Measuring and interpreting point spread functions to determine confocal microscope resolution and ensure quality control

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Published online 10 November 2011; doi:10.1038/nprot.2011.407

This protocol outlines a procedure for collecting and analyzing point spread functions (PSFs). It describes how to prepare fluorescent microsphere samples, set up a confocal microscope to properly collect 3D confocal image data of the microspheres and perform PSF measurements. The analysis of the PSF is used to determine the resolution of the microscope and to identify any problems with the quality of the microscope's images. The PSF geometry is used as an indicator to identify problems with the objective lens, confocal laser scanning components and other relay optics. Identification of possible causes of PSF abnormalities and solutions to improve microscope performance are provided. The microsphere sample preparation requires 2–3 h plus an overnight drying period. The microscope setup requires 2 h (1 h for laser warm up), whereas collecting and analyzing the PSF images require an additional 2–3 h.

INTRODUCTION

Rationale

The use of high-resolution confocal laser scanning microscopy (CLSM) spans virtually all fields of the life sciences, as well as much of the physical sciences. In order to collect valid information from the most accurate image representations acquired with these increasingly sophisticated systems, they must be maintained and optimized for ideal performance. In order to collect diffraction-limited fluorescent images, optical components must be clean, certain optical elements such as differential interference contrast (DIC) prisms must be removed from the light path, and objective lenses must be of exceptionally high quality. Sub-resolution point sources, such as fluorescent microspheres, are ideal for assessing the performance of a confocal microscope system and the quality of its most important optical element, the objective lens.

There are two major methods used to determine the resolution of an objective lens and, in effect, of the microscope itself. A mirror slide with an appropriately mounted cover slip can be imaged and the resolution measured directly from the image[1]. Alternatively, an image stack can be collected of sub-diffraction sized fluorescent microspheres, generally referred to as point spread functions or PSFs. Although both methods measure resolution, they are not equivalent. Imaging the mirror slide results in measurements that are based on a relatively higher frequency of light (excitation laser wavelength), and results in the formation of an image that is completely coherent, containing constructive and destructive interference. In contrast, imaging fluorescent microspheres results in measurements based on a relatively lower frequency of light (fluorescence emission wavelength), an image which is completely incoherent and an image that contains no interference[2]. As the purpose in this paper is the measurement of the resolution of the imaging system from a specimen point of view, we selected the PSF method.

Diffraction, the Airy pattern and the PSF

The light originating from a sub-resolution fluorescent microsphere travels though the optics of the microscope, including the objective lens, and it is diffracted. The result is an image of the point source that is much larger than the actual size of the object (compare **Fig. 1a**,**b**). This larger central point in the image is then surrounded by diffraction rings of many orders (**Fig. 1**). The diffraction rings result from the constructive interference of diffracted light waves originating from the point source, whereas the dark areas between the bright rings are caused by the destructive interference of the light waves. This spreading out of light from a point source by diffraction was first described by Sir George Biddell Airy, an English mathematician and astronomer. The resulting pattern was thus coined the Airy pattern, and its center the Airy disk[3]. As the light from the point source is spread by diffraction, this pattern is more generally referred to as a PSF.

PSF characteristics

The size of the Airy disk and the spacing between the diffraction rings will depend on three things: (i) the wavelength of light being emitted from the fluorescent microsphere, (ii) the numerical aperture of the objective lens and (iii) the size of the object. Shorter wavelengths, such as in the blue region of the visible spectrum will give a smaller image of a sub-resolution point source with closer spacing of the diffraction rings. Longer wavelengths of light, such as in the red region, will give a larger Airy disk with greater spacing between the diffraction rings (http://micro.magnet.fsu.edu/primer/java/imageformation/airydiskbasics/index.html). For example, a 100-nm diameter point source (fluorescently labeled polystyrene microsphere), excited with a 488-nm laser which then emits 530-nm light, results in an image in which the point source will appear to be ~350 nm in diameter (at the full-width at half maximum (FWHM)) when imaging with a ×63 oil-immersion objective lens with a numerical aperture (NA) of 1.

High-NA lenses collect a larger cone of light, including the more highly diffracted light originating from fine features within the sample. They thereby generate smaller PSFs and have an increased resolving power. Diffraction not only causes the light to spread

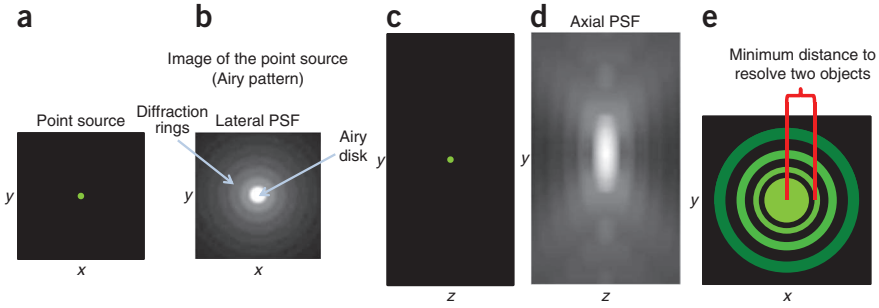


Figure 1 | PSF schematics and theoretical functions. (**a**–**d**) Schematic representation of a 100-nm point source in the *xy* plane (**a**) and the *yz* plane (**c**). Simulated images for a theoretical PSF for a oil-immersion objective lens (1.3 NA) with *xy*-axis pixel sizes of 23 nm and *z*-axis spacing of 20 nm, with an emission wavelength of 557 nm for the *xy* plane (**b**) and the *yz* plane (**d**). The images in **b** and **d** were contrasted with a γ factor of 0.5 to enhance the visualization of the dim diffraction ring patterns. (**e**) A schematic of the diffraction pattern showing the minimum distance that the two objects can be apart in order to resolve them by the Rayleigh criterion. The red lines indicate the center of the Airy disk or the zero-order diffraction spot and the center of the first dark diffraction band within the Airy pattern.

out along the lateral optical axis of the microscope (**Fig. 1a**,**b**), but also along the axial axis in the direction of the propagation of light (**Fig. 1c**,**d**). This spreading or distortion of the light in the axial image plane is even more pronounced than in the lateral image plane (compare **Fig. 1b**,**d**). In fact, the size of the PSF along the axial axis can be estimated at three times that of the lateral axis. Therefore, in the example presented above, the image of the point source would appear to be ~1 μm in diameter (FWHM) in the axial direction (**Fig. 1d**). What this means is that the 3D image of the sub-resolution object is extended along the optical train of the microscope in the *z*-axis image plane (**Figs. 1d** and **2**).

Laser scanning confocal PSFs

In confocal microscopy, the pinhole is used to block out-of-focus light, thus improving the resolution and the contrast in 3D image volumes. The majority of the diffracted light will come from out-of-focus planes; therefore, small pinhole settings (e.g., 1 Airy unit) eliminate the diffraction rings, resulting in a PSF with a compact shape (**Fig. 2a**,**c**). However, at large pinhole settings (e.g., 4–5 Airy units) the PSF has a much broader shape (**Fig. 2b**) and diffraction rings above and below the in-focus image plane are visible (**Fig. 2d**).

Resolution

The resolution of an optical system is its ability to distinguish two objects from one another, when they are in close proximity. We use the Rayleigh criterion, which states that in order to resolve two points they must be no closer together than the distance from the center of the PSF to the first destructive interference band in the diffraction pattern (**Fig. 1e**). The measured resolution, in the lateral and axial planes, is the FWHM of the 3D PSF generated from sub-resolution fluorescent microspheres. Although there are several different theoretical resolution formulae, the resolution for the confocal microscope can be calculated on the basis of the following formulae, which represent the Rayleigh criterion[4,5]:

$$\text{Lateral resolution} = \frac{0.51\lambda_{exc}}{NA} \quad (1)$$

$$\text{Axial resolution} = \frac{0.88 \quad \lambda_{exc}}{(n - \sqrt{n^2 - NA^2})} \quad (2)$$

where λ_{exc} is the excitation wavelength of the laser, *n* is the refractive index of the immersion medium and NA is the numerical aperture of the objective lens. There are many expressions in the literature for the lateral and axial resolution of the confocal microscope. Some are relatively straightforward and some are highly complex, taking into account many other factors that affect image resolution (e.g., emission wavelength, sample thickness and pinhole size). We have chosen the two expressions above, because they appropriately estimate the image resolution and they are relatively straightforward to understand and calculate.

From our experience, for a high-resolution optical system with a high-quality objective lens (under ideal conditions), the size of the PSF from a sub-resolution fluorescent microsphere should be within 10–40% of the theoretical resolution of the microscope. The shape of the PSF can also be used to determine the quality of the entire imaging system, including the objective lens being used and the quality of the microsphere sample preparation.

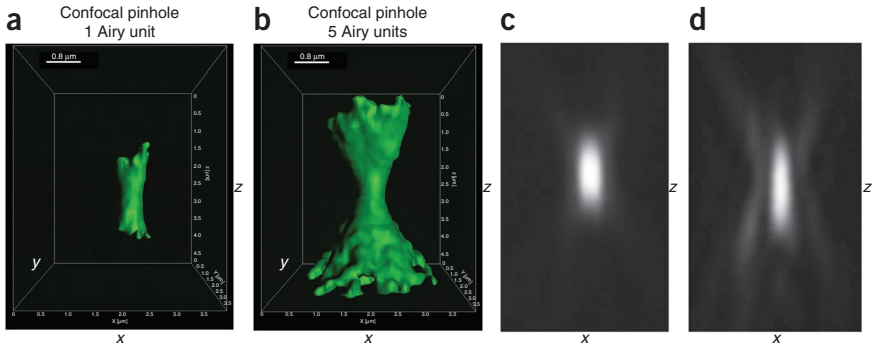


Figure 2 | Microsphere images and isosurfaces from a confocal microscope. Confocal images of a 100-nm-diameter green microsphere collected using a ×63/1.4 NA oil-immersion objective lens. (**a**–**d**) With a small pinhole (1 Airy unit), the 3D isosurface (**a**) and the image (**c**) of the PSF are compact and the diffraction rings outside of the focal volume are mostly absent. With a large pinhole (5 Airy units), the PSF is much larger and the diffraction pattern outside of the focal volume is evident from the hourglass shape of the PSF 3D isosurface (**b**) and image (**d**). The confocal microscope has a much higher resolution along the *z* axis when the pinhole is set to 1 Airy unit. Scale bar, 0.8 μm.

PSF theory, measurements and applications

Detailed publications regarding theoretical PSF calculations and experimental measurements are available in the literature[6–8]. Theoretical and measured PSFs can also be used for a variety of other applications.

For example, the PSF can be used to estimate the excitation volume of the focused laser beam in the confocal microscope. This volume estimate can be used in combination with correlation microscopy techniques in order to convert correlation function amplitudes into absolute protein, lipid or fluorophore concentrations[9–13]. In addition, the PSF measurements can be used as an input for 3D image deconvolution algorithms, allowing for highly accurate 3D image deconvolution[14–17]. Finally, the PSF can be used to assess the quality of a microscope's wide-field illumination sources, including newer light-emitting diodes[18].

This protocol provides instructions for preparing sub-resolution fluorescent microsphere samples, guidelines on how to collect 3D PSF image volumes of these samples and information on how to measure the optical resolution of your microscope from these PSF images. Although it was developed on a Zeiss 710 confocal microscope, the procedure is applicable to any optical imaging system, including wide-field microscopes. There is also a section on how to troubleshoot what may be wrong with your optical system if the shape of the PSF is not symmetric and/or its size is not close to the theoretical resolution of the system. Finally, a set of detailed **Supplementary Methods** are provided. These methods outline procedures for making PSF measurements on confocal laser-scanning microscopes from all of the major manufacturers (**Supplementary Methods**).

MATERIALS

REAGENTS

- Green-yellow microspheres, 100 nm (Invitrogen, cat. no. F8803)
- Green-yellow microspheres, 175 nm (Invitrogen, cat. no. 7220)
- Microspheres sampler kit (Invitrogen, cat. no. T-7284)
- Microspheres, 100 nm, various colors (Invitrogen; see REAGENT SETUP)
- Cover slips (18 mm × 18 mm, certified to be 0.170 ± 0.005 mm, Carl Zeiss, cat. no. 474030-9000)
- Cover slips (no. 1.5, 22 mm × 22 mm, thickness between 0.160 and 0.190 mm, Fisher Scientific, cat. no. 12-520B)
- Microscope slides, Fisherbrand (Fisher Scientific, cat. no. 22-178-277)
- ProLong Gold mounting medium (Invitrogen, cat. no. P36934; see REAGENT SETUP)
- Cytoseal mounting medium (Thermo Fisher Scientific, cat. no. 8310-16; see REAGENT SETUP)
- Cargille immersion oil, type LDF (Cargille Labs, cat. no. 16241; see REAGENT SETUP)
- Tiffen (Royal Photo) or Ross (SPI Supplies) lens tissue
- Lens cleaner diluted 1:5 with dH_2O (e.g., Glass Plus or equivalent cleaner that does not contain ammonia)
- Ethanol
- Distilled water (dH_2O)
- Fluorescent plastic slide (Chroma Technology)
- Green dye, e.g., Alexa Fluor 488 (Invitrogen)

EQUIPMENT

- Zeiss 710 laser-scanning confocal microscope or equivalent (×63/1.4 NA, oil-immersion objective lens) attached to a Zeiss AxioObserver motorized inverted microscope on a vibration isolation air table; Carl Zeiss)
- Argon ion laser (Melles Griot; see EQUIPMENT SETUP)
- Zen software (Carl Zeiss)
- Fiji software (http://fiji.sc/wiki/index.php/Fiji) with MetroloJ plug-in (http://imagejdocu.tudor.lu/doku.php?id=plugin:analysis:metroloj:start)
- Computer (Pentium 4 or higher running Windows XP, with 2GB or more of RAM)
- Conical tubes, 15 ml
- Beakers
- Bunsen burner
- Forceps
- Sonicating water bath
- Kimwipes (Kimberly-Clark)
- Latex or nitrile gloves
- Aluminum foil
- Cotton swabs

REAGENT SETUP

Fluorescent microspheres We use green-yellow microspheres of 100-nm or 175-nm diameters. The 175-nm microspheres come as part of a microscope point source kit that includes blue, green, orange and deep red microspheres. This kit also contains a non-hardening mounting medium for making up to 100 slides, although hardening mounting medium can be used. Microspheres should be no larger than 175 nm for testing high-NA immersion-objective lenses. Different-colored microspheres or slides with mixed populations of microspheres can also be used. Recommended microspheres include various colors of 100-nm-diameter carboxylate-modified microspheres from Invitrogen: blue (350/440; cat. no. F8797), orange (540/560; cat. no. F8800) and red (580/605; cat. no. F8801). Larger 500-nm-diameter microspheres can be used for low-NA lenses (< 0.6), such as TetraSpeck four-color (Invitrogen, microspheres sampler kit) or single-color (yellowish-green—Invitrogen, cat. no. F-8813) microspheres. These large microspheres can be mixed with the smaller microspheres to make finding the 100- or 175-nm microspheres easier.

Mounting medium We use either ProLong Gold or Cytoseal hardening mounting medium. After curing, ProLong Gold has a refractive index of 1.46, whereas Cytoseal is slightly higher at 1.48. These match the refractive index of the glass and immersion oil (1.515) well. Having matching indices of refraction increases the resolution of the microscope by reducing the loss of highly diffracted light from refraction at the interface between the glass cover slip and the mounting medium. Other types of medium can be used, but the protocol should be tested, as the microspheres are not soluble in all types of medium. It should be noted that the refractive index of the microspheres may not perfectly match that of the mounting or immersion medium, which can cause spherical aberrations or distortions within the PSF.

Immersion oil We have previously used Cargille oil, type DF. However, Cargille Labs has stopped manufacturing the DF series of oils. They do offer a new replacement oil for room-temperature (23 °C) work (type LDF). If you are working at 37 °C, then use high-temperature oil (type 37, cat. no. 16237). Mismatching the oil and the application will cause losses in resolution due to aberrations[19]. Cargille Labs has not generated an LDF-equivalent oil for use at 37 °C. Be aware that the 37 °C HF oil may be autofluorescent in the UV range, so it is not well suited for working with blue live-cell dyes, such as Hoechst, which are excited in the 350–400-nm range.

EQUIPMENT SETUP

Lasers, filters and mirrors Excite the 100- or 175-nm yellow-green microspheres with the 488-nm laser line of a 25-mW argon ion laser. Direct the laser onto the sample using a 488-nm main beam splitter (also referred to as a dichroic mirror). Collect fluorescence emission from 500 to 600 nm with one of the photomultiplier tube (PMT) detection channels. Collect images using the Carl Zeiss Zen software.

PROCEDURE

Preparation of fluorescent microsphere slides ● TIMING 14 h 45 min

1| Vortex the bottle of microspheres.

▲ CRITICAL STEP Any microsphere sample that is used must be sub-resolution for the objective lens being tested.

2| In a 15-ml conical tube, dilute 100 µl of microsphere solution to 10 ml with dH_2O for a 1:100 or 10^2 dilution factor.

3| In a second 15-ml conical tube, dilute 100 µl of the 10^2 diluted solution from Step 2 to 10 ml with dH_2O. This will be a second 1:100 dilution for a total stock dilution of 10^4.
▲ **CRITICAL STEP** These dilution steps are specific for the 100-nm microspheres from Invitrogen, which come as 2% solids in solution. Different dilution factors may be needed for other fluorescent microsphere sources.

4| Place the conical tube from Step 3 (10^4 stock dilution) in a sonicating water bath for 20 min. This will break up any aggregated microspheres and avoid the presence of microsphere clusters in the slide preparations.
▲ **CRITICAL STEP** Sonication is required, or clusters of microspheres within the sample may make it difficult to find individual microspheres for imaging. If microsphere clusters are imaged, the size of the PSF will be overestimated, resulting in an underestimate of the microscope resolution.

5| In a third 15-ml conical tube, dilute 100 µl of the sonicated 10^4 stock dilution to 10 ml with 9 ml of dH_2O and 900 µl of 70% (vol/vol) ethanol. This will be the final microsphere solution, a 10^6 stock dilution. If desired, larger 0.5-µm microspheres can be added to the suspension to aid in finding the cover slip surface.

6| Wash microscope slides with 70% (vol/vol) ethanol and wipe dry with a Kimwipe. Label the microscope slides appropriately.
▲ **CRITICAL STEP** Latex or nitrile gloves should be worn for this step and the remainder of the preparation of slides. This will avoid any oil from fingerprints getting on glass surfaces and potentially affecting image quality.

7| Wash no. 1.5 cover slips by placing them in a small beaker filled with 70% (vol/vol) ethanol. Lift them out of the beaker with pointed forceps and flame them with a Bunsen burner. This will create a hydrophilic surface on the glass, thus allowing the microsphere droplets to spread and create an even distribution of microspheres on the glass.
▲ **CRITICAL STEP** Most nondipping, high-quality immersion objective lenses are specifically corrected for 0.170-mm-thick cover slips. Therefore, no. 1.5 cover slips (or cover slips as close to 0.170 mm in thickness as possible) should be used.

8| Vortex the final microsphere solution immediately before use and pipette 10–20 µl directly onto the cover slip.
▲ **CRITICAL STEP** The microspheres must be placed directly onto the cover slip so that they are as close as possible to the objective lens when imaging. If the microspheres are placed on the microscope slide, spherical aberrations will be high, as the light must travel through a layer of mounting medium to reach the microspheres with this sample architecture. Most objective lenses are corrected for spherical aberration at or just below the cover slip; therefore, the PSF measurements will be of the best quality with the microspheres placed and imaged near the cover slip. In addition, the fluorescence emission light must travel back through the mounting medium again before being collected by the detector.

9| Cover the cover slips with aluminum foil in order to prevent dust from settling onto the samples. Let the solution dry for 1–2 h.
■ **PAUSE POINT** The solution can be left to dry for a longer period of time or even overnight.

10| To aid in finding the microspheres on the microscope, a permanent marker can be used to draw a circle around the dried spot on the opposite side of the cover slip.

11| Place a 15-µl drop of ProLong Gold or another appropriate mounting medium onto the microscope slide.
▲ **CRITICAL STEP** For optimal PSF measurements, the mounting medium must have a refractive index that is as close as possible to the refractive index of the glass cover slip and the immersion medium (1.515).

12| Pick up the cover slip by hand (with gloves on) or by using forceps and place it at a 45° angle to the vertical, and then let it fall onto the drop of mounting medium.

13| Lightly press down on the middle of the cover slip with a cotton swab to force any air bubbles in the mounting medium to the edges of the cover slip.

14| Place the samples in the dark overnight to allow the ProLong Gold to cure.

15| Check the samples for the correct microsphere density—i.e., enough microspheres to get many in a microscope field of view without any substantial clustering.
■ **PAUSE POINT** Store the slides at 4 °C. With ProLong Gold or Cytoseal they can last for months or longer.

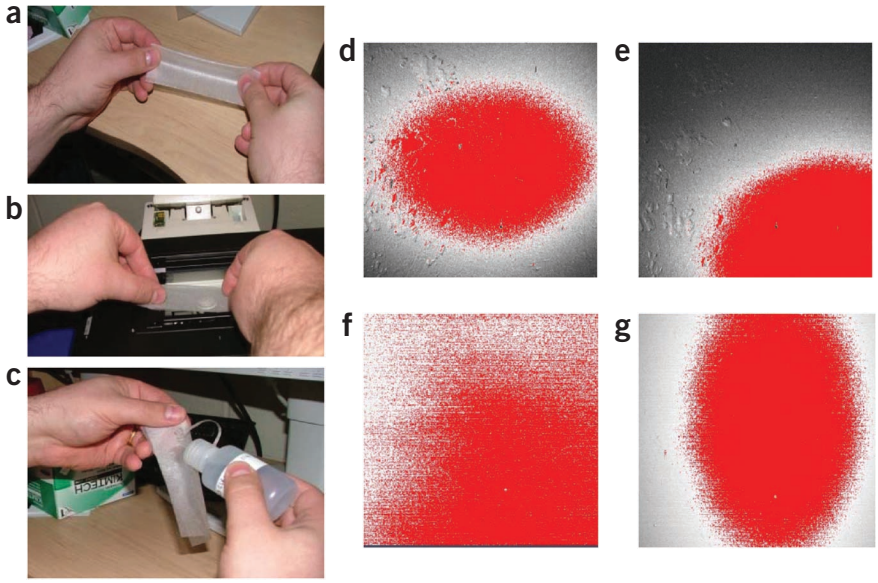


Figure 3 | Lens cleaning protocol and pinhole and laser alignment. (**a**,**b**) When cleaning immersion objective lenses, fold a piece of lens paper along its long axis (**a**), hold it at the edges and move it gently across the lens surface to remove excess oil (**b**). (**c**) Repeat the process with a fresh piece of lens tissue containing cleaning solution and then a third piece containing dH_2O. (**d**,**e**) Fluorescence images generated using a uniform fluorescence plastic slide, showing the fluorescence image generated when the pinhole is aligned (**d**) or misaligned (**e**). (**f**,**g**) Transmitted light images showing the intensity image when the microscope condenser is not in Köhler alignment (**f**) and when it is in Köhler alignment with a well-aligned laser (**g**). (**d**–**g**) The intensity distribution in these images is emphasized by showing saturated pixels in red.

Microscope setup ● TIMING 1 h 45 min

16| Turn on the microscope and allow the laser to warm up for at least 1 h.

▲ **CRITICAL STEP** Ensure that all DIC optical elements are removed from the confocal light path.

17| Clean your objective lens. This can be done while the laser is warming up. Remove any excess oil on the lens by taking a piece of lens paper and folding it three times into a long rectangle (**Fig. 3a**). Hold the paper at the edges between your thumb and index finger. Sweep the paper across the lens three times, moving the paper sideways after each sweep ensuring that a clean part of the lens paper contacts the lens at all times (**Fig. 3b**). It is important to use a fresh area of the paper with each sweep to avoid transferring any dirt or dust from the paper onto the lens, which could damage the lens during a subsequent sweep. Repeat this process two more times: once with lens cleaner on the lens paper (**Fig. 3c**) and once with dH_2O to remove any residue from the lens cleaner.

▲ **CRITICAL STEP** If the objective lens is not clean there are likely to be distortions in the measured PSF. Holding the lens paper at the edges ensures that no pressure is applied directly on the front lens.

18| If the lens has a correction collar (e.g., correction for immersion medium, temperature, cover slip thickness), ensure that it is properly adjusted.

▲ **CRITICAL STEP** If there are problems with the geometry of the PSF, it could be a result of the correction collar adjustment. For example, if the cover slip is specified to be 0.170 mm in thickness but is slightly thinner or thicker, the correction collar needs to be adjusted accordingly. If there is no way to measure the actual thickness of the cover slip, this adjustment can be done manually using the shape of the PSF to determine the ideal setting.

19| If the confocal pinhole is user adjustable, then use a green fluorescent plastic slide to align it. Start by setting up to image the plastic slide as you would for any green dye. You will have to use a very low laser power and low detector sensitivity (e.g., 0.5% laser power or ~8 µW and PMT gain of 500 V). Use a low zoom setting (approximately 1–2) and a fast scan speed (approximately 7–9). To help in seeing small differences in signal across the image, set the image lookup table (LUT) in the software to 'range indicator'. This will set up the image display so that any pixels reading maximum intensity (i.e., saturated) will appear one color (red in the Zeiss software) and any pixels reading zero intensity will appear another color (blue in the Zeiss software). This LUT is sometimes called a 'Hi-Lo' LUT. Set up the laser power and PMT voltage so some of the pixels at the center of the bright region are showing saturation, as in the example shown (**Fig. 3d**). If the pinhole is well aligned, a circular region of bright fluorescence should be seen in the center of the image (**Fig. 3d**). If the maximum brightness in the image is not centered (**Fig. 3e**), then adjust the pinhole. If it is difficult to center the bright region, the laser may be misaligned.

20| Perform Köhler alignment of the transmitted light condenser in order to verify that the laser is well aligned. Köhler alignment assures that both the condenser lens and the objective lens are focused at the same focal plane[20]. If the condenser is not properly aligned, then the transmitted light intensity image will not be representative of the laser alignment (**Fig. 3f**) and this test will not be valid.

21| Set up for imaging using the transmitted light detector. Imaging with the transmitted light detector generates an image of the laser light being transmitted through the sample. The range indicator LUT can be used again. If the laser is well

aligned you will see a bright region centered in the image (**Fig. 3g**). If the bright region is not centered on the transmitted light detector (**Fig. 3f**), verify that the transmitted light condenser is aligned. If so, then the laser is misaligned. If you know how to align the laser, then adjust the alignment to center the bright region of excitation. If you do not, then call your microscope service technician to do this for you.

22| Clean the microscope slide with lens cleaner to remove any buffer solution and clean any oil from the sample using a 70% (vol/vol) ethanol solution.

23| Ensure that the microscope focus is stable and that the system is not in an area prone to temperature shifts.

24| Move the objective lens to be tested into position on the microscope.

25| If the lens is an immersion lens, place a drop of oil, water or other appropriate immersion medium on the lens.

26| Place the 100- or 175-nm green microsphere sample on the microscope stage with the cover slip side facing toward the objective lens. Place it facing up for an upright microscope stand and down for an inverted microscope stand.

Instrument setup ● TIMING 30 min

27| Focus on the 100- or 175-nm microsphere sample. If you need to measure a PSF for a lower-resolution lens (<0.6 NA), then a sample of 0.5 µm microspheres should be used.

▲ **CRITICAL STEP** This is the general instrument setup and microsphere imaging procedure. Detailed procedures with screen-shots are provided for five major laser scanning microscope platforms (Zeiss LSM710; Zeiss LSM510; Olympus FV1000; Nikon A1R; Leica SP5) in the **Supplementary Methods**.

28| Set up the confocal light path for imaging a green dye (e.g., Alexa Fluor 488/GFP). For example, the light path could consist of the 488-nm laser line from an argon ion laser, a 488-nm main beam splitter (dichroic mirror) and an emission filter (barrier filter) collecting light from ~500 to 600 nm. If you are using a Zeiss 710, Zeiss 510, Olympus FV1000, Nikon A1 or Leica SP5, see the **Supplementary Methods** for platform-specific details helpful for performing Steps 29–47.

29| Set the image acquisition to scan an image frame of 1,024 × 1,024 pixels at a moderate scan speed (pixel dwell time of 5–25 µs per pixel). For optimal intensity information it is best to collect 12-bit images. Line or frame averaging can be used to reduce pixel noise. Start with a zoom factor of 2–3 in order to achieve an appropriate pixel size; however, note that this value can be fine tuned later in the protocol.

▲ **CRITICAL STEP** Set the instrument for unidirectional scanning, not bidirectional or raster scanning. If bidirectional scanning must be used, the scanning must be carefully calibrated in order to avoid problems with the registration of adjacent scan lines along the *y* axis, which can lead to image artifacts.

30| Zoom in on the microspheres at the center of the field of view for the best PSF characterization.

▲ **CRITICAL STEP** Artifacts in the PSF shape can be observed when imaging at the periphery of the field of view.

31| Set the PMT detector gain. This setting will vary among manufacturers, but typically a value of 600–750 V is ideal. Refer to the **Supplementary Methods** for more information on specific confocal platforms.

32| Set the detector offset. Most confocal manufactures have a software setting for the detector offset. This function tells the software that a PMT intensity readout below a certain digital threshold is noise and it sets any signal below this value to zero. This setting is often called a digital offset, black level or background level (see **Supplementary Methods** for more platform-specific information). It is best to set the offset using a range indicator–type LUT to make sure that no pixel within the image reads zero intensity units (**Fig. 4a**,**b**). This ensures that accurate intensity information is collected and low-intensity data clipping is avoided (compare **Fig. 4a**,**b** with **Fig. 4c**,**d**).

33| Adjust the detector (PMT) gain and laser power so that the average microsphere intensity is approximately 75% of the maximum image intensity (~3,000 intensity units in a 12-bit image). Choose the 488-nm laser line and start with a laser power of ~0.5% (~8 µW). Use the continuous scanning mode to ensure that there are no saturated pixels within the image. Do not use the fast scanning mode, as noise within the images may make it difficult to properly adjust the settings. Similarly to the black level settings, if the laser power or detector sensitivity is set too high then the fluorescence signal can saturate the detector, thus clipping the high-intensity data (compare **Fig. 4a**,**b** with **Fig. 4e**,**f**). These data points show up

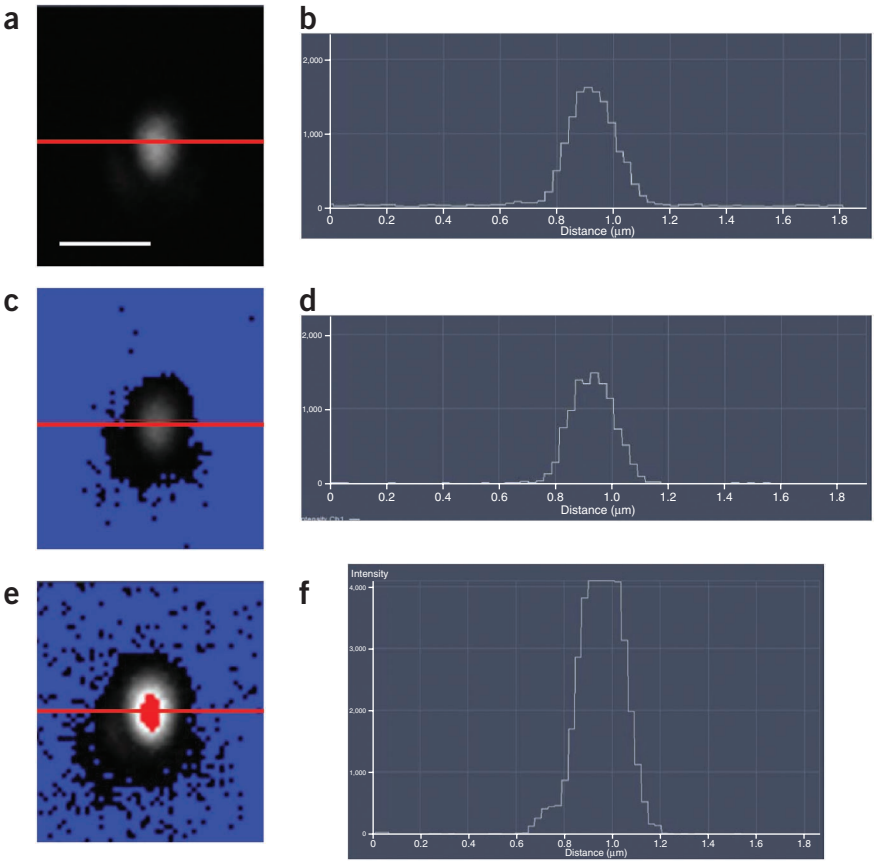


Figure 4 | Image acquisition settings to avoid data clipping. (**a**,**b**) Confocal image showing a fluorescence microsphere and the intensity profile along a line across the microsphere with well-adjusted image acquisition settings. (**c**,**d**) When the offset value is set too high, the image shows blue pixels representing pixels reading zero intensity (**c**), thereby causing low-intensity data clipping in the intensity profile (**d**). When the intensity of light coming from the sample is too high, the detector saturates. (**e**) Saturated pixels within the image are shown in red. (**f**) With both high- and low-intensity data clipping, the bead shape is misrepresented in the intensity profile. Scale bar, 0.5 µm.

as red pixels in the range indicator LUT (**Fig. 4e**). If there are saturated pixels showing up, reduce the laser power until the red pixels disappear. If the minimum laser power is reached and there are still saturated pixels within the image, then reduce the PMT gain until the saturation disappears.

34| Most confocal microscopes have a digital gain feature. Set the digital gain to 1 (i.e., off). If other gain settings are to be used, then they should be maintained across all experiments.

35| Set the pinhole to 1 Airy unit. If your confocal software does not express the pinhole in Airy units, then you should contact your confocal microscope's manufacturer to determine the relationship between the software's pixel size and the Airy unit.

36| Scan an image of the microspheres.

37| Verify the image acquisition settings using the range indicator LUT. A high signal-to-noise ratio is helpful for visualizing and interpreting the PSF; therefore, lower detector gain and higher laser power settings than typically used for imaging biological samples may be required.

38| Once the instrument settings are optimized, take an image of the microspheres.

Microsphere imaging ● TIMING 1 h 30 min

39| Collect images with the proper sampling frequencies in *x*, *y* and *x*. In order to accurately measure the PSF, the pixel size needs to be ~3 times smaller than the resolution of the objective lens. This is a good approximation of the Nyquist limit or the Nyquist sampling frequency[21]. The largest acceptable pixel sizes for accurately determining the PSF are shown in **Table 1**. These values can also be calculated directly from the lateral (equation (3)) and axial (equation (4)) resolution equations.

$$\text{Lateral sampling frequency} = \frac{0.51\lambda_{exc}}{3\text{NA}} \quad (3)$$

$$\text{Axial sampling fequency} = \frac{0.88 \cdot \lambda_{exc}}{3(n - \sqrt{n^2 - \text{NA}^2})} \quad (4)$$

Where λ_{exc} is the excitation wavelength, n is the refractive index of the immersion medium and NA is the NA of the objective lens. Use the zoom and pixel number features of the software to make sure that the image pixel size is approximately at the recommended value, but no larger. This will ensure that the sampling is high enough in order to determine the PSF accurately

TABLE 1 | Resolution and maximum PSF sampling size.

Objective NA	Lateral resolution (nm) for 488-nm excitation light	Axial resolution for 488-nm excitation light	Lateral *xy* sampling (nm)	Axial *z* sampling (nm)
0.60[a]	415	2,147	138	716
0.65[a]	383	1,789	128	596
0.70[a]	356	1,502	119	501
0.75[a]	332	1,268	111	423
0.80[a]	311	1,074	104	358
0.85[a]	293	907	98	302
0.90[a]	277	761	92	254
0.95[a]	262	624	87	208
1.00[b]	249	948	83	316
1.05[b]	237	836	79	279
1.10[b]	226	737	75	246
1.15[b]	216	649	72	216
1.20[b]	207	568	69	189
1.25[c]	199	648	66	216
1.30[c]	191	579	64	193
1.35[c]	184	515	61	172
1.40[c]	178	455	59	152

Note: Calculations for axial resolution were based on the following: [a]Air (n = 1.0), [b]Water (n = 1.33), [c]Oil (1.51) immersion medium.

for the objective lens being tested. The sampling we typically use for a ×63/1.4 NA or ×100/1.4 NA lens is 50 nm in the *xy* plane and 100 nm in the *z* plane.

▲ **CRITICAL STEP** It is important to collect the microsphere images according to the proper sampling frequencies in *x*, *y* and *z*. If the sampling frequency is too low, the shape of the PSF cannot be determined accurately along the lateral (**Fig. 5a**, 80 and 170 nm) and the axial (**Fig. 5b**, 250 and 500 nm) directions. If the sampling frequency is too high the microspheres can bleach, image collection can take a long time and image data sets can be large, without any significant gain in resolution or information about the PSF (compare **Fig. 5a** with **Fig. 5b**).

40| Select the dimmest microspheres within the image to analyze, as these are more likely to be individual microspheres. If the microsphere sample is well prepared then there should be many microspheres of similar intensity. Brighter spots correspond to aggregates of microspheres that are still sub-resolution, but should be roughly double, triple or four times (and so on) brighter than the dimmest microspheres. If these aggregates are used to measure the PSF, there could be some broadening because of the size of the aggregate and the microscope resolution will be underestimated. Note that the confocal software packages often interpolate pixel values to smooth out the data, thereby producing a 'pretty' picture that is not an accurate representation of the measured PSF (compare **Fig. 5b**,**d**).

? TROUBLESHOOTING

41| Crop, zoom or use a region of interest to choose a single microsphere (**Fig. 6a**,**b**).

42| Set up the *z*-stack acquisition within the software.

▲ **CRITICAL STEP** Use the values in **Table 1** or calculations based on equation (4) to set the spacing (i.e., interval) between the *z*-stack images.

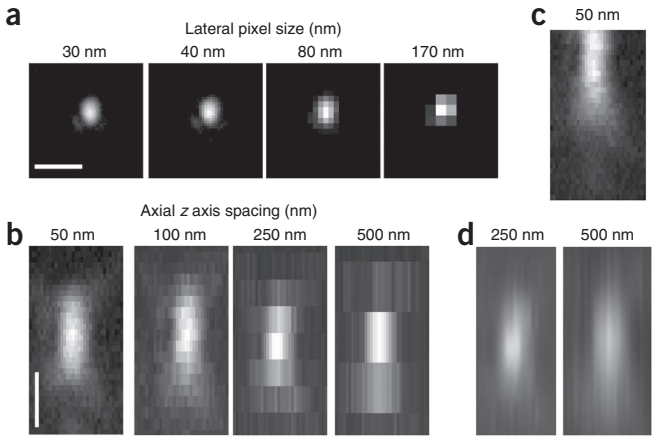


Figure 5 | Over- and under-sampling when measuring the PSF. Images of a 100-nm yellowish-green microsphere taken on a confocal microscope with a ×63/1.4 NA oil-immersion lens. (**a**) Images of the microsphere in the *xy* image plane showing ideal sampling (40 nm), over-sampling (30 nm) and under-sampling (80 and 170 nm). (**b**) Images in the *xz* plane showing ideal (100 nm), oversampling (50 nm) and under-sampling (250 and 500 nm). (**c**) Image of a microsphere in the *xz* plane in which the z-stack parameters were not set correctly, resulting in incomplete imaging of the PSF. (**d**) Many confocal software platforms will interpolate under-sampled data, making microspheres look blurred in the orthogonal view. Images were taken from the Zeiss 710 Zen software. Compare the images of the actual data in **b** with the smoothed data in **d**. Scale bar, 0.5 µm.

43| Set the *z*-axis limits. As a general rule, it is good to set the *z*-axis limits to at least the height of the PSF above and below the plane of focus. Ideally, to ensure that the entire function is imaged, two times the PSF height is best. For example, if the PSF is 2 µm high along the *z* axis, then the minimum stack size should go to 2 µm above the in-focus plane and 2 µm below the in-focus plane.

44| Use the fast or continuous scanning mode and set the *z*-stack options in one of two ways: first/last (option A) or center (option B).

(A) First/last

(i) Focus below the microsphere of interest and mark the first plane when you see no intensity in the image.

(ii) Focus above the microsphere of interest and mark the last plane when you see no intensity in the image.

(B) Center

(i) Focus on the center of the microspheres and click on the center button.

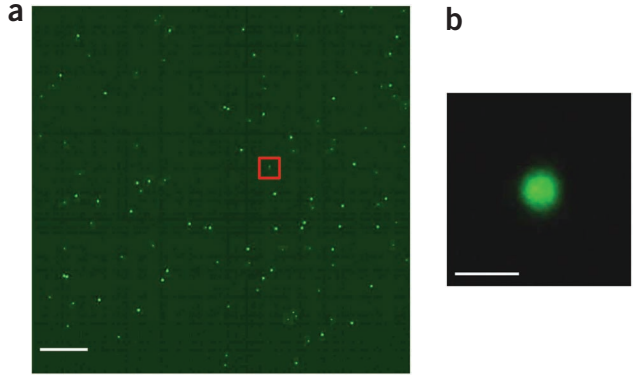
(ii) Enter the total number of slices to be imaged or the distance to image above and below the center image plane.

45| Perform the *z*-stack acquisition.

46| Use the orthogonal viewer to look at your microsphere PSF data in order to ensure that the image of the entire PSF has been captured (**Fig. 5b**) and that the *xz* or *yz* image does not show a partial function (**Fig. 5c**). **Figure 7a** shows a sample microsphere PSF orthogonal view in which the confocal pinhole is set to 1 Airy unit. These data were collected with 100-nm microspheres and a ×63/1.4 NA objective lens. One microsphere is much brighter than the other, and so it is likely to be a microsphere aggregate. A rainbow LUT is used to see the dim diffraction bands within the PSF image (**Fig. 7b**). The PSF data from the 1 Airy unit setting are used to determine the microscope's resolution.

47| Save the data in the microscope manufacturer's proprietary format to maintain the metadata (e.g., pixel size, scan speed, laser power) and also save it as a single 12- or 16-bit multi-TIFF file or a series of single TIFF files (.tif file extension).

▲ **CRITICAL STEP** Make sure the TIFF format is 12-bit or 16-bit and that there is no compression of the image data when saving the files.



48| Collect data for at least five individual microspheres.

49| Repeat the procedure with the pinhole setting at 4–5 Airy units.

▲ **CRITICAL STEP** With a larger pinhole setting, the *z*-stack range may have to be larger because there will be more out-of-focus light (**Fig. 7b**). The laser power may also have to

Figure 6 | Using region tools to image single microspheres. (**a**) Image at Zoom 3 of 100-nm microspheres taken with a ×100/1.4 NA oil-immersion lens. (**b**) Image at Zoom 3 of a single microsphere enlarged for visualization. Scale bar in **a**, 10 µm; scale bar in **b**, 0.5 µm. Images were taken on a Zeiss 710 using the Zen software and settings representative of those found in the **Supplementary Methods**.

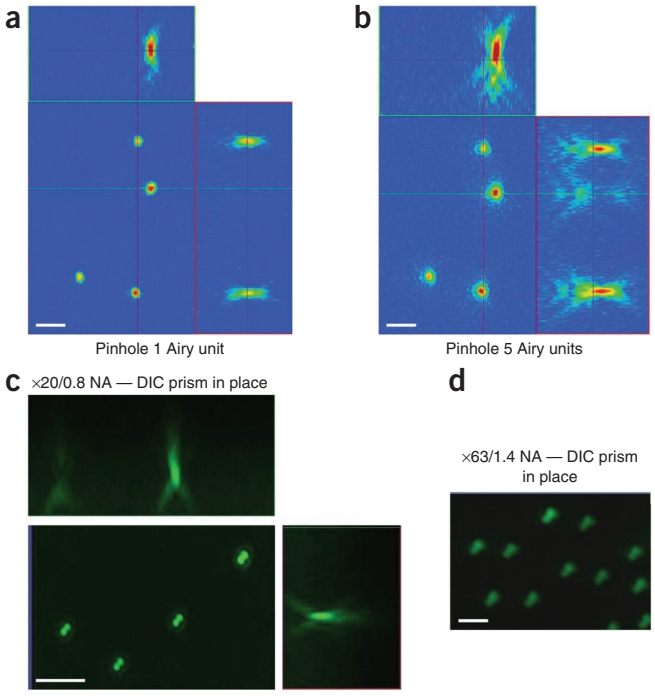


Figure 7 | Ideal and distorted PSF measurements. (**a**,**b**) Representative PSF orthogonal views showing the *xy* image plane (lower left), the *xz* image plane (top) and the *yz* image plane (right) for a pinhole setting of (**a**) 1 Airy unit and (**b**) 5 Airy units. Images were taken with a ×63/1.4 NA, oil-immersion objective lens. The microsphere in **a** and **b** that is about one-third of the way down in the *xy* plane and to the right is likely to be a spot containing two sub-resolution microspheres because it has a higher intensity than the other microspheres within the image volume. (**c**) Image of a PSF imaged with a ×20/0.8 NA, air objective lens shows how the DIC prism splits the laser beam into two distinct spots, resulting in a double image of each microsphere in the *xy* image plane and distortions along the *z* axis. (**d**) At ×63, the double image is not as distinct, but the elongation of the PSF in the *xy* image plane is apparent. Scale bars, 1 μm in **a**, **b** and **d**; 5 μm in **c**.

be adjusted because with the larger pinhole more light will reach the detector. Before collecting the PSF images, use the range indicator LUT to readjust the offset (black level), laser power and detector gain in order to ensure that no data clipping or image saturation is occurring.

Data analysis ● TIMING 20 min

50| Open the *z*-stack data files in Fiji and use the MetroloJ plug-in to analyze the PSF data. Fiji is freely available and is maintained and updated regularly. It can be downloaded at http://fiji.sc/wiki/index.php/Fiji. The MetroloJ plug-in, and a detailed manual for using it can be found at http://imagejdocu.tudor.lu/doku.php?id=plugin:analysis:metroloj:start. Choose 'Plugin-MetroloJ-Generate PSF report' to analyze the microsphere data and determine the size of the PSF.

▲ CRITICAL STEP The MetroloJ plug-in can only analyze one microsphere at a time, so if multiple microspheres are imaged, each one has to be cropped from the others into a separate image stack for analysis. Report data can then be manually averaged for five or more microsphere image stacks.

51| Review the PSF report generated by MetroloJ. The report shows the lateral and axial views of the image of the microsphere (**Fig. 8a**). A summary table shows the theoretical resolution of the lens and the resolution calculated along the *x*, *y* and *z* axes. The former is calculated on the basis of the image collection parameters and the later is based on the data curve fitting (FWHM; **Fig. 8b**). Plots of the intensity data from a line through the center of the microsphere along the *x*, *y* and *z* axes with the curve fitting and the fitting statics are also included in the report (data along the *x* axis are shown in **Fig. 8c**).

? TROUBLESHOOTING

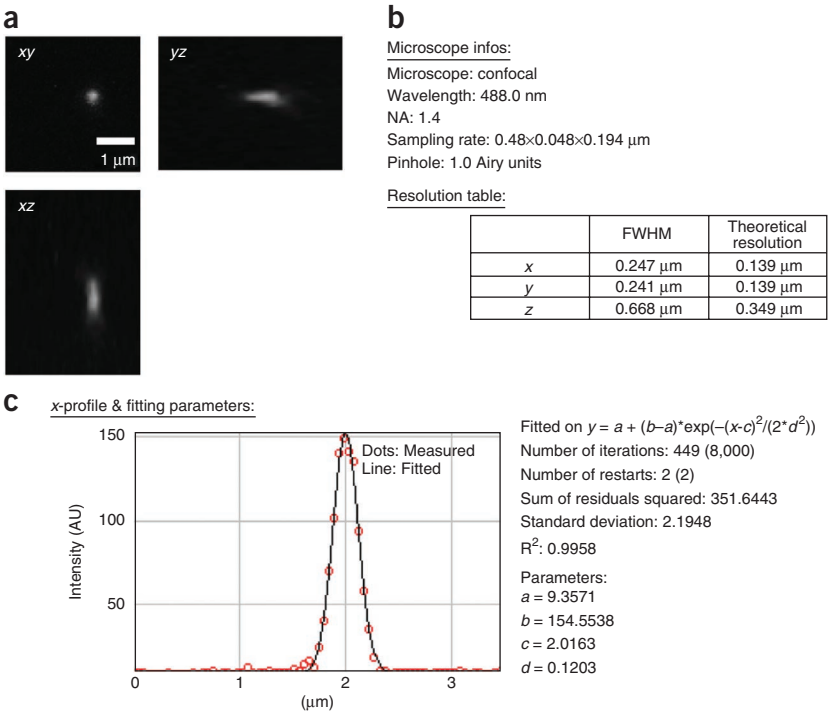


Figure 8 | MetroloJ report summary. (**a**–**c**) The MetroloJ software generates a report that shows the *xy*, *xz* and *yz* images through the center of the microsphere (**a**); the summary of the imaging parameters entered into the software, the measured FWHM from the fit to the PSF in *x*, *y* and *z* and the theoretical resolution of the objective lens in *x*, *y* and *z* (**b**). Finally, the program generates an intensity profile showing the fit to the data and the fitting parameters for the *x*, *y* and *z* resolution determinations (the *x* profile curve and fit is shown in **c**).

52| Check the resolution of your microscope with the objective lens being tested.
▲ **CRITICAL STEP** The MetroloJ plug-in uses a more rigorous equation to define resolution. Thus, the theoretical resolution given by the program is very high. It is preferable to compare the resolution with the values calculated from equations (1) and (2). This is done by multiplying the theoretical resolution from the report by a factor of 1.25.

53| If desired, analyze the PSF in another program or with custom software. The MetroloJ website includes a manual that describes the data analysis in detail. Essentially, the maximum intensity pixel within the entire 3D data set is determined. Data along a straight line though this data point is extracted along the *xy*, *xz* and *yz* axes. These curves are then fitted to a Gaussian function using the built-in ImageJ curve fitting algorithm and the FWHM is determined.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 2**. Additional troubleshooting guidance can be found in reference 8.

TABLE 2 | Troubleshooting table.

Step	Problem	Possible reason	Solution
40	Microsphere intensities are highly variable and there are not many dim spots of similar brightness	Microsphere aggregation	Prepare new microsphere samples; sonicate for a longer period of time; spin down the microsphere sample for a minute or two in a tabletop centrifuge to precipitate out aggregates before preparing new slides
51	PSF is elongated or compressed along the *z* axis	Temperature drift	Isolate the microscope from temperature shifts: cover it, use temperature-controlled chambers
	PSF is elongated or compressed along the *z* axis	Focus drift	Test that the focus of the microscope is stable by imaging a single plane over time. If not, try the solution above for temperature drift. If the problem is still not resolved, ask for a service call for the microscope
	Blurry PSF	DIC optics in place, dirty lens, dirty optical elements, vibrations	Make sure DIC optical elements are removed from the light path; clean the objective lens; clean the optical elements in the light path; check for vibrations by imaging the same microsphere over time at the same *z* plane
	Odd shaped or distorted PSF	Pinhole is misaligned; defective relay optic; problem with galvanometer mirrors	Check pinhole by opening it up, align if necessary. Replace defective optic; try different scan speeds and/or bidirectional scan
	Asymmetric flare on the PSF	Spherical aberration	Clean the objective lens and apply new immersion oil; make sure there are no bubbles in the immersion oil; make sure you used the proper cover slips, try different mounting medium

● TIMING

Steps 1–5, Slide preparation: 45 min
Steps 6–10, Slide preparation: 2 h
Steps 11–15, Slide preparation: 12 h
Steps 16–18, Microscope setup: 1 h (lasers should be warmed up for at least 1 h)
Steps 19–21, Microscope setup: 30 min (may be longer depending on pinhole adjustment)
Steps 22–26, Microscope setup: 15 min
Steps 27–38, Instrument setup: 30 min
Steps 39–42, Imaging: 30 min
Steps 43–49, Imaging: 60 min
Steps 50–53, Data analysis: 20 min

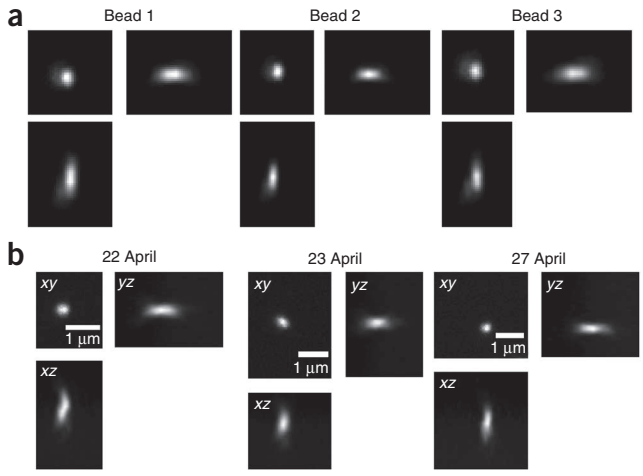


Figure 9 | PSF variability. (**a**,**b**) Representative *xy*, *xz* and *yz* images of sub-resolution 175-nm yellow-green microspheres taken of different microspheres on the same data from the same slide (**a**) and from different microspheres taken on different days, using the same imaging acquisition setup and objective lens (**b**). The variability seen within an experiment is very similar to the variability seen between experiments. Images were generated with a Zeiss Pascal 5 confocal microscope using 100-nm yellowish-green microspheres and a ×63/1.4 NA oil-immersion lens. Scale bars, 1 µm.

ANTICIPATED RESULTS

With the confocal pinhole set to 1 Airy unit, the FWHM, determined from the PSF, is a direct measure of the microscope's resolution for the specific wavelength of light and objective lens being tested. This value is compared with the theoretical resolution. The PSF data shown in **Figure 7a** are representative of a ×63/1.4 NA oil-immersion lens performing close to the resolution limit.

The PSF measurements with the confocal pinhole set to 5 Airy units are meant to test the quality of the objective lens and the sample preparation. The main indicator of a good-quality lens is a symmetric PSF both along the *z* axis (diffraction pattern above and below the focal plane) and on either side of the central optical axis in the *x* and *y* planes (**Fig. 7b**). The example in **Figure 7b** shows the appropriate diffraction patterns below the microspheres near the cover slip. Above the microspheres (right side or upper part of the orthogonal view images), the diffraction pattern is not as apparent. This is likely to be caused by spherical aberrations due to the slight index of refraction mismatch between the immersion oil and the ProLong Gold mounting medium. These distortions in the PSF will worsen when imaging deeper into the sample[8]. Along the optical axis the PSF is very symmetric, indicating a good-quality lens. The point source images should also be very symmetric and circular along the *x* and *y* axes. This symmetry can be visualized in the *xy* image and verified numerically by comparing the FWHM fit values along the *x* and *y* axes in the MetroloJ PSF reports.

TABLE 3 | Variation over time of the average resolution measured for a ×63/1.4 NA lens.

Date	*x*	*y*	*z*
05/02/2011	0.2326	0.2285	0.69575
21/04/2011	0.2034	0.244	0.4886
22/04/2011	0.2366	0.241	0.56
23/04/2011	0.2274	0.2414	0.6476
24/04/2011	0.2256	0.2404	0.5652
25/04/2011	0.229	0.2404	0.5276
27/04/2011	0.2386	0.2496	0.6086
30/04/2011	0.236	0.238	0.543
06/05/2011	0.2306	0.2612	0.6204
07/05/2011	0.23675	0.259	0.5955
09/05/2011	0.24075	0.242	0.69125
Average	0.2307	0.2441	0.5949
S.d.	0.0103	0.0093	0.0661
S.d. (%)	4	4	11

If the PSF data do not show symmetric functions, then go back to Steps 17–22. The most common reasons for poor PSF data are DIC optics in the light path (**Fig. 7c**,**d**); dirty objective lenses; misaligned confocal pinholes; and dirt on the microscope slide, cover slip or within the optical path of the microscope. When the DIC prism is in place, it shears the light into two waves, ordinary and extraordinary, resulting in a double image of the microspheres (**Fig. 7c**,**d**); this will result in a severe underestimate of the instrument resolution. Therefore, DIC optics should always be removed from the light path for high-resolution microscopy applications. To isolate the cause of the PSF distortions, repeat only one step at a time (e.g., realign the pinhole) in the procedure and repeat the PSF measurements. If a poor PSF persists after sequentially repeating Steps 17–22, we recommend repeating the measurement with another objective lens of similar quality. If a second lens has similar PSF abnormalities, there could be an issue with the confocal scanning system calibration or alignment. If the second lens does not show similar abnormalities, contact the microscope manufacturer and have the objective lens inspected to make sure it is not defective or damaged. When receiving new objective lenses, always test their quality using PSF measurements.

In general, PSF measurements will vary from lens to lens, but should be within 30–40% of the theoretical resolution. PSF measurements of different microspheres imaged within the same session typically show a standard deviation of 4–6% for the *x* and *y* axis FWHM values and ~12% for the *z* axis FWHM. For day-to-day measurements, the average PSF

FWHM values show similar standard deviations (4% for the *x*, *y* axis FWHM and 11% for the *z* axis FWHM, **Fig. 9a**,**b** and **Table 3**). Therefore, unless the sample quality degrades over time, sample preparations change, equipment problems arise or objective lenses are damaged, the PSF measurements should not vary substantially. This makes the measurement ideal as a quality control metric. Further suggestions for possible remedies to poor PSF quality are found in an article by Goodwin[8].

Note: Supplementary information is available via the HTML version of this article.

ACKNOWLEDGMENTS We acknowledge the use of the Wadsworth Center's Advanced Light Microscopy & Image Analysis Core Facility, as well as the McGill Life Sciences Complex Imaging Facility for portions of the work presented. We thank R. Stack for help with sample preparation, PSF image stack acquisition and data analysis. We thank B. Northan from Media Cybernetics for generating the theoretical PSF images for **Figure 1**, C. Glowinski for preparing 3D PSF figures using the Bitplane Imaris software for **Figure 2**, A. Spurmanis for providing objective lens cleaning images for **Figure 3**, as well as F. Waharte and M. Thibault for providing **Supplementary Methods** on Nikon and Olympus confocals, respectively. We thank B. Eason and K. Young for critical reading of the manuscript.

AUTHOR CONTRIBUTIONS R.W.C. and C.M.B. conceived of the need for a protocol for testing confocal microscope resolution, confocal microscope quality and objective lens quality. C.M.B. and R.W.C. prepared samples, collected images, analyzed images and interpreted data results. C.M.B. wrote the manuscript and designed the figures with critical reading and editing provided by R.W.C. T.J. collected PSF images, analyzed them and assimilated the data for the time-dependent studies.

COMPETING FINANCIAL INTERESTS The authors declare no competing financial interests.

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