Electronic Supplementary Material (ESI) for Nanoscale

On-chip light sheet illumination enables diagnostic size and concentration measurements of membrane vesicles in biofluids

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1. Supporting methods

1.1. Microscope set-up

Excitation light is coupled into the planar waveguide of the chip by illuminating the waveguide core layer with the output of an optical fiber. The cleaved side of a single mode patch cord fiber (Newport, The Netherlands) with an operating wavelength of 633 nm is used for this purpose. The other side of the patch cord contains an FC/PC connector which is used for coupling laser light into the fiber. Excitation light with a wavelength of 640 nm is provided by a 100 mW diode-pumped Excelsior solid state laser (Spectra Physics, The Netherlands). The fiber core is carefully aligned with the planar waveguide core layer using an ULTRAlign precision linear stage (Newport, The Netherlands). This is possible by using a standard CCD camera to monitor the waveguide exit at the other side of the chip, see **Supporting Figure 1a**. Optimal alignment between fiber and waveguide results in a light sheet in the microchannel, and part of this light enters the second half of the planar waveguide. This situation is thus obtained when a maximum amount of light emerges from the core layer at the waveguide exit, as depicted in **Supporting Figure 1b**.

The chip with fiber is placed under a Nikon Plan Fluorite 40× objective lens with a numerical aperture (NA) of 0.75, using an objective lens inverter (LSM Tech, USA) connected to a Nikon TE 2000-E microscope (Nikon, Belgium), see **Supporting Figure 2**. The fluorescence light coming from the sample is collected by the objective lens, as shown in **Supporting Figure 1a**, and imaged on an electron multiplication charge coupled device (EMCCD) Cascade II:512 camera (Roper Scientific, USA). A pair of achromat lenses is placed in between the camera and microscope side port for an extra 2× magnification of the image on the EMCCD chip so that one pixel corresponds to a distance of 196 nm in the sample. High-speed movies and image stacks are recorded using the Nikon Elements AR imaging software.

1.2. Simulation of the light sheet

Assume a planar waveguide with a core layer of 5 µm thickness surrounded by two cladding layers of 25 µm thick. The core layer has a refractive index n_{co} = 1.595 of SU-8, and both core layers have a refractive index n_{cl} = 1.594 of SU-8 mixed with epoxy resin [1]. Consider further a sample with the same refractive index as water n_{sa} = 1.333 and light with a wavelength of λ = 640 nm. We have simulated the behaviour of the fundamental waveguide mode exiting the planar waveguide into the sample, using the Python eigenmode modelling framework CAMFR (http://camfr.sourceforge.net/), as illustrated in **Supporting Figure 3**.

The Full Width at Half Maximum (FWHM) of the spatial intensity distribution coming from the fundamental TE mode is around 4.6 µm right outside the waveguide. At a distance of 50 µm outside the waveguide, the FWHM is around 5.4 µm, and at distance of 100 µm it has increased to 7.5 µm. The fundamental TM mode behaves similar to the TE mode. These results indicate that the light sheet generated by the planar waveguide based on SU-8 does not strongly diverge over distances that are typical for the field of view in fSPT experiments.

1.3. Experimental characterization of the light sheet

Since out-of-focus light is not rejected in the detection path of the microscope, it is not possible to indirectly characterize the light sheet by simply measuring the intensity of a fluorescent sample in function of the *z*-position. However, if the sample is a dispersion of fluorescent particles, the background corrected intensity I_p - I_b of the particles in focus does not contain out-of-focus contributions, with I_p the intensity of the particle and I_b the local background intensity. Using image processing, the light sheet can thus be characterized by the background corrected intensities of the particles in focus in each image of *z*-stack. The range where the light sheet is located, manifests itself as an intensity peak, and the FWHM of that peak is a measure of the light sheet thickness.

Care should be taken when measuring differences between *z*-positions in microscopy, because it is necessary to account for the possible mismatch between the refractive index of the objective lens immersion medium n_{im} and the refractive index of the sample n_{sa}. In case of a mismatch, the real *z*-position difference d_{re} will not be equal to the observed difference d_{ob}. We have measured the microchannel height to be 56.6 µm with a Dektak 150 profilometer (Veeco, Germany), which is in good agreement with the 55 µm as expected from the fabrication process. On the other hand, the *z*-stacks of images recorded in the microchannel suggest a height of 41.1 µm. This leads to the correction $d_{re} = 1.38 d_{ob}$ for the refractive index mismatch. This is close to the ratio of the refractive index $n_{sa} = 1.333$ of the water, which is the sample medium, and the refractive index of air $n_{im} = 1$, which is the objective lens immersion medium, as suggested for low NA [2]. The reported values in the manuscript are corrected for this effect.

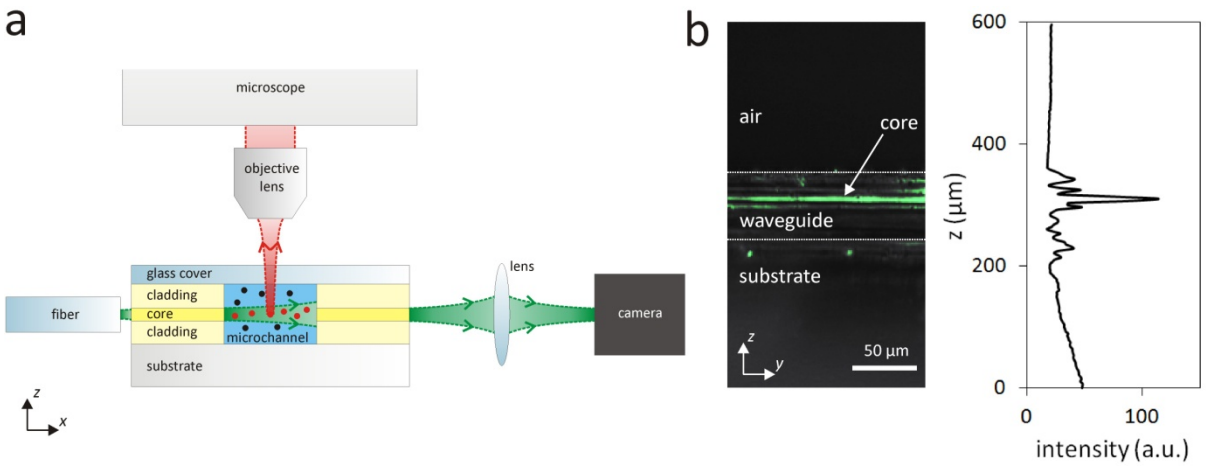
The light sheet is characterized for both the glass and silicon chip. The microchannel is filled with a water dispersion of dark red fluorescent (660 nm excitation peak, 680 nm emission peak) polystyrene 0.2 µm diameter FluoSpheres (Molecular Probes™, Life Technologies Europe, Belgium). A *z*-stack of images with a step of 0.1 µm is recorded throughout the microchannel, with both sheet illumination and epi-fluorescence illumination. In each frame of the stack, the intensity I_p and local background I_b of each particle was determined off line, according to a previously published protocol [3]. In brief, first a suitable intensity threshold is applied to each frame, which allows to identify the particles as the spots in the image that consist of pixels with an intensity that exceeds the threshold. Subsequently the contour of each such particle spot is determined and a second background contour is drawn at a small distance (e.g. 3 pixels) around each particle contour. The intensity I_p of a particle is then calculated as the average intensity of the pixels inside the particle contour, and the local background I_b of the same particle is determined as the average intensity of the pixels along the corresponding background contour. The FWHM of the light sheet peak is obtained by fitting a two-dimensional Gaussian function to the average background corrected intensity I_p - I_b in function of the *z*-position.

1.4. Contrast measurements

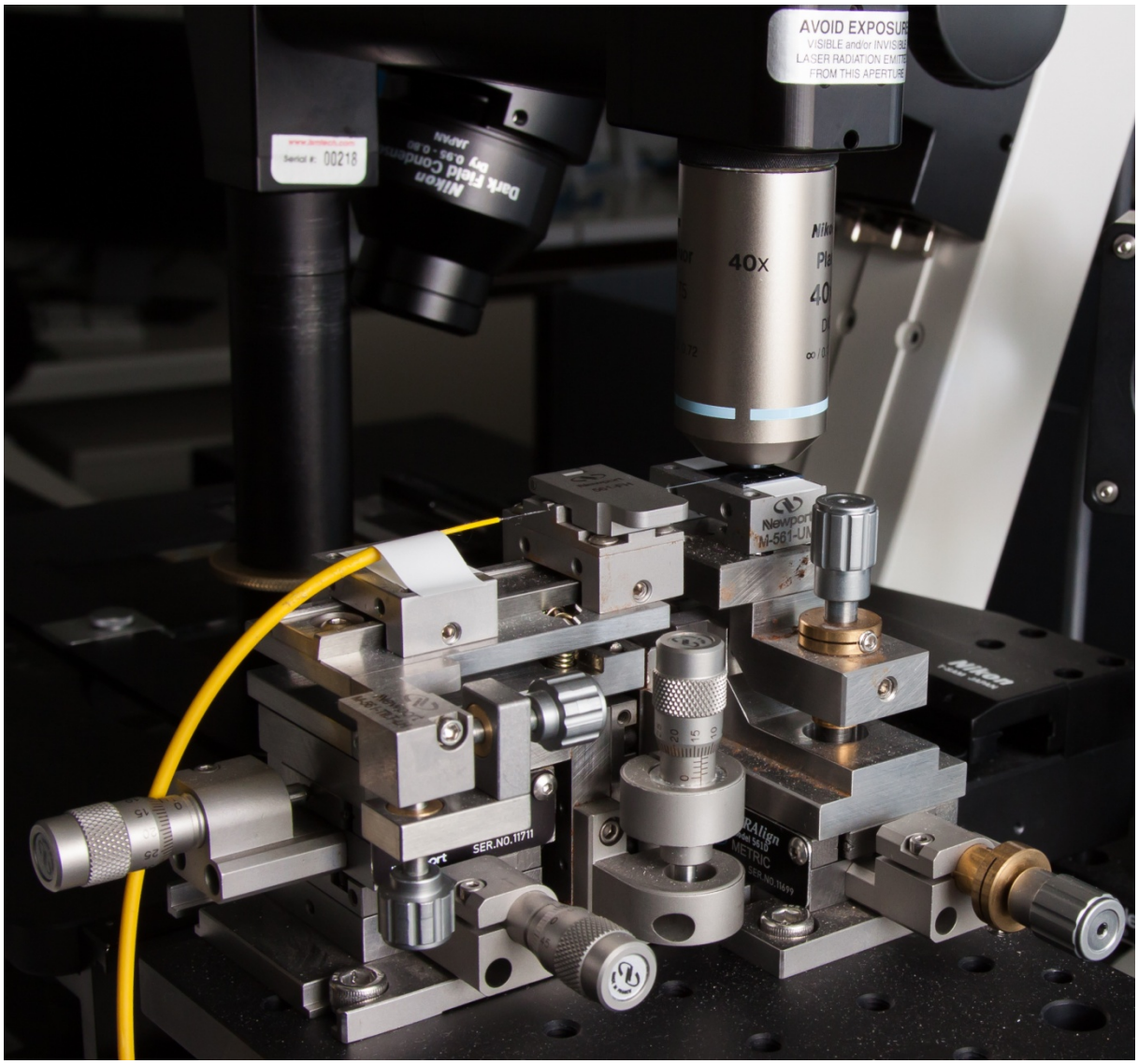
The contrast is measured by recording images of a dispersion of fluorescent particles inside the light sheet. Using image processing, the intensity I_p and local background I_b of each particle is determined (see Section 1.3), and $(I_p - I_b)/(I_p + I_b)$ is used to quantify its contrast [4].

The contrast is measured in both the glass and silicon chip. The microchannel is filled with a water dispersion of dark red fluorescent (660 nm excitation peak, 680 nm emission peak) polystyrene 0.2 μm diameter FluoSpheres (Molecular Probes™, Life Technologies Europe, Belgium). To mimic different fluorescent backgrounds, different concentrations of the red dye Cy5 are added to the water. The microscope was focused at the centre position of the light sheet and several images were recorded with epi-illumination and light sheet illumination. In each image, the average contrast of the nanospheres was calculated off line [3].

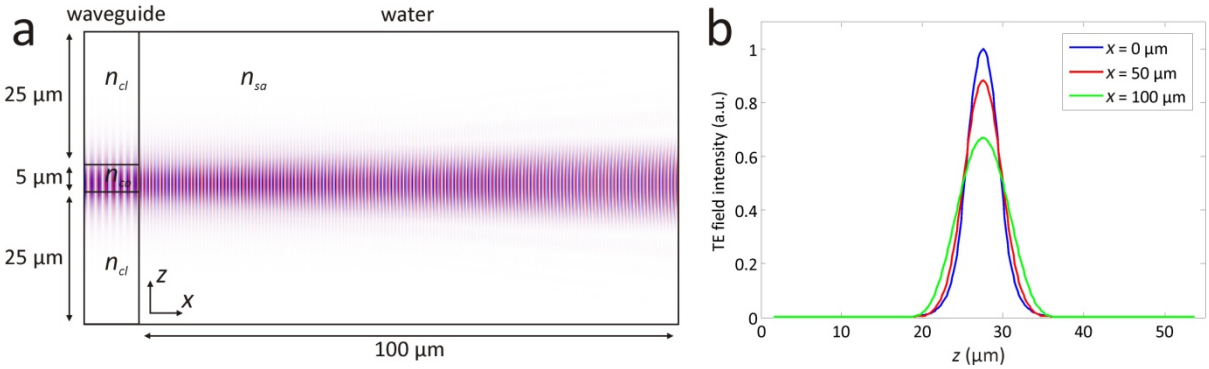
2. Supporting figures



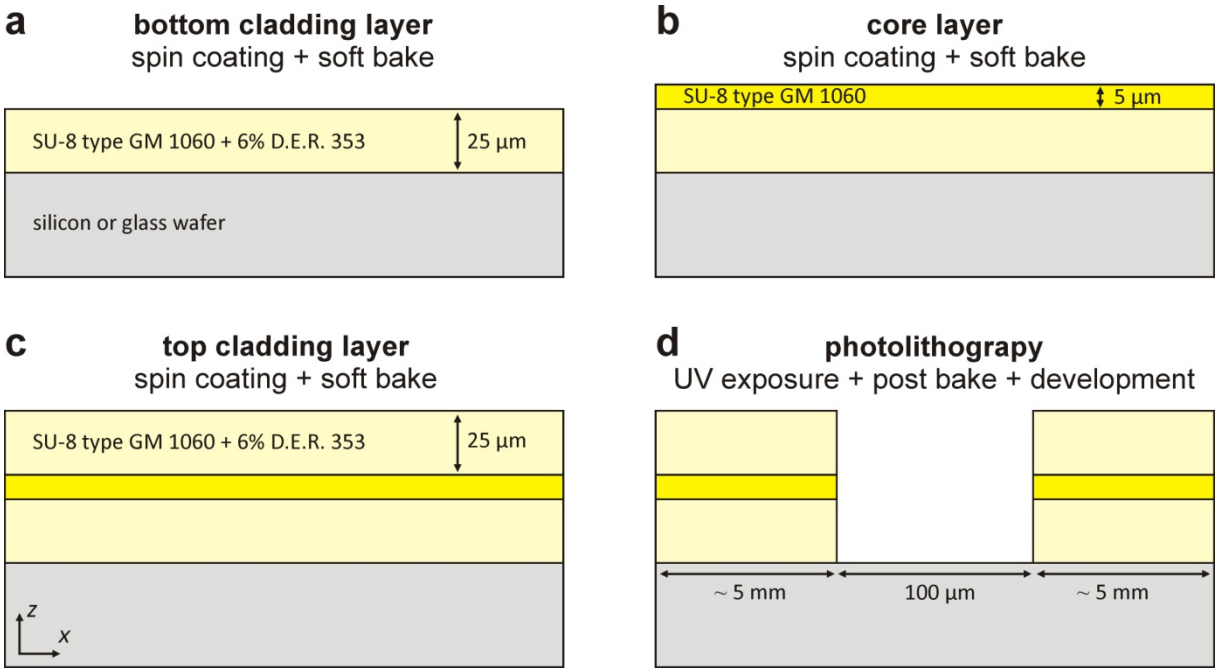
Supporting Figure 1. Coupling the excitation light into the planar waveguide. a) A cross section of the set-up showing the fiber that is used to couple the excitation light into the planar waveguide. The fluorescence light from the sample in the microchannel is detected by the objective lens of the microscope. Successful incoupling of the excitation light into the planar waveguide can be monitored by imaging the other side of the chip. b) In case of successful alignment between fiber and planar waveguide, the waveguide output as seen by the camera exhibits a clear line profile at the centre of the waveguide.



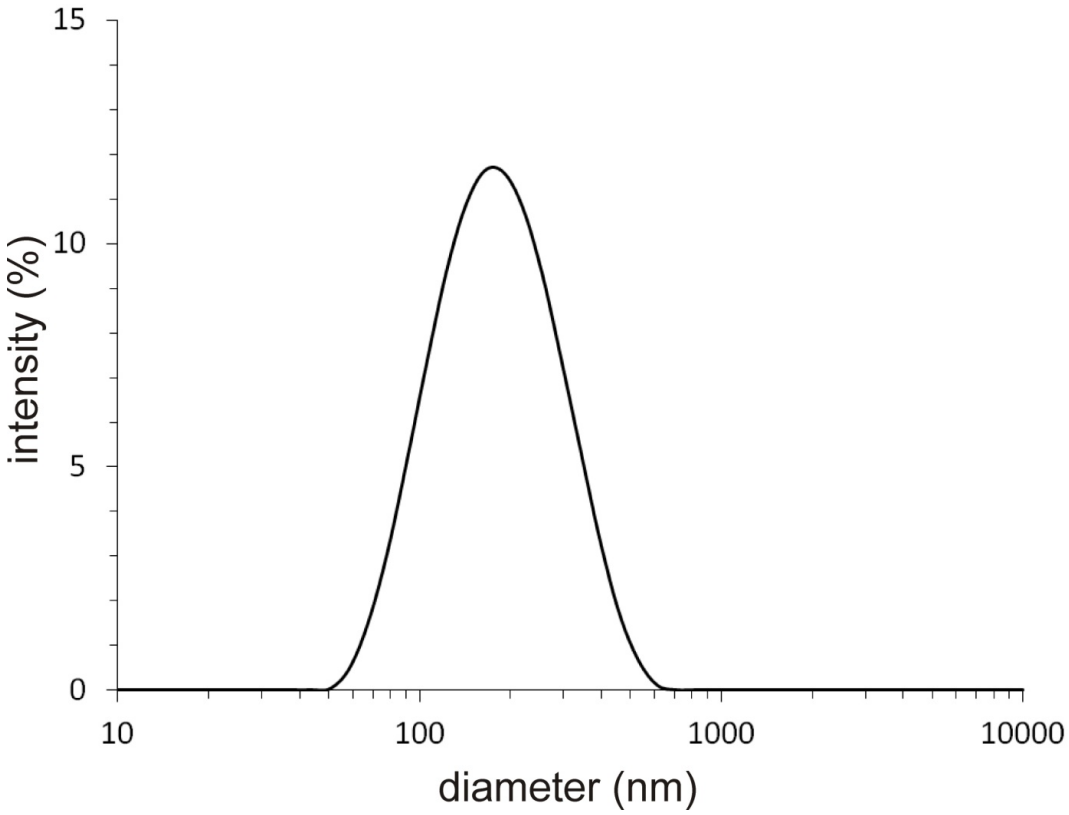
Supporting Figure 2. Photograph of the microscope set-up. A chip with silicon substrate is placed under an objective lens that is attached to an objective lens inverter that sends the fluorescence light to the microscope. The cleaved side of a fiber core is carefully aligned with the core layer of the planar waveguide using precision linear stages.



Supporting Figure 3. Simulation of the light sheet generated by a planar waveguide in a water sample. a) Visualization of the real part of the TE field of the fundamental mode exiting the waveguide. The core layer has a thickness of 5 µm and a refractive index n_{co} = 1.595, the two surrounding cladding layers have a thickness of 25 µm and a refractive index n_{cl} = 1.594, and the sample (water) has a refractive index n_{sa} = 1.333. b) The spatial intensity distribution of the light sheet along the *z*-axis directly outside the waveguide at x = 0 µm has a FWHM = 4.6 µm. At a distance x = 50 µm from the waveguide this becomes FWHM = 5.4 µm, and after x = 100 µm it becomes FWHM = 7.5 µm. The behaviour of the fundamental TM mode is similar.



Supporting Figure 4. Illustration of the chip fabrication process. a) In the first step, the bottom cladding layer that consists of SU-8 type GM 1060 mixed with 6% of D.E.R. 353 is spin coated on a glass or silicon wafer and soft baked. b) In the second step, the core layer that consists of pure SU-8 type GM 1060 is spin coated on the bottom core layer and soft baked. c) In the third step, the top cladding layer that consists of SU-8 type GM 1060 mixed with 6% of D.E.R. 353 is spin coated on the core layer and soft baked. d) In the fourth step, the 3-layer structure is exposed to UV light using a mask, post baked, and developed, in order to create a microchannel of 100 µm width.



Supporting Figure 5. Cell-derived membrane vesicle size distribution obtained by dynamic light scattering. Size distribution measurements are performed by dynamic light scattering on cell-derived MVs in cell culture medium. The MVs have a diameter between 50 nm and 700 nm with a peak around 170 nm.

3. Supporting tables

Chip fabrication process		
Process step		**Details**
1. bottom cladding layer	1.1 spin coating	• 10 s acceleration at 100 rpm/s • 40 s at 1000 rpm • 1 s acceleration at 400 rpm/s • 1 s deceleration at 400 rpm/s • 5 s at 1000 rpm • 10 s deceleration at 100 rpm/s
	1.2 soft bake	• 25 min increase at 4°C/min starting from 20°C • 25 min decrease at 4°C/min starting from 120°C
2. core layer	2.1 spin coating	• 5 s acceleration at 100 rpm/s • 5 s at 500 rpm • 30 s acceleration at 100 rpm/s • 40 s at 3500 rpm • 1 s acceleration at 400 rpm/s • 1 s deceleration at 400 rpm/s • 5 s at 3500 rpm • 35s deceleration at 100 rpm/s
	2.2 soft bake	idem to step 1.2
3. top cladding layer	3.1 spin coating	idem to step 1.1
	3.2 soft bake	idem to step 1.2
4. photolithography	4.1 UV exposure	• 13.5 s at 10 mW/cm^2 • 10 s pause • 13.5 s at 10 mW/cm^2
	4.2 post exposure bake	• 40 min increase at 2°C/min • 40 min bake at 95°C • uncontrolled cooling down for 90 min
	4.3 developing	• 3 min in PGMEA bath • 2 min in other PGMEA bath • N_2 drying

Supporting Table 1. The process for fabrication of the microfluidics chip with integrated planar waveguide consists of 4 basic steps. Details on spin coating and subsequent post baking for each SU-8 layer are given in step 1-3. Details on the UV exposure and subsequent post bake and development for the 3-layer structure are given in step 4.

4. Supporting videos

Supporting Video 1. A *z*-stack through the microchannel of the glass chip containing a dispersion of nanospheres. The microchannel of a glass chip is filled with a water dispersion of dark red fluorescent (660 nm excitation peak, 680 nm emission peak) polystyrene 0.2 μm diameter nanospheres. A *z*-stack of images with a step of 0.1 μm is recorded throughout the microchannel, with both epi-fluorescence illumination (left) and sheet illumination (right).

Supporting Video 2. On-chip fSPT size and concentration measurements of cell-derived MVs with light sheet illumination. The microchannel is filled with the dispersion of fluorescently labelled cell-derived MVs, and the objective lens is positioned so that the focal plane coincides with the intensity peak of the light sheet. Subsequently, a movie with a duration time of 10 seconds is recorded with a frame rate of 27.6 frames per second, an image acquisition time of 20 ms, and an image size of 450 pixels in the *x*-direction, and 192 pixels in the *y*-direction, with a pixel size of 196 nm (above). Particle trajectories are calculated off line and shown on top of the movie (below). Only trajectories of minimally 5 positions are included in the size and concentration analysis.

5. Supporting references

1. J. M. Ruano-Lopez, M. Aguirregabiria, M. Tijero, M. T. Arroyo, J. Elizalde, J. Berganzo, I. Aranburu, F. J. Blanco and K. Mayora, *Sensor Actuat B-Chem*, 2006, **114**, 542-551.
2. S. Hell, G. Reiner, C. Cremer and E. H. K. Stelzer, *J Microsc-Oxford*, 1993, **169**, 391-405.
3. K. Braeckmans, D. Vercauteren, J. Demeester and S. C. De Smedt, in *Nanoscopy and Multidimensional Optical Fluorescence Microscopy*, ed. A. Diaspro, CRC Press / Taylor & Francis Group, Boca Raton, 2010.
4. J. G. Ritter, R. Veith, J. P. Siebrasse and U. Kubitscheck, *Opt Express*, 2008, **16**, 7142-7160.