Einsatz von TetraSpeck Microspheres 0.1 µm zur Messung der PSF J. Kacza / BCF 10.07.2020

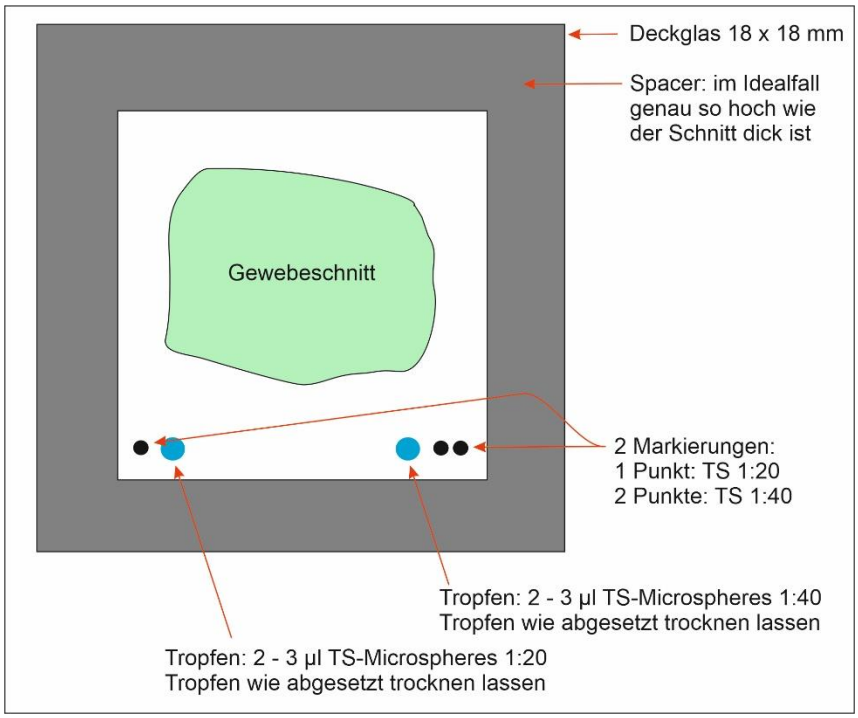
Allgemeine Hinweise

TetraSpeck Microspheres (TSM) 0.1 µm werden vom Hersteller in einer Konzentration geliefert, die für die einfache Herstellung von Präparaten zur Messung der PSF nicht bzw. nur unter Aufwand zu nutzen ist. Neben der Option, eine unverdünnte TSM-Lösung beim Trocknen auszustreichen ist es ggf. einfacher, die Original-TS-Lösung vor dem Einsatz mit Reinstwasser zu verdünnen. Dabei ist bei allen Schritten, angefangen bei Herstellung bzw. Abfüllen des Reinstwassers, Verdünnen der TSM-Lösung und Auftragen der verdünnten TMS auf das Deckglas akribisch darauf zu achten, dass keine Verunreinigungen in den Lösungen bzw. auf dem Deckglas entstehen. Verdünnungen der TSM-Original-Lösungen mit Reinstwasser im Verhältnis 1: 20 und 1:40 haben sich als geeignet erwiesen, um LSM-Aufnahmen von diskret verteilten TSM zu ermöglichen.

Vorbereitung der Deckgläser

Das hier vorgestellte Protokoll bezieht sich auf die Verwendung von TSM bei mit Deckgläsern eingedeckten Präparaten bzw. Schnitten. Hinweise zur Messung der PSF in nachfolgender Dokumentation von SVI.

- Sauberes Deckglas auf der dem Präparat abgewandten Seite mit zwei Punktmarkierungen versehen.
- Jeweils einen Tropfen verdünnte TSM-Lösung auf der dem Präparat zugewandten Seite des Deckglases neben die Markierungen pipettieren.
- TSM-Tropfen bei RT unter Lichtabschluss und staubfrei trocknen lassen (1 – 2 h).
- Präparat / Schnitt mit vorbereitetem Deckglas eindecken. Dabei darauf achten, dass Markierungen und TSM-Tropfen nicht vom *Spacer* (zwischen Deckglas und Objektträger) abgedeckt werden.
- Bei Verwendung von Glyzerin oder Wasser zum Eindecken das Deckglas am Rand vollständig versiegeln (z.B. mit Nagellack, etc.).



Recording beads to obtain an experimental PSF

Background

Beads as PSFs

To measure the Point Spread Function (PSF) of a microscope, one or more 3D images of Sub Resolution beads are needed. For a typical confocal system that means that the bead *should* have a diameter below 50 nm. So far we have not seen images from such small beads which had a sufficient Signal To Noise Ratio to extract a PSF directly, nor a sufficient signal to accurately determine their position so as to average them.

Quantum dots have been considered for this purpose, but good preparations of these are hard to obtain as they easily form aggregates, thus not being "single points" anymore.

If your beads are not point-like they **can not be used *directly* as PSFs**. In current Fluorescence Microscopes this happens with almost any bead, unless you are using quantum dots. They are **too large**, and if you use them as PSFs in DeConvolution you will wash out from your data any feature that is of a size comparable to the bead image, or smaller. Moreover, the remaining data will suffer from Over Restoration.

A single bead image will also have a lot of Photon Noise, thus an averaging procedure using many beads is in any case very much recommended.

Method

One solution is to use large beads with a diameter from 100 nm to 200 nm, and to correct for the bead size. The correction is done with a inverse deconvolution run. Given a model of the bead shape, the PSF is computed 'distilled' which its convolution with the bead model matches the measured bead image. (See Huygens Deconvolution for more details).
In case single bead images have insufficient signal for the PSF extraction, the individual bead images can be accurately aligned and averaged to enhance the signal and increase the Signal To Noise Ratio. All this is automatically done with the Huygens Essential Psf Distiller.

Very important

- Beads should be recorded with **the same** Microscopic Parameters that you will use later to image your specimens, and at least at the Ideal Sampling (see FAQ *Should the sampling density used in PSF measurement be equal to the sampling of the specimen?*).
- Record 3D stacks, not only a single 2D plane. Do not to restrict the number of ***z-sections*** to the exact size of the beads, but make a good recording also a little away from them (***about 10 extra planes above and below***).
- Do not use too large beads, see the FAQ *Can we use 1 micron beads for PSF measurements?*, they lack sufficient high spatial frequency components. See Sub Resolution.
- Do not use **too dense** preparations: beads should be far away from each other to properly probe the PSF. (See images below).

Admittedly, these points are difficult to achieve. How much leeway is there with respect to the microscopic parameters? See Parameter Variation).

Procedure outline

Briefly, the procedure has the following sequence:

1. Prepare a specimen containing fluorescent latex beads.
2. Let the embedding medium harden. Note that the refractive index can change over time.
3. Record a number of images at the microscope settings you also use when recording the images you want to restore.
4. Align and average the individual bead images from single or multiple files using the Huygens PSFDistiller
5. Reconstruct the PSF from the averaged bead image.
6. Store the resulting PSF in ICSfileFormat for later usage.

This post describes steps 1 and 2 of the procedure. To see steps 3-5 using the Huygens Software, go to Psf From Beads.

Practical beads

- Beads for Widefield PSF
- Beads for Confocal PSF
- Beads for STED PSF

Multicolor beads are very useful to calibrate also the → Color Shift.

The beads are appropriately diluted, absorbed to a coverslip, embedded in your usual embedding medium and placed on an object slide. When prepared properly, you should hardly see any Bleaching Effects during the recording of an image. This allows you to use a high averaging count during image acquisition.

Sample preparation

(From an Invitrogen Product Information Sheet)

Experimental protocols depend somewhat on the instrument and software used; please refer to the materials applicable to your particular instrument. The following serves as a guideline for mounting Tetraspeck microspheres on microscopic coverslips.

1. Use clean coverslips with a thickness for which your microscopic objectives are corrected. Special cleaning is usually not required. It is advised to coat the coverslip with for example Poly-L-lysine to immobilize the beads.
2. If desired, the beads in suspension can be diluted with distilled water before use. Before sampling, be sure that the beads are uniformly suspended by mixing on a vortex mixer or by sonicating.
3. Apply 5 µl of the Tetraspeck bead suspension to the surface of a coverslip and spread with the pipette tip. Wait for the droplet to dry and then apply ~15 µl of glycerol, water, immersion oil, or other embedding mounting medium over the dry sample of beads. Some immersion oils may gradually extract dye from the microspheres, resulting in diminished bead fluorescence and increased

background fluorescence. Consequently, the durability of bead samples prepared using oil may be limited.
4. Place the coverslip with the beads and embedding medium down on an object slide. If needed, seal the coverslip with nail polish, quick-drying glue or melted paraffin.
5. Note, that certain embedding meda continue to harden over time during which the refractive index changes. You may want to check out the specifications of your embedding medium.

N.B.: as the beads should be measured under the same conditions as you will use later for your samples, the same mounting medium should be used as well!!!

Record images

To record bead images, follow these steps:

1. Set the recording parameters to the **same values** you will use for recording the object you want to restore. **Take care that you do not undersample the image.** Recomended Sampling Distances are about 50 nm lateral sample interval and 100 nm axial sampling interval for confocals, the double for widefields. (But see *Imaged volume* below for details!!!).
2. Average the image as much as possible, for instance 16 or 32 times. **Avoid conditions where the intensities of the image before averaging are clipped.** Clipped Images are obtained when the Dynamic Range of the input signal exceeds that of the analog to digital converter (ADC). In practice this means that the lowest values in the input image are actually negative, and/or that the highest values should in fact be represented by a number greater than 255. Negative values are usually converted to 0; values above 255 to 255. (Some microscopes have ADCs with a larger range than 0-255).

- Confocal data: Record a number of images of lateral size in the order of for example 512x512 and 40-50 sections. Use the Nyquist calculator to acquire the images at optimal sampling intervals. There should be 1-10 beads in each image. If the beads are too close to each other or to an edge they will be rejected at a later stage. In addition you can cut away empty areas in an image later on, so it is not necessary to search for images with say 5 equally spaced beads. In total you need between 5 to 10 usable beads.
- Widefield data: You should be able to obtain a strong signal from a single bead with not too much bleaching. Make sure that it is indeed a single bead and not a cluster. Use the Nyquist calculator to acquire the images at optimal sampling intervals. With an objective with Numerical Aperture NA = 1.3 50-100 sections at 200 nm distance should be sufficient, to cover at least 12 µm along Z. Although you have just a single bead, you still need to apply the Average bead tool to centre the bead and to remove the BackGround.

Imaged volume

If the number of planes recorded along Z is too low, you will get an error when distilling the PSF: "the axial image size is too small". It can also happen that this error is not displayed but no usable bead is found in your image.

What matters for a correct PSF distillation is not (only) the number of slices, but the total physical volume that is actually imaged. A good bead image includes information of the cone of blur around it, that specially in Wide Field Microscopes and in low Numerical Aperture (NA) cases can be very large. For example, when using a 0.95 NA objective in a widefield microscope the PSF is very large along Z, and a larger volume must be imaged in this direction than in the NA = 1.3 example above to register all the relevant information. You could indeed acquire more planes, but you can also combine that with a reduction of the Sampling Density along Z. The ideal sampling rate (that depends on the NA) for normal imaging can be found using the Nyquist Calculator, but when recording beads some OverSampling is recommended.

If you ask the calculator to show also a PSF, you can find out how large it is expected to be, both in real

volume and in number of samples when imaged at the Nyquist Rate. (Experimentally you can record some less planes along Z than that is shown there, because it is not recommended to acquire very low intensity regions much affected by noise. But that theoretical PSF is a good guide to see how much volume is necessary to distill a PSF).

For a 0.95 NA typical widefield microscope the beads should be recorded in a 3D image that covers at least ~ 21 µm along the optical axis. The Nyquist Rate along Z for these conditions is one plane every ~ 700 nm. If we record the beads with some OverSampling (recommended), at one plane every 500 nm, we need at least 42 planes to cover that volume. At that rate, despite we recorded less planes than in the NA = 1.3 example above, we have covered much more 3D volume!!!

Bead density

If the number of beads in the image is of an order much larger than 10, chances are that they are too close to each other. (Therefore their blurred images overlap, and it is no longer possible to disentangle their PSF's). The Psf Distiller will reject beads that are too close to each other. You can make its criterion less strict by using the "reduce PSF size" parameter, but there is a limit for this to work properly.

Examples

- This could be an example of a bead image, is a top view Fast Mip. The concentration of beads is quite good (the beads are well and evenly spread), but an even lower concentration would be even better: a low concentration will minimize the chance of bead clusters and/or overlapping PSF's.

Beads for measuring a confocal PSF

Recording Beads is a necessary first step in distilling an Experimental Psf, but sometimes it is difficult to find the correct fluorescent beads to use in a Confocal Microscope.

Some recommended Tetraspec beads (Invitrogen) work fine for Wide Field Microscopes, but users reported that for confocals they are not bright enough. The 200 nm beads are already a challenge. Especially when the slides are old. Green tends to bleach real fast. .

Invitrogen has special beads for PSF measurements: PS-Speck(tm) Microscope Point Source Kit with single color beads. The Tetra speck beads will be less bright. There is less dye of a given color in each bead, this minimizes cross-signal for each color. Also, this means that each color will probably be less bright than their single-color counterparts. The only beads made for point-source work are the ones in the PS-Speck kit.

Users at the Erasmus Medical Center in Rotterdam report that 100 nm green and red beads from Duke Scientific (now Thermo Scientific) are very bright and can be scanned perfectly with low laser settings.

They also advise the following:
Be generous when putting beads on a glass. It's an absolute pain to try to find the focal plain when they are too widely spread. And there is always a spot on the glass where they are nicely spread and you can have about 6 usable beads in a field of
view. Good for averaging and saves time scanning many fields for adecent psf.

It is not wise to do a 2 colour bead scan (even multi-track) of 100nm bead mix for PSF reconstruction. The red are too "green" and leak in the 505-550 spectrum too much leaving just enough intensity for Huygens to decided that it is a green bead and gives false intensities. Especially when a red and a green bead are laying on top op each other or are touching each other you will have a big problem distilling a correct PSF.

Experimental PSF with the Huygens PSF distiller

By default, Huygens uses a **theoretical PSF** to deconvolve your image data, so no extra work is needed. When a deconvolution run is started the theoretical PSF will be automatically calculated from the microscopy parameters and used "on the fly".

Huygens also offers the option to load and use an **experimental PSF**. With the Huygens PSF Distiller Option, you can even make your own experimental PSF from bead images (see Recording Beads). The PSF Distiller is a wizard-based option that assist in properly distilling a PSF from single and multiple channel data. This data can be multiple beads recorded as separate images, multiple beads in the same image, or both.

Chromatic shift, if present, will be reported by the PSF Distiller wizard, and can subsequently be corrected with the Chromatic Aberration Corrector option.

The PSF distiller uses a Non Linear Iterative Method to measure a PSF when your beads are not small enough to provide a direct measurement of the PSF (i.e. they are not Sub Resolution in terms of Band Width).

To learn how it works, refer to Psf From Beads. For a practical tutorial see Using The Psf Distiller.

You can Test The Software to see how it works.