NANOMETROLOGY

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Summary

Methods and protocols are described when using fluorescence metrology to determine the average nanoparticle (np) size in colloids in the range of 1-10 nm. The technique is based on determining the rotational correlation time of the np from the decay of fluorescence anisotropy of a dye that is electrostatically or covalently attached to the np as it undergoes Brownian rotation. The np size is then calculated from the Stokes-Einstein equation. The exemplar of silica nps is presented, but the approach can also be applied to other types of nps.

Key words: Fluorescence anisotropy decay, fluorescence lifetime, nanoparticle, silica, nanometrology, LUDOX®, sol-gel, TCSPC

1. Introduction

Measurement science on the nanoscale (nanometrology) is widely seen as a key to progress in the many burgeoning applications of nanotechnology. These applications span not only science and technology, but also engineering and medicine. Global interest in the metrology of nanoparticles (nps) in particular

has undergone quite a renaissance in recent times. This is because of the development of novel manifestations of nps such as carbon nanotubes, noble metal, diamond and semiconductor quantum dots. Irrespective of physical and chemical differences between nps there is a certain common concern in respect of the potential toxicological and environmental issues surrounding not only new types of nps, but also older structures. Carbon nps from combustion and metal oxide nps such as silica and titania are some of the latter that immediately spring to mind. As a paradigm here we consider only silica. Also, we confine ourselves to the 1-10 nm np range as this is where there is a particularly urgent need for globally accepted standards and precise measurement techniques. Such small nps are known to traverse cellular membranes with ease, are at the limit of present-day measurement techniques and yet are most appropriate for study in a colloid using fluorescence nanometrology. Traditional methods for nanoparticle metrology, such as small angle x-ray and neutron scattering, are much more expensive, complex and difficult to use when compared to fluorescence nanometrology, as we will describe here.

Fluorescence nanometrology can be defined as the use of fluorescence to determine nano scale lengths. When it comes to tracking rapidly changing phenomena involving nps on the scale of super-molecular dimensions, fluorescence decay time techniques have few equals *(1)*. This is because they can be performed in-situ, have an appropriate time-scale (ps to ns), offer high sensitivity (down to the single-molecule, single-photon level) and benefit from the wide-range of well-characterized fluorescence probes with which to customize a study. The associated equipment is also now extremely easy to use, reliable and relatively inexpensive. However, the interpretation of data still benefits from skill and experience. Here we describe the use of the decay of fluorescence anisotropy to determine np size.

The measurement approach is depicted in **Figure 1** for the case of dye molecules partitioned between dual Brownian motions relating to when bound to a np and when rotating freely.

The rotational correlation time of the free dye of known radius gives the local viscosity of the solution using the Stokes-Einstein equation. When the viscosity is combined in the same equation with the

rotational correlation time of the dye bound to nps this leads to determination of the np radius. Both stable *(2)* and changing *(3)* np sizes can in principle be measured (*see* **Note 1**).

2. Materials

Chemical Preparation

As fluorescence is an extremely sensitive technique we stress that all glassware must be sufficiently cleaned before use (*see* **Note 2** and **Note 3**). Prepare and store all reagents at room temperature (unless indicated otherwise). Use distilled water (unless indicated otherwise). Cap all chemicals and create an additional seal with parafilm (unless indicated otherwise). Label each chemical/sample being used (*see* **Note 4**) and preferably store in the dark. Diligently follow all waste disposal regulations when disposing of waste chemicals.

2.1 Stöber synthesis of silica nanoparticles

The Stöber process is the ammonia-catalysed reaction of an orthosilicate e.g. tetraethylorthosilicate (TEOS: $Si(OR)_4$; $R = C_2H_5$) with water in low-molecular-weight alcohols to produce monodispersed, spherical silica nanoparticles (*see* **Figure 2**). This method, as specified by Green et. al. *(4)*, produces a narrow size distribution of spherical particles as in the case of LUDOX®.

1. 10 ml sols will be produced using a conical flask.
2. The solutions required for their synthesis are 33 % ammonia in water solution (*see* **Note 5**), water, tetraethyl orthosilicate (TEOS) and methanol. (*see* **Note 6**).
3. We recommend making a tertiary diagram like our **Figure 3**. From this calculate the ratio of ammonia, water and TEOS for the desired sample. For accuracy we recommend calculating the mass of each component you will require for your sample (*see* **Note 7**).

4. Make a water-ammonia solution of desired concentration. This solution should be made in a separate glass container which is placed on a balance and zeroed. The water should be first added by using a pipette. The 33 % ammonia solution should be quickly added to the water using a Pasteur pipette. The container should be immediately capped and shaken to ensure that it has completely mixed (*see* **Note 8**).
5. Place the 10 ml conical flask on the balance and zero it. Add the desired volume of TEOS using a Pasteur pipette.
6. Fill the conical flask up to the half-way mark with methanol and re-zero it again (*see* **Note 9**).
7. Next add the water-ammonia solution and fill up to the 10 ml mark using methanol.
8. Add a 5 mm stirring bar and cap (*see* **Note 10**).
9. Place the conical flask on a magnetic stirrer for 24 hours at a gentle setting.
10. Decant the solution into a glass container and store at room temperature (*see* **Note 11**).

2.2 Fluorescent dye preparation

Like all fluorescence measurements an appropriate dye must be selected. The dye must meet two criteria, first it must attach to the np under investigation and second have appropriate photophysical properties (e.g. fluorescence decay) for tracking the np rotation. For anisotropy measurements of nanoparticles, the dye may attach to the system electrostatically or covalently. Before working with a new dye it is important to record the steady state absorption and emission spectra so one has a basic understanding of the photophysical properties of the dye. We will take Rhodamine 6G as an example here.

1. Prepare a stock solution of dye by dissolving the dye in methanol or some other common solvent at a concentration of ~10-50 µM (*see* **Note 12, Note 13**, **Note 14** and **Note 15**).
2. Pipette 3 ml of solvent into a cuvette. Pipette 50 µl of stock solution (*see* **Note 16, Note 17** and **Note 18**).
3. Record a steady state absorption spectrum like that of **Figure 4** using a spectrophotometer. In our case we used a Jasco V660 spectrophotometer. A second identical, and preferably matched, cuvette filled with only the pure solvent should be used in the reference channel.

4. From the absorption spectra you can verify the dye concentration in the cuvette using the Beer-Lambert law **eqn. {1}** (*see* **Note 19**)

$$A = \log\left(\frac{I_0}{I}\right) = \varepsilon c l \qquad \{1\}$$

where A is the absorbance, I_0 and I are the intensity of the incident and transmitted light respectively, c is the concentration in $\mathrm{Mol\ l^{-1}}$, l is the path length in cm and ε is the molar extinction coefficient in $\mathrm{Mol^{-1}\ l\ cm^{-1}}$.

5. From the concentration in the cuvette and the dilution factor, you can calculate the concentration of the stock solution (*see* **Note 20** and **Note 21**).
6. From the absorption spectra, select a suitable pulsed light source for excitation. This should have a peak wavelength in a region where the dye has a high absorbance. In our case we selected an IBH 503 nm DeltaDiode.
7. Record a steady state emission spectrum using a spectrofluorometer and select the excitation wavelength to match that of your selected light source (*see* **Note 22**). In our case we used a HORIBA Fluorolog 3 spectrophotometer.
8. Normalise and overlay both absorption and emission spectra as our **Figure 5**.
9. Measure the Stokes shift and select an appropriate long pass filter or monochromator wavelength for later use with lifetime measurements (*see* **Note 23**).

2.2.1 Electrostatic labelling

Due to electrostatic interactions cationic dyes will readily bind to negatively charged silica colloids in alkaline solution (*see* **Note 24**) and vice-versa.

1. Measure the pH of the colloid.
2. Prepare an oppositely charged dye that will affix to the np by electrostatic interaction.
3. In this case Rhodamine 6G as prepared above will be used as an example.

4. Add a small quantity of the dye to the nanoparticles using a pipette, mix the solutions using the pipette and place a lid on the cuvette (again *see* **Note 16, Note 17, Note 18** and **Note 19**)
5. Measure the absorbance spectra to have a peak at ~0.1 (again *see* **Note 20**).
6. Prepare a similar dye that does not bind to the np, for example Rhodamine B, in exactly the same manner as Rhodamine 6G.
7. A dye such as Rhodamine B that does not bind to the np can be used to determine the microviscosity. We recommend preparing both dyes for a comparison. *See* **Figure 6** for the chemical structures of both dyes.

2.2.2 Covalent labelling

For covalent labelling a dye needs to be selected with not only appropriate properties as listed previously, but must also possess chemical linkers for binding to the np *(5)*. In this example Fluorescein 5(6)-isothiocyanate (FITC) will be bound to 3- Aminopropyl trimethoxysilane (APS). Such a labelling approach has previously been used with silica nanoparticles *(6,7,8)*. *See* **Figure 7** for chemical structures and the reaction mechanism.

1. Dry a glass vial under nitrogen (*see* **Note 25**).
2. Measure out 110 mg of FITC and add to this to a 10 ml glass vial (*see* **Note 14**).
3. Add 10 ml of anhydrous ethanol to the glass vial and a magnetic stirrer (*see* **Note 25**).
4. Seal and stir at room temperature at a high speed for 30 minutes.
5. Add 50 µl of APS (*see* **Note 26**).
6. Dry the top of the vial with Nitrogen.
7. Seal and stir at a high speed at room temperature for ~24 – 72 hours.

2.3 Adding the dye to the nanoparticle

1. Pipette 3 ml of the nps into a glass or quartz cuvette (*see* **Note 17** and **Note 18**).

2. Add a small quantity of your covalent dye or electrostatic dye (*see* **Note 16** and **Note 19**).
3. Measure the absorbance spectrum, aim for a peak at ~0.1 (*see* **Note 20**).
4. Measure the emission spectra, excited by the appropriate wavelength from the light source.

2.4 Preparation of a scattering colloid for recording the excitation pulse.

1. Add 17 ml of water to a 30 ml glass vial.
2. Add 2 ml of 0.1 M sodium hydroxide.
3. Add 1 ml of LUDOX SM30.
4. Pipette 3 ml of your scatter colloid into a plastic, glass or quartz cuvette (*see* **Note 17, Note 18** and **Note 27**).
5. The scatter colloid is quite stable if left sealed but we recommend replacing it every month (*see* **Note 28**).

3. Methods

Decay Time Techniques

3.1 Acquisition of data

It is generally accepted that anisotropy decay time measurements are best performed in the time domain using time-correlated single photon counting techniques (TCSPC) *(9,10)*. A schematic of such a fluorometer is shown in **Figure 8**. In our case we have used a FluoroCube from Horiba Jobin Yvon IBH Ltd, Glasgow.

3.1.1 Fluorescence decay time measurement

The fluorescence lifetime is the average time a fluorescent dye remains in an excited state before emission of a photon (*see* **Note 29** and **Note 30**).

1. Choose an excitation source with a compatible wavelength for exciting the dye (*see* earlier notes about absorption spectra). The most appropriate light sources are pulsed laser diodes sources or pulsed light-emitting diodes (LEDs) *(11-13)* which operate ~ 1-10 MHz repetition rate (*see* **Note 31**)
2. Choose a suitable detector with a compatible wavelength response and single-photon timing sensitivity for your sample. In our case a compact photomultiplier in a T0-8 package (Hamamatsu Model R7400) (*see* **Note 32**).
3. Ensure the light source and the detector are at right angles to each other in L-format orientation (*see* **Figure 8** and **Note 33**).
4. Choose a time to amplitude range which suitably includes all the fluorescence decay of the dye as in (*see* **Note 34** and **Note 35**). A logarithmic view helps to achieve this.
5. Use a scattering colloid to simulate the optical geometry of the sample (*see* **Note 28**).
6. Set the excitation monochromator to the required excitation wavelength (*see* **Note 36** and **Figure 9**).
7. Set the emission monochromator to the excitation wavelength (*see* **Note 37** and **Figure 9**).
8. Set the excitation polarizer to the vertical position (*see* **Note 38**).
9. Set the emission polarizer to the vertical position.
10. Change the delay lines as appropriate (*see* **Note 39** and **Figure 10**) to find a suitable time range for measurement.
11. Add a neutral density filter if necessary before the sample (on the excitation arm) or iris diaphragm to ensure that the photon count rate is kept ≤ 2 % of the source repetition rate (*see* **Note 40** and **Figure 8**).
12. Acquire the instrumental pulse, typically to 10^4 counts in the peak at a channel width of ~ 100 ps.
13. Replace the scattering colloid with the colloid which contains the labelled nps of interest and tune the emission monochromator to the peak wavelength of fluorescence (*see* **Note 42**). Given

the different optical densities of the scattering colloid and the fluorescent sample it is usually necessary to adjust the focusing of the collection optics in order not to waste fluorescence counts (*see* **Note 42**).

14. Set the emission polarizer to the magic angle (54.7º) (*see* **Note 43**).
15. Acquire the fluorescence decay typically to 10^4 counts in the peak at a channel width of ~ 100 ps (*see* **Note 44**).
16. Fit to the fluorescence decay to obtain the fluorescence decay times using reconvolution analysis in order to correct for the finite duration of the excitation pulse.
17. Figure 11 illustrates the principle of reconvolution whereby each time channel of the instrumental pulse (i.e. the measured excitation pulse) is treated as a δ function and the sum of the fluorescence impulse responses is then fitted to the measured fluorescence decay using non-linear least squares (NLLS). In NLLS analysis the sum of the squares of the weighted residuals are minimized and the fit to the data assessed using statistical criteria. In the first instance these are usually the trend in the residuals and the reduced chi-squared (χ^2) (*see* **Note 45**).

3.1.2 Anisotropy decay time measurements

1. Your experimental apparatus should be set up exactly in the same manner as for the lifetime measurement.
2. Insert the scattering colloid and record a prompt as instructed for the lifetime measurement
3. Replace the scattering sample with the colloid which contains the labelled nps of interest and again tune the emission monochromator to the peak wavelength of fluorescence.
4. Set the excitation polarizer to the vertical position (*see*
5. Figure 11).
6. Record the polarized fluorescence decay curves by alternating the emission polarizer between the vertical and horizontal positions (e.g. every 30 s) using automated polarizers (*see* **Note 46**).
7. The key statistic to take under consideration is the peak number of counts in the difference between these curves which may vary over 10^3-10^5 counts depending on the precision required.

8. Record the relative optical throughput of the vertical and horizontal emission polarizations, the so called G-factor.
9. To do this place the excitation polarizer in the horizontal orientation and record the decay curves for both vertical and horizontal emission polarizer orientations. 10 cycles of 30 s is usually sufficient (*see* **Note 31** and **Note 47**).
10. When taking anisotropy measurements again ensure that the photon count rate is kept ≤ 2 % of the source repetition rate, use neutral density filters on the excitation arm as required (*see* **Note 40** and **Figure 8**).
11. Data analysis is performed using non-linear least squares (NLLS) iterative reconvolution using a library of fluorescence and anisotropy decay functions. In our case IBH DAS 6.6 software.
12. First fit to the fluorescence decay $I_{VV}(t)+2GI_{VH}(t)$ i.e. the denominator of **eqn. {2}** as outlined in 3.1.1 (*see* **Note 47**).
13. Next the difference curve $I_{VV}(t)-GI_{VH}(t)$ i.e. the numerator in **eqn. {2}** is fitted using different models for the anisotropy decay $R(t)$, in order to obtain the best fitting rotational parameters. Selection of the most appropriate model is aided using the χ^2 goodness of fit criterion.
14. The anisotropy decay curves are recorded for typically up to a few hours as necessary to acquire 10,000 counts in the peak, at 28 ps per channel *(5)* (*see* **Note 48**). The errors are best quoted as 3 standard deviations.

3.2 Analysis of data

By recording vertically and horizontally polarised fluorescence decay curves, $I_{VV}(t)$ and $I_{VH}(t)$, orthogonal to vertically polarised excitation, generate an anisotropy function $r(t)$ *(14)* i.e.

$$r(t)=\frac{I_{VV}(t)-GI_{VH}(t)}{I_{VV}(t)+2GI_{VH}(t)} \qquad \{2\}$$

Where $G = I_{HV}(t)/I_{HH}(t)$ is determined for horizontal orientation of the excitation polarizer (*see* **Note 49** and **Note 50**).

In order to analyse **eqn. {2}** first fit the denominator, which is in fact the fluorescence decay, to the minimum number of decay components needed using NLLS reconvolution of the instrumental pulse *(9,10)* with a chi-sq (χ^2) goodness of fit criterion i.e. as a rule of thumb $\chi^2 < 1.2$ for a good enough fit (*see* **Note 51**). This yields the best fit fluorescence decay parameters τ_{f1}, τ_{f2}, τ_{f3}, etc. associated with the fluorescence impulse response had we been using a δ function for excitation.

Again using NLLS reconvolution of the instrumental pulse fit the product $r(t)\times\left[I_{VV}(t)+2GI_{VH}(t)\right]$ to the difference data $I_{VV}(t)-GI_{VH}(t)$ where $I_{VV}(t)+2GI_{VH}(t)$ is now described by the fluorescence impulse response parameters and $r(t)$ describes the rotational kinetics in terms of the minimum number of rotational correlation times needed to describe the difference data.

The simplest case occurs for a spherical rigid rotor in an isotropic medium such as a solvent *(14)*. If all the dye is rigidly attached to the np, the decay of $r(t)$ describes fluorescence depolarization due to Brownian rotation of the np according to:

$$r(t) = r(0)\exp\left(-\frac{t}{\phi_p}\right) \qquad \{3\}$$

where $r(0)$ is the initial anisotropy with a maximum value of 0.4 for one photon excitation (*see* **Note 52**) In this case ϕ_p is the np rotational correlation time described by the Stokes-Einstein equation:

$$\phi_p = \frac{\eta V}{kT} \qquad \{4\}$$

where η is the microviscosity, V the hydrodynamic volume = $4\pi r^3/3$ prescribed by the np, T the temperature and k the Boltzmann constant.

The radius (**Note 53**) of the particle is then given by:

$$R_p = \left(\frac{3kT\phi_p}{4\pi\eta} \right)^{1/3} \quad \{5\}$$

In the case where the dye partitions between being bound to the np and freely rotating we have:

$$r(t) = (1-f)r(0)\exp\left(-\frac{t}{\phi_d}\right) + fr(0)\exp\left(-\frac{t}{\phi_p}\right) \quad \{6\}$$

where *f* is interpreted as the fraction of fluorescence due to dye bound silica nps rotating with a correlation time ϕ_p and $1-f$ to dye molecules unbound in the colloid and rotating faster with a correlation time ϕ_d (3). Substitution of ϕ_d and the dye hydrodynamic radius in **eqn. {4}** yields the microviscosity η. This, when combined with ϕ_p, gives the particle radius again through use of **eqn. {4}** and **eqn. {5}** (*see* **Note 54**).

If there are probe molecules also bound to aggregates of the "fundamental nanoparticles" and of similar composition, but a much larger size, then we can similarly approximate this by:

$$r(t) = (1-f-g)r(0)\exp\left(-\frac{t}{\phi_d}\right) + fr(0)\exp\left(-\frac{t}{\phi_p}\right) + gr(0) \quad \{7\}$$

where $gr(0) = r(\infty)$ can also effectively describe a very slow rotation, i.e. *g* is the fraction of fluorescence derived from dye attached to the aggregate and $1-f$ describes free dye or bound dye wobbling on a nanoparticle or a compromise between the two. When dealing with more complex kinetics (*see* **Note 55**, **Note 56** and **Note 57**).

3.3 Typical results

Table 1 shows typical np measurements using electrostatic and covalent labelling for silica nps.

4. Notes

Note 1. The fluorescence lifetime of the dye τ_f should be; (i) mono-exponential as this simplifies the decay analysis, although since the dye fluorescence tracks the rotation irrespective of the decay profile this is not too critical; (ii) of a value with respect to the np rotational correlation time ϕ_p given by $\phi_p/10 \leq \tau_f \sim \phi_p$; (iii) stable during the course of the measurement. In fact ϕ_p / τ_f ratios up to 50 have been used successfully *(2)*.

Note 2. Fluorescence is an extremely sensitive technique and all glassware should be cleaned thoroughly. Our cleaning procedure involves rinsing all glassware out with hot tap water and scrubbing with a bristled brush. All glassware is then steeped overnight in a 5 l polypropylene beaker containing 3 % DECON 90 solution. After the glassware has steeped it is then rinsed out once again with hot water (ensuring all detergent bubbles are removed). Finally the glassware is rinsed with a wash-flask of cleaning alcohol (methanol or ethanol) and a wash-flask of distilled water and allowed to dry.

Note 3. For cuvettes (and smaller items) a similar cleaning procedure is used. To scrub the inside and outside of cuvettes cotton buds are used (pick cotton buds which don't fragment heavily when wet). The cuvettes are rinsed with a wash-flask of water and steeped overnight in a 100 ml polypropylene beaker containing 3 % DECON 90 solution. Polypropylene beakers are preferentially used over glass beakers to prevent glass to glass scratching. Cuvettes are then rinsed as with a wash-flask of cleaning alcohol (methanol or ethanol) and a wash-flask of distilled water (again ensuring all detergent bubbles are removed). Cuvettes are generally dried using nitrogen gas.

Note 4. Marker pens may be used to scribble on temporary glassware but for glassware kept in use for more than one day it is recommended to use a label printer. If a splash of alcohol runs on the outside of a label with a marker pen the ink will run and the label will become illegible. Brother P-Touch labels are chemical resistant and don't present such a problem.

Note 5. Ammonia at 33 % concentration, tends to bubble away rapidly, we recommend checking the concentration of ammonia by titration before nanoparticle synthesis.

Note 6. Other low molecular weight alcohols such as ethanol and propanol may be used as solvents, in addition other orthosilicates may be used. The nanoparticle sizes will be different than those produced with methanol, water, ammonia and TEOS *(15)*.

Note 7. When calculating the ammonia and water content recall that the ammonia solution is 33 % ammonia and 67 % water.

Note 8. 33 % ammonia solution is very volatile; it is extremely difficult to measure small amounts. To achieve greater accuracy we upscale it so the water-ammonia solution is ~30 ml.

Note 9. Filling the container up to the halfway mark before addition of the water-ammonia solution prevents an uncontrolled initial reaction from taking place.

Note 10. Ensure that the stirring bar is added after the conical flask is filled up to the 10 ml mark.

Note 11. We have found the Stöber silica nanoparticles produced via this method to be very stable and have observed no changes in them for up to a month when sealed and left at room temperature. Samples left for over a year were found to form gels.

Note 12. Fluorescent dyes are generally shipped in powder form; you will need to dissolve some dye in solvent to make a stock solution. Skip this step if you purchased the dye as a solution.

Note 13. Since there is usually only a small quantity of fluorescent dye used per measurement (~50 µl of dye stock solution), it is not necessary to make huge volumes. In general we make the stock solution in 3 ml volumes.

Note 14. We recommend using a 4 ml amber-glass container for storing and making dye stock solutions as fluorescent dyes can be extremely photosensitive. If you don't have amber glass vials, wrap each vial up with dye in aluminium foil.

Note 15. Some dyes will not readily dissolve in solvent and we recommended trying to sonicate these at ~37 Hz for 30 minutes at room temperature to induce them to dissolve. It should be noted that some dyes will be insoluble in particular solvents and hence a range of solvents needs to be available for making the stock solution.

Note 16. Pipette liquid up and down to mix the dye with the sample. To ensure thorough mixing, cap the cuvette and gently tip the cuvette up and down a few times. The cuvettes should remain sealed as repeated exposure to atmospheric CO_2 has the effect of lowering the pH, eventually resulting in auto-gelation of silica sol.

Note 17. Plastic cuvettes may not be suitable for methanol or ethanol solvents as they will become dissolved by such solvents if left exposed for a prolonged time. Plastic cuvettes may also have strain induced birefringence that will affect the polarization.

Note 18. When taking absorption spectra, be wary of the wavelength range your cuvette can transmit. Glass and plastic cuvettes for example will transmit at ~350 – 800 nm while quartz ones will work from 200 – 800 nm. In addition some solvents will transmit at certain wavelength ranges. The baseline measurement is a transmission measurement and from this you can directly observe the range your cuvette and cuvette/solvent will work at. Although in general you will be given the specification of all the optical components (e.g. neutral density filters, long pass filters and cuvettes) that you order, it is worthwhile verifying them experimentally. Absorption measurements recorded outwith the working transmission ranges are meaningless.

Note 19. For the majority of dyes the molar extinction coefficient ε can be found in their supplied datasheet in addition to normalised spectra.

Note 20. We often just use the absorption spectra to verify the dye concentration as this is often easier than attempting to measure out minute quantities of dye powder.

Note 21. We generally calculate the volume of dye that has to be added to 3 ml of solvent to generate an absorbance of 0.1 and label the vial accordingly. An absorption of ~0.1 is sufficiently strong enough to generate a good fluorescence signal but not strong enough for dye-dye interactions such as self-quenching to become a major issue.

Note 22. Record the steady state emission spectra just after the excitation wavelength; if you have narrow slits in your spectrofluorometer, then you may begin 1-2 nm after your excitation wavelength. If however you have broader slits then you should start 5-10 nm after the excitation pulse. Otherwise you will observe a peak from your excitation wavelength which will be much

larger in magnitude than your fluorescence emission. There is no point in measuring the wavelengths before the excitation wavelength.

Note 23. A suitable wavelength is one where there is strong emission and weak self-absorption.

Note 24. An example of those already demonstrated would be negatively charged colloidal silica nps such as those in Dupont LUDOX® *(16)* at pH 10 and a cationic range of methoxyquinolinium dyes or acidic hydrogels and certain xanthene dyes such as rhodamines can be bound to both anionic and cationic nps and provide free dye for determining the microviscosity.

Note 25. APS is very reactive with water and once it reacts with water it will lose its ability to bind to the np. The mechanism is the same as the Stöber reaction for np preparation. APS does not readily dissolve in methanol or ethanol which is why the reaction needs to be left mixing for such a long period of time.

Note 26. A 1 mol FITC – 1 mol APS ratio should be made. In practice we find lumps of APS at the bottom, leaving a significant proportion of free dye (~40 % bound and ~60 % free). To bind all the dye we have attempted to add APS in 10 molar excess however in this case adding the dye to the nps becomes similar to adding more TEOS and results in the formation of silica flakes. These can be removed from the sample via centrifuge however the absorption peak becomes much weaker going from ~ 0.1 to ~0.012. Similar anisotropy results were found via both methods however it should be noted that there is a trade-off between optimizing the synthesis of FITC-APS and disturbing the np by adding too much APS. Ideally the reaction to create FITC-APS should be carried out with a huge excess of APS, later a high molecular weight molecule should be added in even larger excess and with appropriate chemistry to bind to the remaining free APS in order to isolate the FITC-APS by centrifuge.

Note 27. We recommend you to match the cuvette type with that used for the sample.

Note 28. LUDOX SM30 *(16)* when purchased is generally too concentrated to use directly as a light scattering colloid. It will aggregate under acidic conditions and the sodium hydroxide is necessary to ensure the pH is kept between 10 and 12 when diluting. Other colloids can also be used e.g. when exciting at visible wavelengths, diluted milk may be used. Chalk is also commonly used right down to the UV measurements.

Note 29. The term “lifetime” is strictly only applicable to a one-component fluorescence decay and the term “decay times” rather than “lifetimes” is better when describing multi-component behaviour.

Note 30. The decay time measurement is an inherent part of the anisotropy measurement described in section 3.1.2 but the separate description given here serves to check out the instrument in a simpler mode of operation before proceeding to the np measurement.

Note 31. Note most suitable pulsed light sources display some level of polarization.

Note 32. Avalanche photodiodes with single photon timing capabilities are an alternative to photomultipliers but they have a stronger temporal dependence on the wavelength which can adversely affect reconvolution analysis, and less active area of response than a photomultiplier.

Note 33. L format orientation reduces the transmission of scattered excitation light by the emission arm.

Note 34. In order to optimize data collection, it is often necessary to alter the range of the time to amplitude convertor (TAC). The TAC range should be altered to closely match the lifetime of the fluorophore; if the TAC range is much smaller than the lifetime of the fluorophore under investigation then much of the fluorescence decay will be missed, if on the other hand one has a much larger TAC time than the dyes lifetime, the resolution will be significantly reduced (*see* Figure 10).

Note 35. Ensure that the light source repetition rate isn’t too high for the TAC range being used otherwise the dye will be excited twice in the measurement window and a second lifetime decay may overlap with the end of the first.

Note 36. LEDs emit light over a broader spectrum in comparison to laser diodes and can sometimes generate weaker emissions at longer wavelengths than their fundamental wavelength band *(11-13).* For these reasons the excitation was selected using an optical monochromator (in our case a Seya-Namioka geometry).

Note 37. In the case where a long pass filter is being used to separate the fluorescence emission from the scattered light, insert no emission filter during the measurement of the excitation pulse.

Note 38. Both dichroic and prism optical polarizers can be used successfully to select polarization however dichroic polarizers are unsuitable for measurements in the UV.

Note 39. The time delay at the stop signal should be varied, so that the prompt (excitation pulse) is measured near the start of the TAC. This can be done by running a prompt measurement with a scatter sample and altering the delay lines accordingly. Care should be taken to ensure that the prompt is not at the very edge due to non-linear effects which occur at the edge of the TAC range (*see* **Figure 10**). Linearity can be checked using uncorrelated pulses e.g. photomultiplier noise.

Note 40. In all the measurements keep the photon count rate to ≤ 2 % of the source repetition rate to eliminate data pile-up *(9,10).* A neutral density filter or iris diaphragm may be inserted before the sample to ensure that this requirement is met. Use the pulsed source to generate "stop" signals and the detector to generate "start" signals in order to minimize dead time. This is known as running the TAC in reverse mode. Although the measurement is ran in reverse mode, the data can be reversed on the computer and later analyzed in the conventional way.

Note 41. Alternatively use a long pass filter on the emission arm. The choice between optical monochromator and filter is determined by the count rate available, the monochromator always being preferable if a fluorescence count rate of ~ 2% can be achieved. To reduce the detection of scattered excitation light both components are sometimes used.

Note 42. Motor driven lenses are preferable to manual adjustment as they enable the excitation and emission focusing to be automatically computer-optimised in order to maximise the data collection rates for samples of differing optical density.

Note 43. Fluorescence lifetimes are measured at the magic angle (54.7 º) to eliminate polarization bias in the instrument *(9,10)* e.g. due to the diffraction grating response in the monochromator.

Note 44. We would recommend testing your kit with a few reference samples. e.g. fluorescein in methanol, rhodamine 6G in water. The lifetimes of these (and of other common dyes) are well covered in the literature. Compare your results to the literature values to verify your electronics (linearity and calibration) are working correctly. Time calibration can be checked using a crystal oscillator to generate start and stop signals or using calibrated delay lines for the same signal

input to the start and stop channels. A series of calibration points of known time difference can then fitted to using least squares analysis.

Note 45. Non-Linear Least Squares (NLLS) is an iterative process with the fluorescence impulse response adjusted until a good fit to the data is obtained. A good fit is taken to be when the reduced χ^2 is ~ 1.0 (reduced means χ^2 divided by the number of channels of data minus the number of fitted parameters). Because the data is statistically imperfect due to the effects of systematic errors (such as scattered excitation light) in practice this figure is often taken to be ≤ 1.2. In the first instance iteration usually means increasing the number of decay components. However, care should be taken not to over-parameterise as this can give an anomalously low χ^2 value i.e. use the minimum number of decay parameters and do not increase their number when doing so produces little change in the χ^2. An extra fitted parameter, which needs to be included in reconvolution analysis, is that of a time shift. This corrects for the quantization of the time channels and provides a 1st order approximation to wavelength dependent timing response in the photomultiplier. The latter arises from the effect on the photoelectric effect caused by differences in wavelength between excitation and fluorescence.

Note 46. Continuous alternation of the emission polarizers between horizontal and vertical orientation will correct for any drift in light intensity. Motorised control is recommended for optimal accuracy and ease. If motor control is however unavailable manual optimization of optical throughput can be used.

Note 47. This procedure can be used in place of the fluorescence decay time measurement described in section 3.1.1.

Note 48. The measurements in our early work *(2)* were taken using the IBH NanoLED which had a repetition rate of 1 MHz. Measurements with the newer more intense IBH DeltaDiodes have a repetition rate of 10s MHz, significantly reducing measurement acquisition time.

Note 49. *G* is just a number and the decay curves $I_{HV}(t)$ and $I_{HH}(t)$ can simply be integrated to obtain numbers of counts to calculate their ratio.

Note 50. $I_{VH}(t)$ denotes the excitation polarizer to be orientated in the vertical (V) direction and the emission polarizer to orientated in the horizontal (H) direction.

Note 51. As a rule of thumb $\chi^2 < 1.2$ for a good enough fit. It is essential to obtain a good fit to the fluorescence decay as any errors here will translate into the anisotropy determination.

Note 52. Higher $r(0)$ values and hence higher dynamic range can be achieved using two photon excitation *(17)*, though at a significant increase in measurement complexity.

Note 53. The cube root reduces the effect of errors in ϕ_p and η on R_p.

Note 54. A different and non-binding dye can also be added to determine the microviscosity *(18)*, *see* section 2.2.1.

Note 55. If $\phi_p >> \phi_d$ then the second term in **eqn. {6}** becomes a residual anisotropy and ϕ_p is immeasurable. If the dye is not free but bound and wobbling on the np this can also be approximated by **eqn. {6}** or if both free and wobbling on the np then $1-f$ and ϕ_d describes an average of free and wobbling dye and the effect of the associated different viscosities. It should be noted that only if the fluorescence quantum yield of the probe is the same when unbound and bound to silica particles, does the fraction f exactly describe the probe partition ratio between the nanoparticle and fluid. Also, τ_f for unbound and bound probe needs to be similar for **eqn. {6}** and **eqn. {7}** to be valid. Moreover a single rotational correlation time description is only strictly valid for a spherical rotor of fixed radius of rotation. Hence in the case of a non-spherical or a distribution of nanoparticle sizes the analysis becomes more complicated and the above treatment becomes more approximate *(17)*.

Note 56. The determination of size distributions is generally not possible as such samples don't yield unique solutions to reconvolution analysis.

Note 57. The presence of other depolarization mechanisms can be sources of error e.g. diffusion of the dye on the surface of the np, homo fluorescence resonance energy transfer between dye molecules. These can however be corrected for to an extent by incorporation of additional terms in **eqn. {6}**.

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6. Figures and Tables

Figure 1 Depicts a dye rotating free (ϕ_d ~ 100 ps) and rotating bound to a nanoparticle (ϕ_p ~ 10-100 ns) in a colloid.

Figure 2 Schematic of the Stöber synthesis of nps.

Figure 3 Ternary diagram of silica colloid compositions.

Figure 4 Absorption spectra of rhodamine 6G in methanol. In this example the path length was 10 cm and therefore the concentration in the cuvette is ~ 0.1 μM. One can clearly see the most suitable wavelength region for excitation.

Figure 5 Normalised absorption and emission spectra of rhodamine 6G, excited at 503 nm. From this one can calculate the Stokes shift and see what wavelength range is the best for measuring the fluorescence.

Figure 6 Chemical structure of (A) rhodamine 6G and (B) rhodamine B. Note the electrostatic attraction circled green for both dyes and the electrostatic repulsion from the carboxylic acid group circled red for rhodamine B.

Figure 7 (A) Fluorescein 5(6)-isothiocyanate (FITC) is bound to (B) 3-aminopropyl trimethoxysilane (APS) via the mechanism (C) to create (D) FITC-APS.

Figure 8 Schematic of the fluorometer used for fluorescence anisotropy decay measurements. Fluorescence photons are detected using a single photon timing detection system. Fluorescence decay data is accumulated using the FluoroHub electronics which incorporate a time-to-amplitude converter (TAC), operated in reverse mode to minimize dead time, and multichannel analyser. The operation of all functions is fully controlled under Microsoft Windows software.

Figure 9 Polarizer orientations for a fluorescence decay measurement. Typically the axes would be displayed using semilogarithmic scales with *x* being the time axis and *y* the counts (log) however in this case a linear scale is used to emphasise the differences between the decay curves.

Figure 10 Adjustment of the TAC range and delay lines so the measurement window covers the area of interest. The axes in this are semilogarithmic with *x* being the time axis and *y* the counts (log). Examples illustrated are too small a TAC range, too large a TAC range, too small a delay, too large a decay, adjusting the delay and a correct measurement. Purple lines; indicate likely non-linear regions of the TAC at the beginning and end of the time range.

Figure 11 Reconvolution analysis of fluorescence decay. The axes in this are semilogarithmic with *x* being the time axis and *y* the counts (log).

Figure 12 Polarizer orientations for an anisotropy measurement. Typically the axes would be displayed using semilogarithmic scales with *x* being the time axis and *y* the counts (log) however in this case a linear scale is used to emphasise the differences between the decay curves.

Table 1. Typical results for nanoparticle metrology via fluorescence anisotropy measurements on silica colloids.

Sample	Measured Particle Size via Other Methods (nm)	Measured Particle Size by Fluorescence Anisotropy (nm)
$M4_{R6G}$	4.2 ± 0.2 *(4)*	3.5 ± 0.1 *(5)*
$M4_{FITC-APS}$	4.2 ± 0.2 *(4)*	3.8 ± 0.1 *(5)*
LUDOX® SM30	3.5 *(16)*	4.0 ± 0.4 *(2)*
LUDOX® AM30	6.0 *(16)*	6.4 ± 0.5 *(2)*
LUDOX® AS40	11.0 *(16)*	11.0 ± 1.6 *(2)*