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 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.

If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

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 s. 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 s 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:

where *N* is the number of scatterers in the irradiated volume and is the self-intermediate scattering function (Ref. 4). If is more than 10, which is the case for our setup, the second term is almost negligible. Please note that we cannot measure when 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 ( = 50 m) 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 m).

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, , is estimated as follows:

where , , *r* are the length scale of irradiated volume (~ 10 m), the length scale of the beam size at the detector (~ 100 m), and the distance between the detector and the lens in front of the detector (~ 10 cm). Thanks to the small irradiated volume, 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 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).