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Measurement of Particle Size Distribution in Turbid Solutions by Dynamic Light Scattering Microscopy

--Manuscript Draft--

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Abstract:	A protocol for the measurement of the polydispersity of concentrated polymer solutions using dynamic light scattering is presented. Dynamic light scattering is a technique used to measure the size distribution of polymer solutions or colloidal particles. Although this technique is widely used for the assessment of polymer solutions, it is difficult to measure concentrated solutions using this technique due to multiple scattering or strong light absorption. Therefore, it is generally accepted that concentrated solutions should be diluted before measurement with dynamic light scattering. Owing to the implementation of confocal optical system in dynamic light scattering microscope, this difficulty can be resolved. Using such a microscope, both transparent and turbid systems can be analyzed under the same experimental setup without dilution. As a representative example, the measurement of the size distribution in a temperature-responsive polymer solution was performed. The size of the polymer chains in an aqueous solution was several tens of nanometers at a temperature below the lower critical solution temperature (LCST). In contrast, the size increased to more than 1.0 μm above LCST. This result is consistent with the observation that the solution turned turbid above LCST. Details of the protocol and data analysis are presented.
Author Comments:	I'd like to ask about the setup. We did the experiment introduced in this manuscript by using the setup in this manuscript. However, we modified some parts for the current project and the setup is a little bit different from the attached photo. Is it better to return the setup shown in the manuscript at the time of filming?
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April 22, 2016

Dear Editors,

We are submitting our manuscript entitled "***Dynamic Light Scattering Microscope: Measurement of Particle Size Distribution for Turbid Solutions***" to Journal of Visualized Experiments.

In this study, a protocol for the direct measurement of particle size distributions of concentrated solutions by using a dynamic light scattering microscope is presented. Dynamic light scattering is a technique to measure a size distribution of polymer solutions. Although this technique is widely used for the assessment of polymer solutions, it is difficult to measure concentrated solutions due to the existence of multiple scattering or strong light absorption. Therefore, it has been common knowledge that the concentrated solution should be diluted before the measurement of dynamic light scattering. A dynamic light scattering microscope, which has been developed recently, overcomes this difficulty by using a confocal optical system. By using this microscope, we can measure both transparent and turbid systems under the same experimental setup without dilution. As a representative example, a measurement of the size distribution of a temperature-responsive polymer solution was performed. The size of the temperature-responsive polymer in an aqueous solution is the order of several tens nanometer at the temperature below their lower critical solution temperature (LCST). In contrast to this, the size became more than 1 □m above LCST. This result is consistent with the fact that this solution becomes turbid at the temperature above LCST. Details of the protocol and data analysis are presented.

We believe that these protocols will be helpful for many researchers who are trying to implement this technique. We confirm that this manuscript has not been published elsewhere and is not under consideration by another journal. We would be grateful if the manuscript could be reviewed and considered for publication in Journal of Visualized Experiments.

Sincerely yours,

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TITLE:
Measurement of Particle Size Distribution in Turbid Solutions by Dynamic Light Scattering Microscopy

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KEYWORDS:
dynamic light scattering, confocal microscopy, polydispersity, colloidal solution, backscattering, heterodyne

SHORT ABSTRACT:
A protocol for the direct measurement of particle size distribution in concentrated solutions using dynamic light scattering microscopy is presented.

LONG ABSTRACT:
A protocol for measuring polydispersity of concentrated polymer solutions using dynamic light scattering is described. Dynamic light scattering is a technique used to measure the size distribution of polymer solutions or colloidal particles. Although this technique is widely used for the assessment of polymer solutions, it is difficult to measure the particle size in concentrated solutions due to the multiple scattering effect or strong light absorption. Therefore, the concentrated solutions should be diluted before measurement. Implementation of the confocal optical component in a dynamic light scattering microscope[1] helps to overcome this barrier. Using such a microscopic system, both transparent and turbid systems can be analyzed under the same experimental setup without a dilution. As a representative example, a size distribution measurement of a temperature-responsive polymer solution was performed. The sizes of the polymer chains in an aqueous solution were several tens of nanometers at a temperature below the lower critical solution temperature (LCST). In contrast, the sizes increased to more than 1.0 μm when above the LCST. This result is consistent with the

observation that the solution turned turbid above the LCST.

INTRODUCTION:

Particle size is one of the most fundamental properties of colloidal and polymer solutions. Numerous techniques are used to measure the particle size. Particle sizes of 1.0 μm or larger can be measured directly using an optical microscope. For smaller particles, alternative techniques, such as laser diffraction, electron microscopy, or atomic force microscopy, are used[2,3]. Dynamic light scattering is a commonly-used technique for the measurement of particle size distributions in solutions[4]. The results obtained using this technique are not derived from images of the particles but from the characteristic time of the fluctuations in scattered light intensity. These fluctuations originate from Brownian motion, which is characterized by a diffusion constant. The size distribution is obtained from the distribution of diffusion constants using the Einstein-Stokes equation. Due to its simplicity, dynamic light scattering is widely used for the routine assessment of solutions, such as paints and food colloids.

Pretreatment is required for most of the techniques used for the particle size measurement of solution samples. In the case of electron microscopy and atomic force microscopy, the sample must be analyzed under vacuum conditions. Therefore, it is difficult to observe the samples in their native forms. Furthermore, for laser diffraction and dynamic light scattering, only diluted samples that are free from multiple scattering and light absorption can be measured. To overcome this difficulty, several new techniques have been proposed for the measurement of dynamic light scattering from undiluted, concentrated solutions, such as cross-correlation spectroscopy[5,6], low-coherence dynamic light scattering[7,8], diffusing-wave spectroscopy[9,10], and differential dynamic microscopy[11,12].

We have developed a new apparatus called a dynamic light scattering microscope[1]. This apparatus enables us to measure turbid samples without dilution by means of a confocal optical system in which multiple scattering is eliminated using a pinhole. However, the measurement procedure and data analysis are slightly more complicated than those of commercially-available instruments. This video explains the measurement procedure and data analysis in detail using the analysis of the temperature-responsive polymer, poly(*N*-isopropylacrylamide), as an example.

PROTOCOL:

1. Sample Preparation

1.1) Purification of temperature-responsive monomers

1.1.1) Dissolve 20 g of *N*-isopropylacrylamide (NIPA) in 100 mL of toluene.

1.1.2) Filter the solution under suction to eliminate dust.

1.1.3) Mix the filtrate with 500 mL of petroleum ether.

1.1.4) Place the reaction vessel in an ice-water bath.

1.1.5) Stir the solution until the monomers are precipitated (typically 30 min).

1.1.6) Filter the solution under suction to obtain the precipitated monomers.

1.1.7) Dry the monomers under reduced pressure (100 Pa) overnight.

1.2) Preparation of the temperature-responsive polymer solution

1.2.1) Degas 20 mL of deionized water for 1.0 min using a diaphragm pump.

1.2.2) Dissolve 780.8 mg of the purified NIPA in 9.5 mL of degassed and deionized water.

1.2.3) Place the reaction vessel in an ice-water bath.

1.2.4) Shield the reaction from light by covering the apparatus with aluminum foil.

1.2.5) Stir the solution gently for 10 min while introducing a moderate flow of Ar gas gently via a pipette tip attached to the gas cylinder with a tube.

1.2.6) Add 11.9 µL of *N*,*N*,*N*',*N*'-tetramethylethylenediamine to the solution via a micropipette.

1.2.7) Stir the solution for 1.0 min while introducing Ar gas, as mentioned in step 1.2.5.

1.2.8) While stirring the sample, dissolve 4.0 mg of ammonium persulfate in 0.5 mL of degassed and deionized water.

1.2.9) Mix the sample solution (from step 1.2.7) and ammonium persulfate solution (from step 1.2.8).

1.2.10) Stir the solution for 30 s while introducing Ar gas, as mentioned in step 1.2.5.

1.2.11) Cover the solution with aluminum foil and keep it in a refrigerator (4 °C) overnight.

1.3) Preparation of sample mounts

1.3.1) Place 60 µL of the sample solution (from step 1.2.11) in a cavity slide.

1.3.2) Cover the solution with a circular cover glass. Be careful not to trap air bubbles.

1.3.3) Remove excess solution using a micropipette and laboratory wipes.

1.3.4) Seal the sample with glue. Let the glue dry at room temperature (typically 6 h).

1.3.5) Prepare another slide filled with 0.1 wt% polystyrene latex (100-nm particle diameter) suspension by following steps 1.3.1-1.3.4. This slide is used as a standard.

2. Particle Size Measurement with a Dynamic Light Scattering Microscope

2.1) Optimization of the instrument

2.1.1) Place the polystyrene latex suspension slide (from step 1.3.5) on the stage of the inverted microscope. The cover glass side should face downward.

2.1.2) Place a beam damper in front of the detector (an avalanche photodiode and an autocorrelator).

2.1.3) Apply a laser beam (Ar-Kr ion laser, λ = 514.5 nm, 200 mW, continuous wave) to the sample through an objective lens (×10). A portion of the reflected light goes through a launch mirror of the microscope and is observed by a CCD camera mounted at the side port of the microscope (Figure 1(a)).

2.1.4) Adjust the direction of the laser beam by using two mirrors before the microscope (Figure 1(b), mirrors A and B).

2.1.4.1) First, tilt one mirror mount to move the focal point. In this step, the laser beam is not vertically introduced into the objective lens.

2.1.4.2) Then, tilt the other mirror mount to vertically direct the laser to the objective lens. When the laser is vertically introduced into the objective lens, the position of the reflected image remains unaffected, even when varying the height of the objective lens.

2.1.4.3) While performing these procedures, place the position of the focal point at the center of the objective lens (*i.e.,* at the center of the observed image recorded by the CCD camera).

2.1.5) Adjust the height of objective lens to set the focal point at the sample suspension by shifting the height of objective lens from the low-to-high position. During this procedure, the reflected image is focused three times: at the surface of the cover glass, at the interface between the cover glass and the sample, and at the interface between the sample and the hole-slide glass. Set the focal point between the second and third points.

2.1.6) Attenuate the scattered light intensity by inserting neutral density filters to protect the detector from overcurrent. Typically, two filters with optical densities of 1.0 and 2.0 are inserted.

2.1.7) Introduce the scattered light into the detector by removing the beam damper in front of

the detector. This unit measures the time correlation of the light intensity.

2.1.8) Set a pinhole (φ = 50 µm) between the microscope and the detector to achieve the confocal effect. Adjust the position of the pinhole to maximize the light intensity at the detector. If necessary, remove the neutral density filters.

2.1.9) Measure the time correlation function of scattered light intensity for 30 s by initiating the operation of the correlator via a computer. The measured correlation function is often expressed as $g^{(2)}(t) - 1$, where t is the correlation time[4] and $g^{(2)}(t) = \langle I(0)I(t)\rangle_T / \langle I(t)\rangle_T^2$. Here, $I(t)$ is the scattered light intensity at the time t and $\langle \cdots \rangle_T$ is time averaging. The decay time will be approximately 0.1 ms.

2.1.10) Adjust the focal point to obtain a wide range for the initial amplitude of the time correlation function ($g^{(2)}(t = 0) - 1$).

Note: The initial amplitude is strongly affected by the amount of reflected light. By moving the focal point toward the interface between the cover glass and the sample, the amount of reflected light increases. For strong light scatterers, such as polystyrene latex, the initial amplitude can be altered from 0 to 1. However, it is difficult to set the initial amplitude close to 1 for more common polymer solutions because the intensity of the reflected light is much higher than that of the scattered light.

2.1.11) Apply the inverse Laplace transformation (using the constrained regularization program CONTIN[13,14]) to the obtained time correlation function to acquire the size distribution function. In cases where the initial amplitude is set to less than 0.2, the distribution function of the hydrodynamic radius will show a sharp peak around 100 nm, which is twice the actual radius (see the discussion for details).

2.2) Sample measurement

2.2.1) Set the stage temperature to 25 °C.

2.2.2) Place a slide prepared with poly-NIPA (PNIPA) solution (step 1.3.4) on the stage of the microscope.

2.2.3) Measure the time correlation function of the scattered light intensity by following steps 2.1.5-2.1.9. If the initial amplitude is larger than 0.2, adjust the focal point to make the initial amplitude of the time correlation function less than 0.2 by following step 2.1.10. A small initial amplitude simplifies the analysis.

2.2.4) Set the stage temperature to 35 °C and wait until the solution turns turbid. The lower critical solution temperature (LCST) of PNIPA solution is 32 °C[15].

2.2.5) Measure the time correlation function by following steps 2.1.5-2.1.9. If feasible, adjust

the position of the focal point to make the initial amplitude of the time correlation function less than 0.2. For turbid solutions, their initial amplitudes tend to increase, since the intensity of the scattered light increases while that of the reflected light remains constant.

2.2.6) Apply the inverse Laplace transformation to the obtained time correlation functions to obtain the size distribution functions. Note that the actual size is half the obtained value in cases where the initial amplitude is less than 0.2.

REPRESENTATIVE RESULTS:

Time correlation functions of scattered light intensity for a polystyrene latex suspension (particle radius: 50 nm) were measured at different focal points, as shown in Figure 2(a). These correlation functions were converted into the distribution functions of the hydrodynamic radius by the inverse Laplace transformation (refer to Figure 2(b) and (c)). Using the same procedure, the time correlation functions and distribution functions of the hydrodynamic radius of the PNIPA solution were obtained at 25 °C and 35 °C, respectively. Figures 3(a) and (b) show the time correlation functions of the scattered light intensity and the corresponding size distribution functions of the PNIPA solution below (25 °C) and above (35 °C) the LCST. The size distribution functions were obtained by the inverse Laplace transformation followed by the correction of the partial heterodyne. The average hydrodynamic radius below the LCST is several tens of nanometers, which is typical for polymer solutions. In contrast, the hydrodynamic radius above the LCST is about 1.0 μm. This result is consistent with the fact that the solution is turbid above the LCST. The red and blue lines in Figure 3 represent the size distribution of PNIPA solutions obtained immediately after and 20 min after the solution became turbid, respectively. Figure 3(b) clearly indicates the growth of the aggregation.

Figure Legends:

Figure 1. Schematics of the dynamic light scattering microscope. (a) Schematic of the dynamic light scattering microscope: variable neutral density (VND) filter, pinhole (PH), beam splitter (BS), polarizer (Pol), and avalanche photodiode (APD). (b) Layout of the optical components. Mirrors A and B, which are used for the alignment described in step 2.1.4, are indicated by red letters.

Figure 2. Representative results for a polystyrene latex suspension. (a) Time correlation functions of the scattered light intensity for the polystyrene latex suspension. The nominal radius is 50 nm and the concentration is 0.1 wt%. Two datasets were obtained from different scattering points. (b), (c) Corresponding size distribution functions for the polystyrene latex suspension obtained by the inverse Laplace transformation of Figure 2(a). The red line corresponds to the time correlation function whose initial amplitude is approximately 1.0, and the blue line corresponds to that with an initial amplitude that is approximately 0.2. The horizontal axis was calculated without (b) and with (c) considering the effect of partial heterodyning (PHD) when $A \ll 1$.

Figure 3. Representative results for a PNIPA solution. (a) Time correlation functions of scattered light intensity for the PNIPA solution. (b) Corresponding size distribution functions for

the PNIPA solution obtained by the inverse Laplace transformation of Figure 3(a). The horizontal axis was calculated considering the effect of partial heterodyning for each data set. The black line represents the data obtained at 25 °C. The red line represents the data obtained just after the solution turned turbid (35 °C). The blue line represents the data obtained after a 20-min measurement of the red line.

DISCUSSION:

The initial amplitude of the time correlation function heavily depends on the focal point, as shown in Figure 2(a). This seemingly contradicts the fact that the solution is homogeneous (except for the thin layer at the interface)[8]. This variation in the initial amplitude is attributed to a variation in the amount of reflected light. Partial heterodyne theory[16] predicts that the initial amplitude, *A*, the scattered light Intensity, I_s, and the reflected light intensity, I_r, satisfy the following equation[1]:

$$A = 1 - \left(\frac{I_r}{I_s + I_r}\right)^2$$

This equation shows that the larger I_r becomes, the smaller *A* becomes. Therefore, *A* is reduced by setting the focal position close to the interface. The apparent diffusion constant D_A can be obtained by fitting the time correlation function in the case of monodisperse solutions:

$$g^{(2)}(t) - 1 = Ae^{-2D_A q^2 t}$$

where $q = 4\pi n \sin\left(\frac{\theta}{2}\right)/\lambda$. Here, *n* is the refractive index of the solvent (water, 1.33), θ is the scattered angle (180°), and λ is the wavelength of light (514.5 nm). Since we applied backscattering geometry, the value of *q* is fixed. However, this point is solved by using different wavelengths of light. Please note that any kind of continuous-wave laser source is available to construct the DLS microscope. Thanks to the small irradiated volume, the coherence factor[17] is estimated to be more than 0.99 and is negligible. For polydisperse solutions, the distribution function of D_A is obtained by the inverse Laplace transformation. Partial heterodyne theory also predicts that D_A is not the same as the actual diffusion constant *D*. These two diffusion constants satisfy the following equation:

$$D_A = \frac{1 - \sqrt{1 - A}}{A} D$$

The diffusion constant D is converted into the hydrodynamic radius R_h using the Einstein-Stokes equation[4]. When $A = 1$, this relationship becomes $D_A = D$. In this case, the data conversion process is the same as that for the common dynamic light scattering. The red line shown in Figure 2(b) corresponds to this case. In contrast, this relationship becomes $D_A = 0.5D$ at the limit of $A \to 0$. Therefore, the size is estimated to be twice as large as the actual size when A is small (practically, less than 0.2), as shown by the blue line of Figure 2(b). If we know that *A* is significantly small, the horizontal axis can be shifted, as shown in Figure 2(c). In principle, we can convert D_A into *D* for any value of *A*. In practice, however, it is better to set the initial amplitude smaller than 0.2, since the simple approximation $D_A \sim 0.5D$ holds true.

The prominent features of the dynamic light scattering microscope technique were demonstrated using a PNIPA solution. The conformation of PNIPA below and above the LCST has been extensively studied using small-angle neutron scattering[15,18]. In contrast, dynamic light

scattering has not been utilized for the analysis of PNIPA above the LCST because of its turbidity[19]. This problem is solved by the dynamic light scattering microscope, as shown in Figures 3(a) and (b). The size of these aggregates is several μm, which cannot be obtained by either small-angle X-ray/neutron scattering or conventional light scattering techniques. Time-resolved measurements using this system give information on the aggregation process during the temperature change.

The drawback of the dynamic light scattering microscope is also illustrated in Figure 3. For the result below the LCST, the time correlation function is strongly affected by the very small amount of dust present (the black lines in Figure 3). For example, the time correlation function does not decay completely, even with correlation times in the order of 1.0 s. This is because the volume irradiated with this apparatus (approximately 1.0 μm) is significantly smaller than that irradiated with the usual dynamic light scattering apparatus (approximately 100 μm). In cases where the intensity of scattered light is weak, the signal is obscured by the noise, such as that caused by small amounts of dust in the solution. Therefore, the three peaks shown in Figure 3(b) may not have quantitative importance although the general order of the size is meaningful. Note that such a weak scatterer can be measured by a conventional dynamic light scattering apparatus.

We have demonstrated that the dynamic light scattering microscope enables us to measure both transparent and turbid samples with the same setup. Since the optical path length in the samples is short, this technique can be applied to strong light-absorbing samples, such as carbon nanotube suspensions[20]. In addition, due to its high spatial resolution, this technique can be applied to biological cells. For its application to biology, this method can also be combined with other imaging techniques, such as fluorescence and Raman imaging. Thus, we believe that the dynamic light scattering microscope is a powerful tool for a wide range of research fields.

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DISCLOSURES:
The authors have nothing to disclose.

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Figure 1

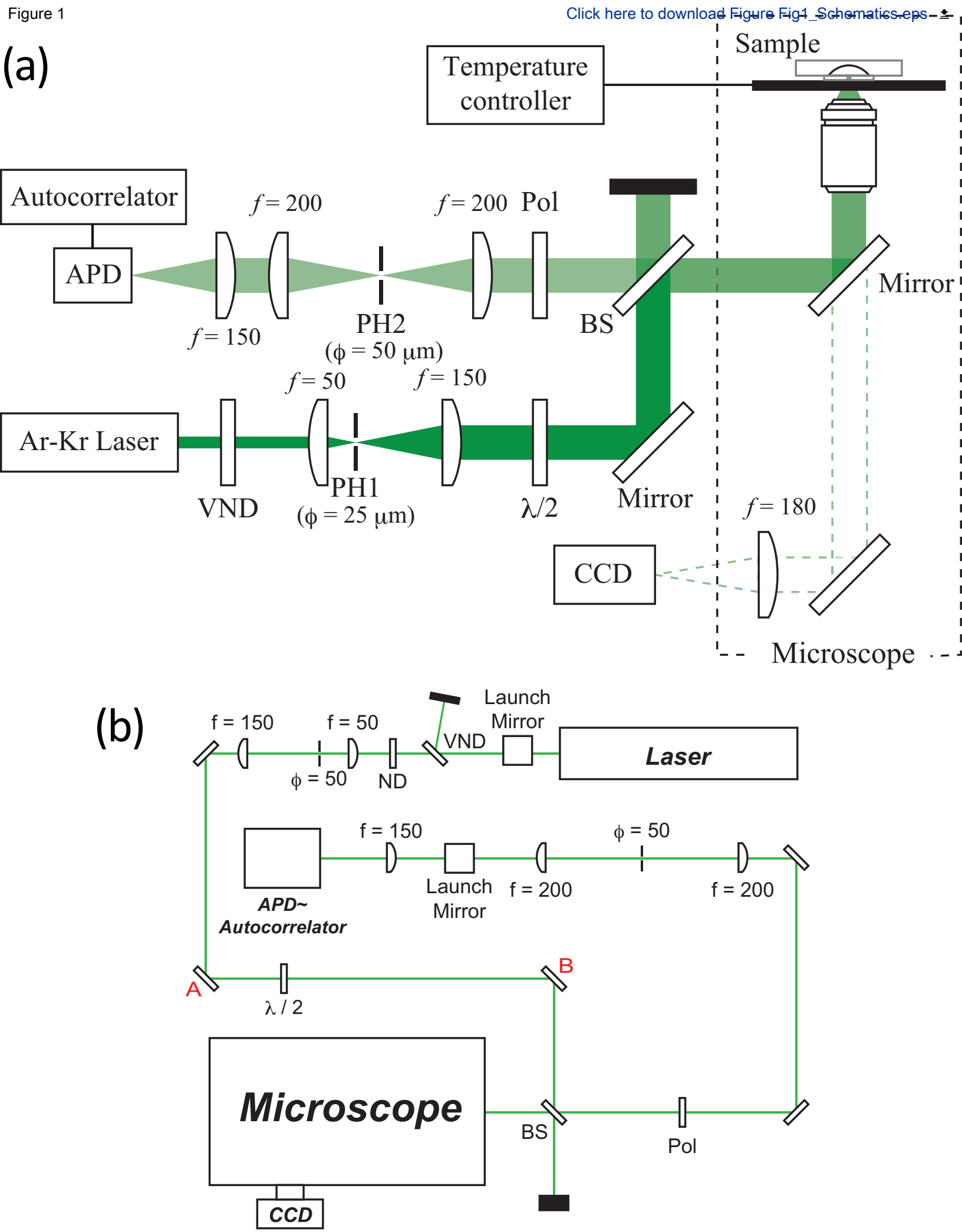
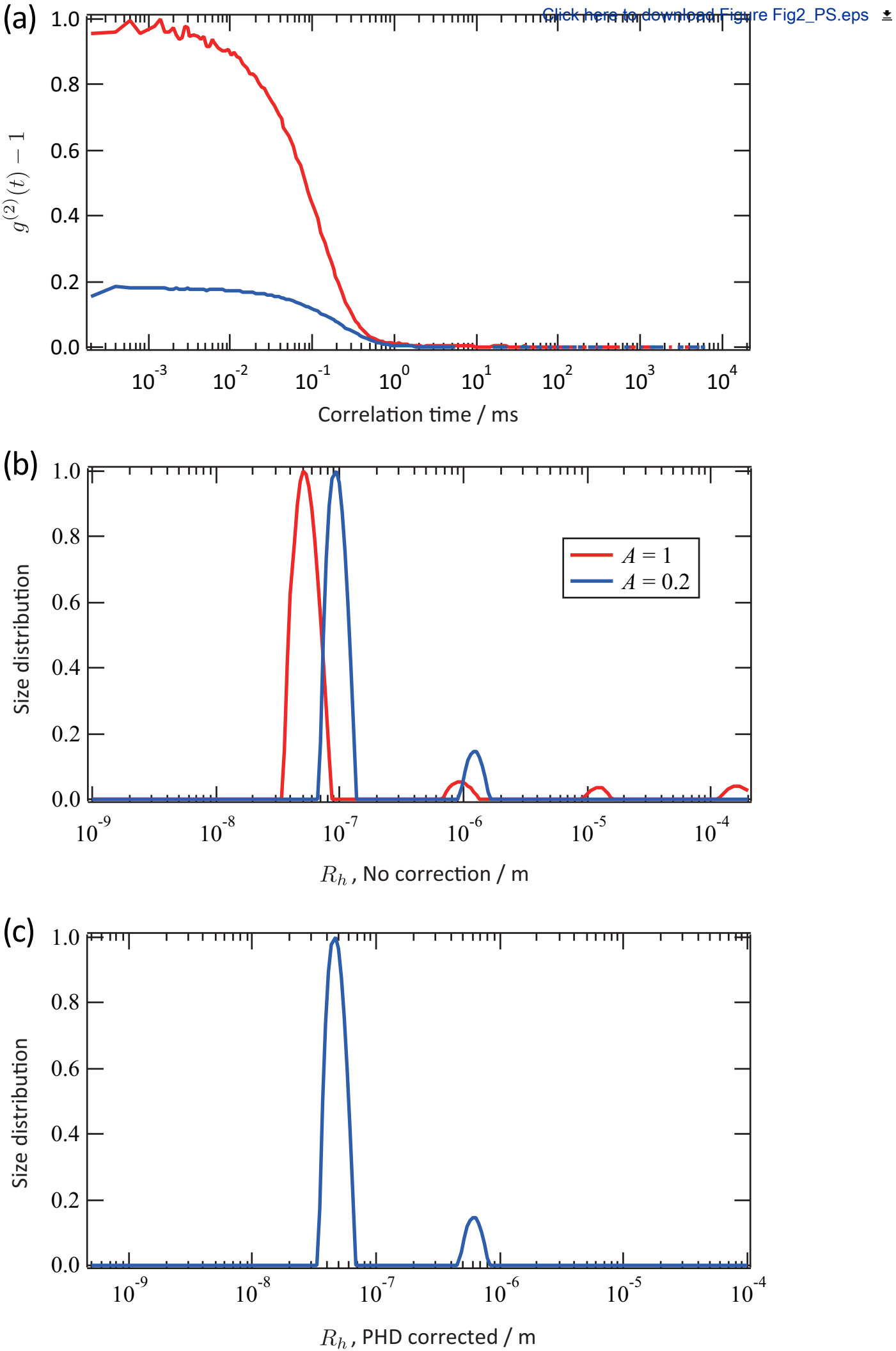
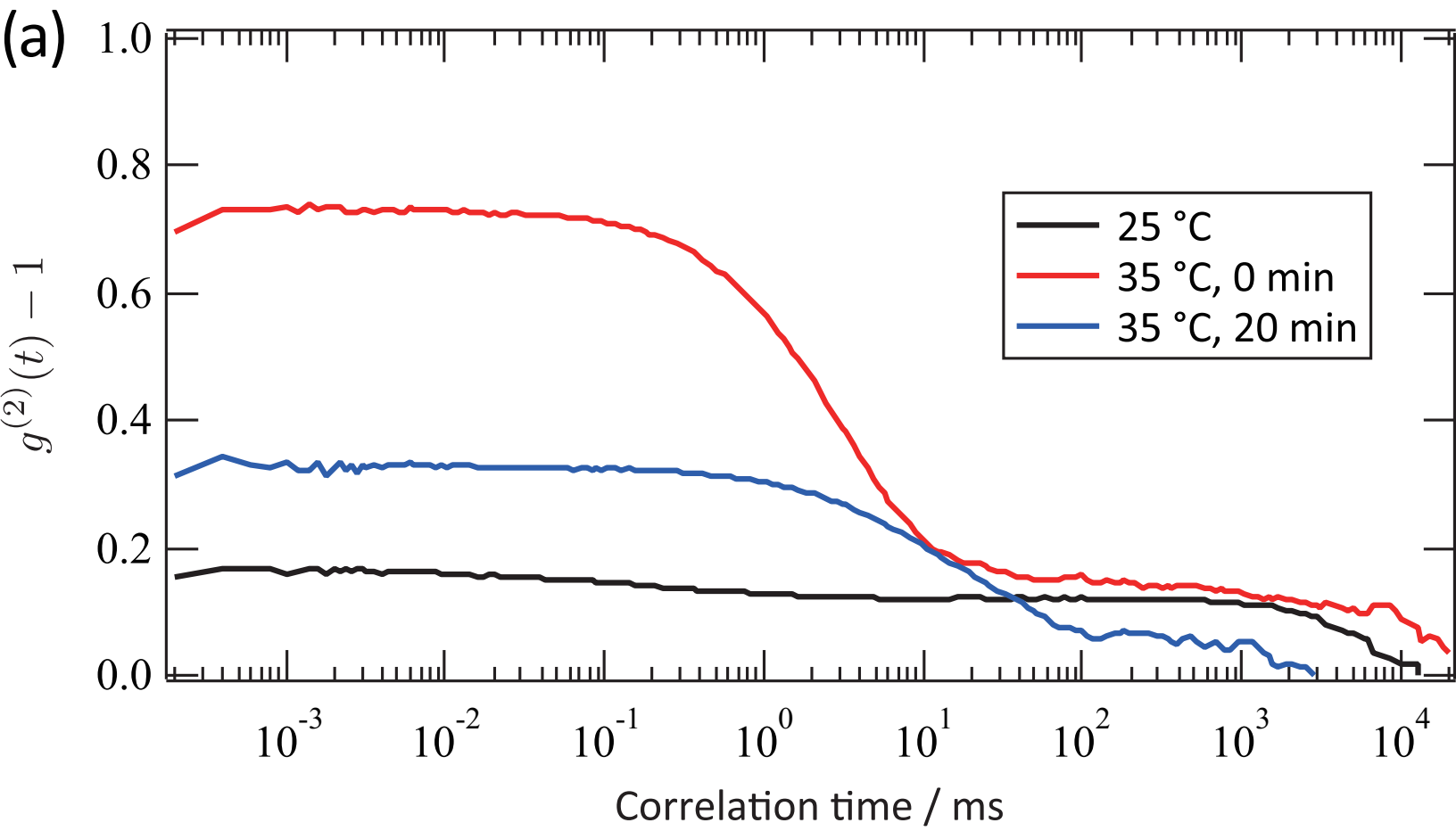
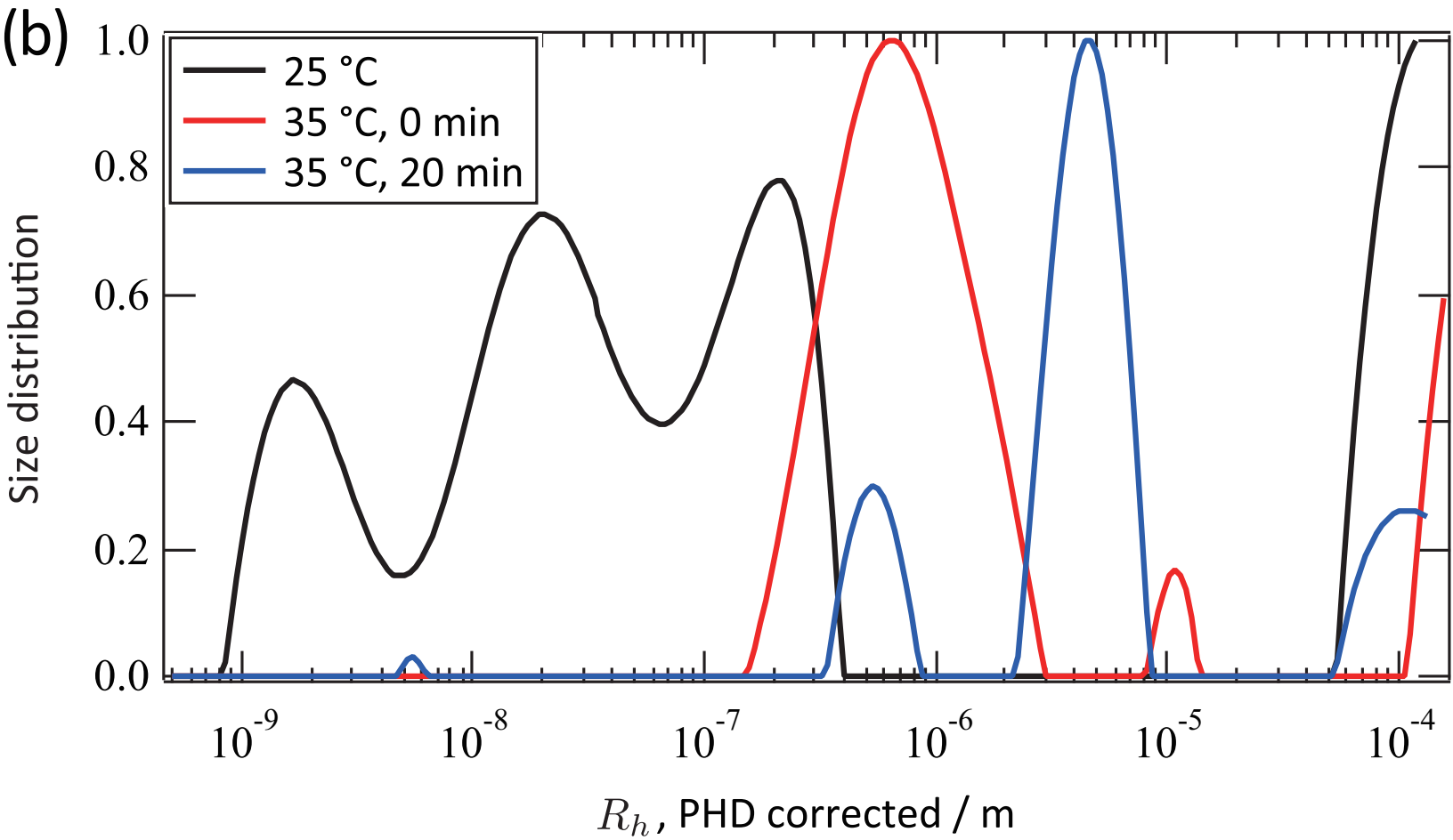


Figure 2







Excel Spreadsheet- Table of Materials/Equipment

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20160518_DLSM_Movie_Reagent_Revise1_02.xls

Name of Reagent/ Equipment	Company	Catalog Number	Comments/Description
N-isopropylacrylamide, 98%	Tokyo Chemical Industry Co., Ltd.	I0401	
toluene, 99%	Wako Pure Chemical Industries, Ltd.	201-01876	
petroleum ether, distillation temperature 30 ~ 60 ° C	Wako Pure Chemical Industries, Ltd.	169-22565	
N,N,N',N'-tetramethylethylenediamine, 99%	Sigma	T9281	
ammonium persulfate, 98%	Sigma	248614	
polystyrene latex suspension, 1 wt%	Duke Scientific Corporation	3500A	
argon	Koike Sanso Kogyo Co., Ltd.		purity > 99.999 vol.%
cavity slide	Matsunami Glass Ind.,Ltd.	83-0336	
inverted microscope	Nikon Instech Co., Ltd.	ECLIPSE Ti-U	

Thermo Plate	Tokai Hit CO.,Ltd	TP-108R-C
Ar-Kr ion laser	Spectra-Physics	Stabilite 2018
avalanche photodiode	ALV-GmbH	ALV-High Q.E. Avalanche Photo Diode
correlator	ALV-GmbH	ALV-5000/EPP

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Title of Article: Dynamic Light Scattering Microscope: Measurement of Particle Size Distribution for Turbid Solutions

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Response to Reviewers

First of all, we would like to thank the reviewers for valuable comments and suggestions. We took all of them into account and made a revision as listed below. Please let us know if further revision is required.

Editorial comments:

1) All of your previous revisions have been incorporated into the most recent version of the manuscript. In addition, Editor may have made minor copy edits to your manuscript and formatting changes to comply with the JoVE format. Please maintain these changes. On the JoVE submission site, you can find the updated manuscript under "file inventory" and download the microsoft word document. Please use this updated version for any future revisions and track all changes using the track changes function in Microsoft Word.
OK.

2) Grammar:
-Please copyedit the manuscript for numerous grammatical errors. Such editing is required prior to acceptance, and some errors are indicated below.
-Line 38 – “of confocal optical system”; “in recently developed dynamic light scattering microscope”
-Line 43 – “in the order of”
-Line 49 – “polymer solutions.,” – punctuation
-2.1.4 – “suspensio”
We fixed these errors.

3) Visualization: Please provide a photographic image of the custom setup, which can be supplied as a supplementary file.
We attached a photo of the image.
In addition, I’d like to ask about the setup. We did the experiment introduced in this manuscript by using the setup in this manuscript. However, we modified some parts for the current project and the setup is a little bit different from the attached photo. Is it better to return the setup shown in the manuscript at the time of filming?

4) Additional detail is required:
-1.2.6 – How is the solution added? Via a syringe or pipette?
Via a micropipette. We added the explanation.

-2.1.6 – How is this done? Does this mean to turn on the light source? Or are they placed somewhere?

It is done by removing the beam damper in front of the detector. We added the step 2.1.2 and also fixed the explanation in 2.1.7.

-2.1.8. 2.1.9 – Please define all terms. What is g?
We added the definition of $g^{(2)}(t)$ explicitly in 2.1.9.

-2.2.3 – How would the focal point be adjusted here?
It is done by following step 2.1.10. We added this statement in 2.2.3.

5) Branding: 2.1.10 – CONTIN, ALV – software name and company should appear in the materials table only.
We deleted the company name from the manuscript. The company name has already written in the materials table. Note that “CONTIN” is not the name of software but the name of algorithm.

6) Results: Please discuss what the results of the analyses are in the results section. That is, what is the interpretation of the data shown? What was the particle size distribution detected? Such interpretation should be moved from the discussion to the results section (see lines 289 – 300).
I’m sorry but I cannot understand the meaning of this point. It seems that the discussion should be done not in discussion section but in result section. Please note that the contents written in lines 289 – 300 (original version) are also written in the results section and figure legends. That is, the interpretation of the data shown in the results is clearly shown. The reason why we wrote the similar contents in two sections is to clarify the flow of discussion.

7) Discussion: Please discuss any troubleshooting/modifications that can be performed.
The most probable modification is the wavelength of light. We added this point in line 274-277.

8) Please disregard the comment below if all of your figures are original.
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N/A

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
This manuscript describes an application of a dynamic light scattering microscope that can be applied to turbid media. In order to extend this technique to opaque samples, a confocal approach has been implemented. As with any confocal system, the reduction of the imaging region to that of a pinhole greatly reduces the imaging region, but will also significant reduce multiple scattering events. As such, this implementation will allow for the acquisition of data from turbid media.

Major Concerns:
With regards to the general applicability of this technique, there are serious concerns. It is very difficult to call this method a dynamic light scattering microscope. DLS is a widely use technique that is typically used in spectroscopy mode where scattered light from a small sample region within a cuvette is acquired and fed into an autocorrelator. The method described in this manuscript is more of an improvement or extension of the spectroscopic method rather than a true extension to a microscope technique. Performing a search in the literature came up with the description of a technique by the same name in a 2004 Biophysical Journal article from Dzakpasu and Axelrod. There, they used a focused line illumination along with a motorized stage to image an entire biological cell and were able to visualize different rates of motion throughout the sample. Here, with the sample region, biological or otherwise, reduced to just a pin hole, a very small and non-representative point cab be acquired, which will limit its scope.
We have cited the article of Dzakpasu and Axelrod in our first report (Ref. 1) but their method is completely different from ours. They utilized the streaking of CCD camera to measure the intensity time correlation function. Thanks to this, they can measure 2D image of the correlation function at once. However, the resolution of correlation time is restricted to 300 μs. This means that they cannot measure small particles less than 100 nm. In contrast to this, we can achieve the high time resolution around 1 μs by using□□□□□□□□□□□□□□□□□□
Please note that this time resolution is typical for conventional DLS apparatus. The new point is that we achieved the measurement of turbid and light-absorbing suspension without loss of time resolution. From this point of view, we believe that the method described in this manuscript is “a true extension to a microscope”.

Minor Concerns:
There are a couple of questions regarding the optical setup that needs to be addressed for clarity, with regards to figure 1:
1. There is both a variable and fixed neutral density filter. Why is the fixed ND used when a variable one is present?
Fixed ND was used during the alignment of the apparatus. During the alignment procedure,

strong light reflection may be introduced to the autocorrelator, which induces fatal damage to the autocorrelator. To protect the autocorrelator from such damage, we inserted fixed ND filter to achieve high attenuation of light intensity, which is not achieved by a variable ND (Procedure 2.1.6).

2. A CCD camera is used to focus the reflected light that is transmitted through the from the sample. It is not clear why this is necessary when the APD is used to collect the scattered light for the autocorrelator.
CCD camera is used for alignment (Procedure 2.1.3). During the measurement, we do not have to use CCD camera. However, we can also measure the position dependence of size distribution functions by combining the image information of the CCD camera and the time correlation functions from the autocorrelator, which is desired for inhomogeneous samples such as biological cells.

Additional Comments to Authors:
N/A

Reviewer #2:
Manuscript Summary:
The manuscript describes a novel technique, dynamic light scattering microscopy, to measure the particle size distribution of turbid samples. The technique combines dynamic light scattering with confocal optics, which allows a scattering volume with small enough optical path to avoid multiple scattering. The technique is demonstrated for PNIPA solutions, which size and turbidity depend on temperature. The manuscript is well written. However, few crucial points are missing.

Major Concerns:
My main concern deals with three aspects:
1. To summarise DLS in a nutshell: DLS measures particle dynamics at a specific length-scale, or more commonly a scattering vector amplitude "q". Thus, the time correlation function g(2)-1 depends on the value of q, and correspondingly the characteristic time tc depends on q. While q is mentioned in the expression of g(2)-1, there is no indication on either its value and/or how to determine its value, which is crucial to extract the diffusion coefficient, and thus particle size distribution.
The magnitude of scattering vector, q, is determined by the wavelength of light (514.5 nm), the refractive index of the solvent (water, 1.332) and the scattered angle (180°). We added the definition of q explicitly (line 273).

2. My second point deal with the focal position. The authors clearly explain that their

technique mainly works near the glass wall interface so that the reflected light is negligible. However, it is well know that particle dynamics, and therefore their diffusion coefficients are affected at a glass wall. Unfortunately, no information is given about the focal position relative to the glass wall, which is again crucial to extract a reliable particle size distribution.
As Reviewer 2 pointed out, the reduction of particle movement at the glass-suspension interface has already reported (For example, Ref. 8). We also reported the position dependence of nominal particle size in Ref. 1 though the result is different from other reports.
As for this manuscript, we measured the data at the position where we do not have to care about the reduction of particle movement. Please note that our technique works not only near the glass but also every point in the suspension thanks to the partial heterodyne method.

3. The authors makes clear that multiple scattering is avoided due to a small optical path of about 1um. However, with such small optical path, one would expect strong effect from number fluctuations, i.e. particle comes and leaves the scattering volume, which again could affect significantly the extracted particle size distribution. A clear discussion on this topic is required so that readers know how to deal with it. There is one sentence that might deal with it (lines 308-310), but requires strong expertise to the field to make the connection.
At this stage, we cannot measure the number fluctuation since the number of particles in the irradiated volume is still large. Like fluorescence correlation spectroscopy, the number of particles in the irradiated volume should be one or two to measure number fluctuation. Quantitatively, the intensity time correlation is proportional to:

$$\langle I(0)I(t)\rangle_T \propto \langle N\rangle^2 \left(\left|g^{(1)}(t)\right|^2 + 1\right) + \langle \delta N(0)\delta N(t)\rangle$$

where N is the number of scatterers in the irradiated volume and $g^{(1)}(t)$ is the self-intermediate scattering function (Ref. 4). If $\langle N\rangle$ is more than 10, which is the case for our setup, the second term is almost negligible. Please note that we cannot measure when $\langle N\rangle$ is around one as mentioned in line 308-310 (current version: line 317-319).

Minor Concerns:
line 327: indicate type of cross-section for the cavity slide, e.g. rectangular, circular?
The cross-section is circular. We don't know the cavity slide whose cross-section is rectangular.

line 163: missing "n" at "suspensio"
Thank you for pointing it out. We fixed this part.

line 176: indicate size of pinhole
Thank you for pointing it out. We added the size (ϕ = 50 μm) in the text and also in Figure 1.

line 177: detail further on how to maximise? what value to maximise by moving pinhole?
What we maximize is the light intensity at the detector. We modified the word “throughput” into “the light intensity at the detector”. We cannot describe how to maximize in greater detail since what we have to do is just moving the position of pinhole as already mentioned.

line 183: the time 0.1ms depends on particle size and value of q. Indicate the corresponding value of q, here.
Of course the characteristic decay time of particles depends on the size of particles. Please note that the corresponding part is focused on the measurement of polystyrene latex suspension whose diameter is 100 nm. We added the explanation of the definition and required parameters for *q* in line 273.

line 188-193: How deep in the sample can one measure? mention potential interactions with wall.
We wrote about potential interaction in the previous comments. In principle, there is no limitation for the depth of the focus point. In practice, the depth is determined by the depth of the cavity (200 μm).

line 265: it would more useful to give A as a function of Is and Ir
Thank you for your suggestion. This is definitely true. We changed the form of equation (line 269).

line 269: give clear information on how to measure q and the range of accessible q values
We added the explanation of the definition and required parameters for *q* in line 273.

line 294: "which typical"
Thank you for pointing it out. We fixed this part.

line 308-310: Do the authors mean effects from number fluctuations here? If so, detail further.
We wrote about this issue in the previous comments.

Additional Comments to Authors:
N/A

Reviewer #3:
Manuscript Summary:
The manuscript Measurement of Particle Size Distribution in Turbid Solutions by Dynamic

Light Scattering Microscopy by T. Hiroi and M. Shibayama describes an apparatus that combines a microscope and a digital autocorrelator+PMT for the determination of the particle size distribution in dense solutions exhibiting multiple scattering. The procedure is carefully described (read below for some exceptions) but I am not sure that an implementation by a potential JoVE reader will be smooth and painless. Nevertheless, before recommending publication I would like the Authors to address the following issues.

Major Concerns:

1) In this work, as in the cited Ref. 10 the Authors have to deal with the interference between the scattered light and the reflected beam (heterodyne detection). I have to say that I am not entirely satisfied of how the authors address this issue in the present manuscript. My major concern is that the zero-time intercept of the intensity autocorrelation function is not solely determined by this effect. It is well known that even in the absence of heterodyning, the intercept of g2-1 can be smaller than 1 because of the overall efficiency of the speckle detection scheme, an effect that can only be made worse by the presence of multiple scattering. The Authors should discuss in more detail this aspect to provide a convincing and unambiguous proof that they are doing the right assumptions. Also, they should guide the potential user very clearly in this respect.

Coherence factor, β, is estimated as follows:

$$\beta = \frac{1}{1 + \left(\frac{2\pi}{\lambda}\frac{R_{DP}R_{SP}}{r}\right)^2/4}$$

where R_{DP}, R_{SP}, r are the length scale of irradiated volume (~ 10 μm), the length scale of the beam size at the detector (~ 100 μm), and the distance between the detector and the lens in front of the detector (~ 10 cm). Thanks to the small irradiated volume, β is estimated to be larger than 0.99 in our setup. This is also experimentally proved that we obtained the time correlation function whose initial amplitude is almost 1 (data not shown). We added this fact in line 277-278.

2) Another point that deserves attention is that the Authors never really show insensitivity to multiple scattering in their measurements. They claim it but they never prove it. It would be very important to show that the samples could not be measured reliably with standard DLS, by showing for instance the results that would be obtained in that case.

Thank you for your suggestion. That point has already been proved in our previous paper (Ref. 1). The main focus of this paper is not to show the validity but to show how to use and analyze the data of the DLS microscope.

Minor Concerns:

1) The Authors should discuss what differences are expected if the interested reader who want to implement the method has a different laser or a different microscope (i.e. not inverted) or a different correlator and so on...
Thank you for your suggestion. The most probable modification is the wavelength of light. We added this point in line 274-277.

2) The relevant quantities should be defined. For instance, I did not find a definition of g2. Please, consider that a potential user would like to have a concise but complete description of the technique, including the theoretical basics.
Thank you for pointing it out. We added the definition of $g^{(2)}(t)$ explicitly in 2.1.9.

3) Figure 2 is quite confusing. I understood it after 10 minutes. The Authors may want to consider an alternative way of showing the data, maybe on two different graph or maybe on the same graph but only after correcting for the effect of heterodyning.
Thank you for your suggestion. We decided to divide the Figure 2(b) into two (Figure 2(b) and 2(c)).

Additional Comments to Authors:
The Authors do not cite or compare with relevant work in which the dynamics of soft matter is determined by using a commercial microscope. In particular, I refer to Differential Dynamic Microscopy (DDM) [1,2] and Confocal Differential Dynamic Microscopy (ConDDM [3]). I assume that the Authors are not familiar with these methods but in a recent review (where their Ref 10 is also cite) they will find a good discussion of the topic [4]. The comparison of [1-4] with the propose method would be very useful, since also DDM and ConDDM have been shown to be very user friendly and, more importantly for this work, quite insensitive to multiple scattering.
[1] Differential dynamic microscopy: Probing wavevector-dependent dynamics with a microscope by R. Cerbino, V. Trappe, Phys. Rev. Lett. 100, 188102 (2008)
[2] Scattering information obtained by optical microscopy: Differential Dynamic Microscopy and beyond by F. Giavazzi, D. Brogioli, V. Trappe, T. Bellini, R. Cerbino, Phys. Rev. E 80, 031403 (2009)
[3] Characterizing concentrated, multiply-scattering and actively-driven fluorescent systems with confocal differential dynamic microscopy by P.J. Lu, F. Giavazzi, T.E. Angelini, E. Zaccarelli, F. Jargstorff, A.B. Schofield, J.N. Wilking, M.B. Romanowsky, D.A. Weitz, R. Cerbino, Phys. Rev. Lett. 108, 218103 (2012)
[4] Digital Fourier Microscopy for Soft Matter Dynamics by F. Giavazzi, R. Cerbino, J. Opt. 16, 083001 (2014)
Thank you for your information. We read suggested papers and they surely deserve citation. We added [1] and [3] as references (Ref. 11, 12).