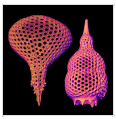

Nov 26, 2018

Working

Simple 3D imaging of biogenic silica structures by fluorescence microscopy

Version 2

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dx.doi.org/10.17504/protocols.io.vuze6x6

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ABSTRACT

This protocol describes how to prepare, stain and mount biogenic silica particles (opaline from diatoms, Radiolaria, sponges spicules, phytolith...) for 3D imaging with fluorescence microscopy. The protocol is fast, convenient and affordable. Hence it could be a relevant alternative to X-ray computed tomography or photogrammetry.

EXTERNAL LINK

doi: 10.7554/eLife.26066

GUIDELINES

The fluorochromes should be protected from long-term light exposure for avoiding photobleaching.
The staining volume and dye working concentration could be optimized empirically according to the amount of surface/particles to stain.
If you notice some fluorescent background onto the coverlsip, it can be reduced by saturating its surface with poly-L-lysine prior mounting the sample and also by improving the washing step (iteration and/or rinsing volume) of your sample after the staining step.

SAFETY WARNINGS

There is no hazardous chemistry or technology.

BEFORE STARTING

This protocol is dedicated to an a posteriori staining of biogenic silica particles (i.e. their surfaces) with a fluorescent dye in order to image the labeled objects by means of fluorescence microscopy, and more specifically, confocal laser scanning microscopy. The staining protocol was designed for a convenient, fast and affordable sample preparation. The mounting strategy is also detailed for improving 3D-reconstruction quality. Biogenic silica can be also stained in vivo during its biosynthesis (eg several protocols are available for diatoms) but this is not detailed here. Other biominerals could be also adressed by this method but the staining efficiciency is lower for calcite.

1 **Preparation of the biogenic silica particles.** If they come from living organisms, they should be cleaned and the organic materials removed.There are many protocols available in the literature which are based on acidic and/or oxidizing and/or detergent and/or solvent treatments.The mineral micro-fossils or sediments can be processed right away after a quick water washing step. If some organics remains are still attached, they will be most likely also stained and they will mask the silica surface.
At the end of this step you should have a suspention of clean particle(s) in double-distilled or ultrapure water (dd-H_2O).

2 **Preparation of the dye.** The staining strategy relies on the electrostatic binding of the polymer poly-L-lysine on silica surface in water at neutral pH. Hence, the poly-L-lysine (PLL) polymer is conjugated with fluorochromes molecules to allow its visualization by fluorescent microscopy. A wide variety of amine-reactive fluorescent dyes are commercially available for conjugation to proteins which allows a high flexibility regarding spectral properties of the fluorochrome moiety. The conjugation reaction on a poly-amine protein is then trivial and the different reagents are affordable.
The preparation of the conjugate is described by Colin et al. (2017), doi:10.7554/eLife.26066. Briefly, in that case, the fluorescent dye moiety was an Alexa Fluor 546 NHS succinimidyl ester (AF546SE, Invitrogen A20002; Thermo Fisher Scientific, MA, USA). The PLL (Sigma-Aldrich

P5899; Merck, Germany) display an average length of 2,340 lysine unit. The conjugation reaction was performed according to the AF546 provider's protocol for protein conjugation. The ratio [R-NH2]/[AF546SE] was calculated to generate the statistical binding of one AF546 per 1500 lysine units. The stock solution of this conjugate was kept at −20°C.

3 **Staining protocol.**

- Few milligrams of silica particles are resuspended in a 1ml of dd-H_2O in 2 ml microcentrifuge tube. In case you work on single isolated object, you might want to adapt lower volume and tube size.
- After the sedimentation of the particles, the supernatant is gently removed. Optionally, liquid removing can be performed using pipette tips capped by a 1 µm mesh piece to avoid particles losses.
- Resuspend the particles in 500 µl of the staining mix (500 µl dd-H_2O + 5 µl of the PLL-A546 conjugate solution prepared in step 2). For single isolated micro-structure, 25 µl of similar staining solution should be large enough.
- Leave the tube at least 1/2 hour, at room temp, in the dark. The tube can be flipped few times for homogenizing the staining media.
- Make sure that the particles are sedimented at the bottom of the tube, remove the staining solution and rinse the particles with 2 ml of dd-H_2O.
- Repeat the washing step at least twice.
- Protect the particles from light in order to avoid the photobleaching of the dye. The labeled particles can be kept in dd-H_2O at 4°C in the dark for several weeks without significant decrease of the labeling intensity.

4 **Mounting protocol.** According to your microscope and objectives specifications, several options are available for mounting such specimens for 3D-imaging. Since the thickness of the objects could be quite large, I would not advise a slide/coverslip mounting. Instead, any petri dish or multi-well plate with a suitable optical bottom (I recommend a high quality glass bottom as close as possible to coverslip #1.5H) can be used with inverted microscope whereas upright microscope equipped with dipping objectives can image directly in the mounting media as far as your observation dish is large enough. For water immersion objectives, a simple mounting in water can be undertaken. It is a fast and straightforward mounting procedure as you can load the suitable amount of your suspension of particles in your observation container and start the imaging directly. However you may have a better 3D-imaging results if the mounting media match the glass refractive index. Indeed you would then be closed of an optimal optical homogeneity. Nevertheless, keep in mind that the refractive index of glass and biogenic silica (opaline) are not exactly similar. In that case, I suggest to load a suitable amount of particles in your observation container, to remove liquid and to oven dry the particles (~50°C, few hours or overnight). Then you can mount your labeled specimens directly in immersion oil or any other mounting media of your choice that matches glass refractive index as far as they are compatible with the stability of the staining (binding and fluorescence). Finally, glycerin immersion objective and glycerin mounting could be eventually a relevant choice as opaline refractive index range from 1.42 to 1.47. For light-sheet microscopy, the mounting can be adapted with hydrogel embedding (agarose, phytagel, polyacrylamide etc...).

5 **Imaging.** Several fluorescence microscopy modalities can propose 3D tomography by optical sectioning. The main solution will be confocal laser scanning microscopy (single point or multipoint scanning methods). Multiphoton microscopy may enable a deeper penetration into thick specimens, 3D-STED nanoscopy may be well suited if higher resolution is needed, and multi-angle light-sheet microscopy may help to adress scattering specimens and facilitate isotropic reconstruction.