and fluorescence measurements under the same flow rate.
- Ensure all necessary controls are analyzed to prevent misinterpretation of results and aid troubleshooting.

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