Superresolution Imaging with Standard Fluorescent Probes

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ABSTRACT

For more than 100 years, the ultimate resolution of a light microscope (~200 nm) has been constrained by the fundamental physical phenomenon of diffraction, as described by Ernst Abbe in 1873. While this limitation is just as applicable to today's light microscopes, it is the combination of high-end optics, clever methods of sample illumination, and computational techniques that has enabled researchers to access information at an order of magnitude greater resolution than once thought possible. This combination, broadly termed superresolution microscopy, has been increasingly practical for many labs to implement from both a hardware and software standpoint, but, as with many cutting-edge techniques, it also comes with limitations. One of the current drawbacks to superresolution microscopy is the limited number of probes and conditions that have been suitable for imaging. Here, a technique termed bleaching/blinking-assisted localization microscopy (BaLM) makes use of the inherent blinking and bleaching properties of almost all fluorophores as a means to generate superresolution images. *Curr. Protoc. Cell Biol.* 60:21.8.1-21.8.17. © 2013 by John Wiley & Sons, Inc.

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INTRODUCTION

Imaging techniques that achieve resolutions surpassing the diffraction limit offer considerable advantages to cell biologists. While the majority of eukaryotic cells are within the resolvable range of traditional light-microscopic methods, many of their organelles and constituent cellular proteins are well out of reach of diffraction-limited microscopy. Traditionally, researchers have been caught between the flexibility and accessibility that light microscopy has to offer (with maximal resolutions of ~200 nm) and the more rigid, less accessible techniques that electron microscopy (with resolutions at the angstrom level) provides. It is no surprise then that superresolution imaging has garnered a substantial amount of interest in recent years, with the number of techniques and applications developed for cell biology growing rapidly.

Techniques such as PALM, or photoactivated localization microscopy (Betzig et al., 2006), FPALM, or fluorescence photoactivation localization microscopy (Hess et al., 2006), STORM, or stochastic optical reconstruction microscopy (Rust et al., 2006), FIONA, or fluorescence imaging with one nanometer resolution (Yildiz et al., 2003), and SHRImP, or single-molecule high-resolution imaging with photobleaching (Gordon et al., 2004), among many others, all rely on localization techniques (Thompson et al., 2002) that fit signals emitted from individual fluorescent molecules to Gaussian curves in an effort to more precisely localize their position. These techniques are in contrast to other superresolution methods like SIM, or structured illumination microscopy

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(Gustafsson, 2001), which makes use of precisely patterned illumination of the sample in order to capture high-frequency information normally diffracted outside of the objective aperture. Different still is STED, or stimulated emission depletion microscopy (Klar et al., 2000), which implements a dual-laser strategy; the first laser excites fluorophores in a diffraction-limited space, while the second, shaped, high-power laser suppresses the fluorescence of molecules which are excited by the outermost periphery of the diffraction-limited excitation beam.

All of the aforementioned techniques (and more) have their own unique benefits as well as limitations when it comes to a given sample, and researchers are urged to consider these when choosing a particular superresolution technique for their experiment. However, many superresolution techniques share a limitation in that they are not suitable for use if proper probes in specified conditions are not used. Among the techniques with the highest potential resolution to date are the localization-based methods (e.g., PALM and STORM), and great effort has gone into probe characterization to determine optimal fluorophore properties for point localization. This is not without good reason, as the degree of certainty with which a probe may be localized to a position is ultimately dependent on signal-to-noise ratio, which translates to number of photons emitted relative to noise sources inherent in the sample as well as the imaging system. Recently, there has been an effort to extend superresolution-based methods to standard fluorophores and sample preparations (Simonson et al., 2011), and it is anticipated that these approaches and their applicability will only increase in the future. The technique described in this unit, termed bleaching/blinking assisted localization microscopy (Burnette et al., 2011b), or BaLM, attempts to bridge the gap between maximal resolution and applicability by extending superresolution methods to standard fluorescent probes and thus to more research applications. First, this unit will briefly describe the setup of a variable-angle TIRF (total internal reflection fluorescence) microscope system, with a few modifications recommended for successful BaLM acquisition and analysis. Second, sample preparation will be described briefly, as this is not substantially different (other than probe density considerations) from what others have routinely reported for standard diffraction-limited fluorescence microscopy. Third, imaging parameters ideal for BaLM analysis, followed by implementation of the computational tools required for BaLM, will be covered.

STRATEGIC PLANNING

As mentioned, superresolution imaging provides a powerful tool for cell biologists. However, there are a range of options emerging, and if considering the path forward using these tools, it is important to take into account what knowledge may be required before starting, as well as some limitations of many of these techniques, including BaLM.

Although superresolution techniques are becoming much more accessible to mainstream laboratories, the researcher should be well versed in diffraction-limited fluorescence microscopy, as well as specialized techniques such as TIRF (*UNIT 4.12*) and spinning-disk confocal microscopy (*UNIT 4.5*), as these are often required for generating superresolution datasets. In this unit in particular, a strong working knowledge of TIRF microscopy is highly recommended. In addition, the researcher is strongly advised to have a good understanding of multidimensional microscopy managed through the use of imaging software. Such dimensions include *x*/*y* position, wavelength (channel), *Z* stack (or axial positioning), and time, although multi-position datasets are possible as well. In addition, the user should be familiar with software control of the automated peripherals of the system such as TIRF angle, laser power, and camera settings.

Sample preparation is another consideration. Signal-to-noise ratio is perhaps the single largest variable when it comes to successful superresolution microscopy. As such, the researcher is encouraged to have an intimate knowledge of the specific sample and preparation with regard to standard diffraction-limited imaging techniques before moving towards superresolution microscopy. Such familiarities may include the overall availability of the protein(s) in question, autofluorescence of the sample itself, background of primary or secondary antibodies (if using immunofluorescence), and stability of the probes over time (applies to both immunofluorescence and fluorescent protein expression). Although general considerations and protocols may be similar for conventional fixation and staining, attention to probe density in relation to the intended target's structure is of particular importance. Successful imaging is dependent on many variables; however, they all assume that there are enough fluorescent molecules to be imaged to adequately resolve a given structure. More on this is discussed in Basic Protocol 2.

Keeping some of the above considerations in mind before planning superresolution experimentation such as BaLM can help avoid common mistakes when entering the field, and more accurately line up an important biological question with an appropriate technique. In addition to prior knowledge requirements, current limitations of superresolution microscopy (including BaLM) are also an important consideration for researchers entering the field. For a more detailed description of such limitations, see the Commentary section at the end of this unit.

SETUP OF MICROSCOPY HARDWARE FOR BaLM ACQUISITION

BASIC PROTOCOL 1

In principle, acquisition of images for BaLM analysis can be accomplished using a variety of microscope hardware configurations. However, the inherent accuracy of the eventual localization is dependent on the signal-to-noise ratio (SNR) achieved during image acquisition (Thompson et al., 2002), similar to other localization-based methods. There are many strategies to enhance SNR, starting with the proper choice of microscopy technique. Equally important are the choice of detector (and thus pixel dimensions) and measures to mitigate the many sources of noise inherent in the imaging hardware as well as the sample itself. For example, we have demonstrated that both TIRF microscopy and spinning-disk confocal microscopy are capable of generating datasets suitable for BaLM at different sampling frequencies. In addition, multi-color BaLM is achievable using an array of different probes (Fig. 21.8.1). The best microscopic approach depends on the applicability to a given sample, and the researcher is urged to have a good understanding of these techniques (and their samples) before committing to a certain setup. Rather than speaking in general terms and trying to account for all possible scenarios, the authors will describe the recommended configuration of a variable angle TIRF microscope optimized for BaLM acquisition of fluorescent microtubules in COS-7 cells. However, the reader should understand that other methods (such as spinning disk confocal microscopy) could be sufficient for data acquisition for BaLM, depending on the requirements of the sample. As will be described in Basic Protocols 3 and 4, the researcher will learn strategies for changing acquisition parameters to adapt this protocol for use in other types of samples as well.

Since there are several commercially available configurations for TIRF microscopy, many researchers do not undertake the burden of building the system from the ground up. If more detailed knowledge is desired, the reader is encouraged to review protocols on design of these systems (*UNITS 4.12 & 4.21*). The major difference between the setup of a BaLM-capable microscope and configurations routinely used for TIRF or spinning-disk confocal microscopy, for example, is the amount of secondary magnification added to the system. Such addition of magnification results in higher spatial sampling frequency than would be required for most diffraction-limited or superresolution imaging configurations.

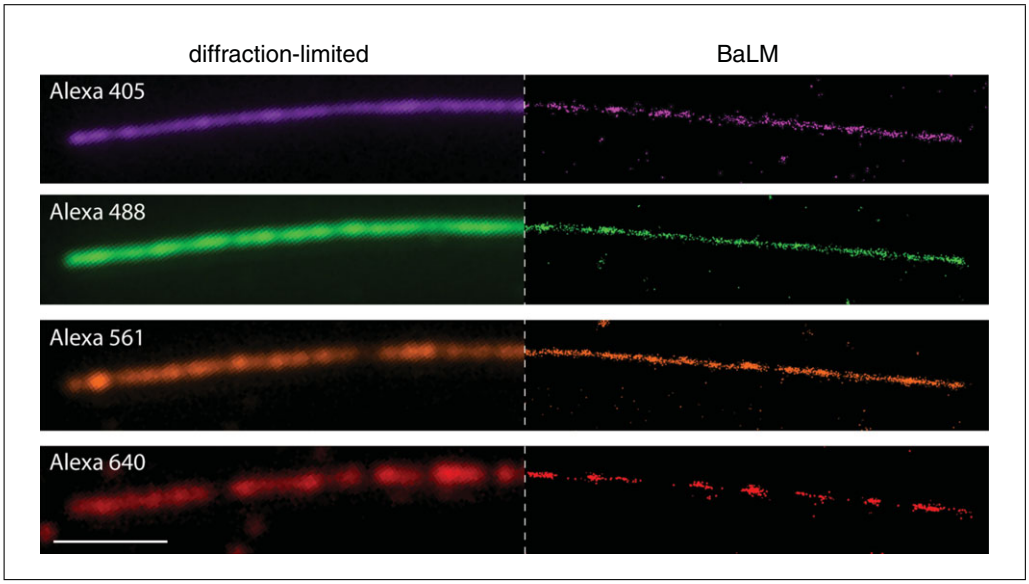

Figure 21.8.1 Multicolor BaLM. Microtubules were fixed and immunostained for α-tubulin with a spectrum of Alexa Fluor conjugated secondary antibodies including Alexa Fluor 405 (purple), Alexa Fluor 488 (green), Alexa Fluor 561 (orange), and Alexa Fluor 640 (red). Diffraction-limited images of a microtubule acquired by TIRF microscopy (left) is juxtaposed with the corresponding BaLM image (right). Images were acquired in the following order: Alexa Fluor 640, Alexa Fluor 405, Alexa Fluor 488, and Alexa Fluor 561. Scale Bar: 2 μm. Image adapted from Burnette et al. (2011b). For the color version of this figure, go to *http://www.currentprotocols.com/protocol/cb2108*.

However, it is important to note that this resultant oversampling does not contribute to additional resolution beyond the diffraction limit. Classical sampling theory requires a minimum number of pixels per unit area to properly sample an image produced by a given lens, a number that ultimately is a function of the objective's numerical aperture. Oversampling beyond this number of pixels is generally detrimental to image quality, as smaller pixels are required, resulting in decreased sensitivity and a corresponding loss in SNR. Counter-intuitively, most of the superresolution imaging techniques (using point-localization) do not require such spatial sampling, and slightly larger pixels are thus relied upon for better signal due to the fact that activated fluorophores are more sparse and statistically much farther apart than the diffraction limit of the utilized objective. The limitation to this, of course, is that enough pixels are required to sufficiently sample the point spread function (PSF), which in turn allows for a properly fit Gaussian curve and thus precise localization of the molecule's position. As described previously for isolated PSFs (Thompson et al., 2002), the ideal pixel size is one that matches the standard deviation in the PSF itself. However, BaLM is different in that it does not utilize low activation energies to limit the number of fluorophores excited, but rather excites the entire field within range of a given technique (e.g., within the evanescent wave in TIRF applications) and relies on post-processing to reveal the stochastic nature of dynamic probe phenomena such as bleaching and blinking. The result is, first, a potentially large dynamic range of intensities in any given frame of a BaLM dataset, and second, higher background due to adjacent bleach/blink events. As a result, without additional magnification, the large pixel size would average out signal from single-molecule events, and in combination with the large dynamic range would result in poor localization precision. As such, the secondary magnification spreads the higher dynamic range to more pixels and reveals single-molecule phenomena once the image subtractions have been completed. A theoretical model depicting signal, background, and sampling frequency is illustrated in Figure 21.8.2. Beyond magnification, differences in BaLM systems (relative to a diffraction-limited TIRF system for example) are revealed only in

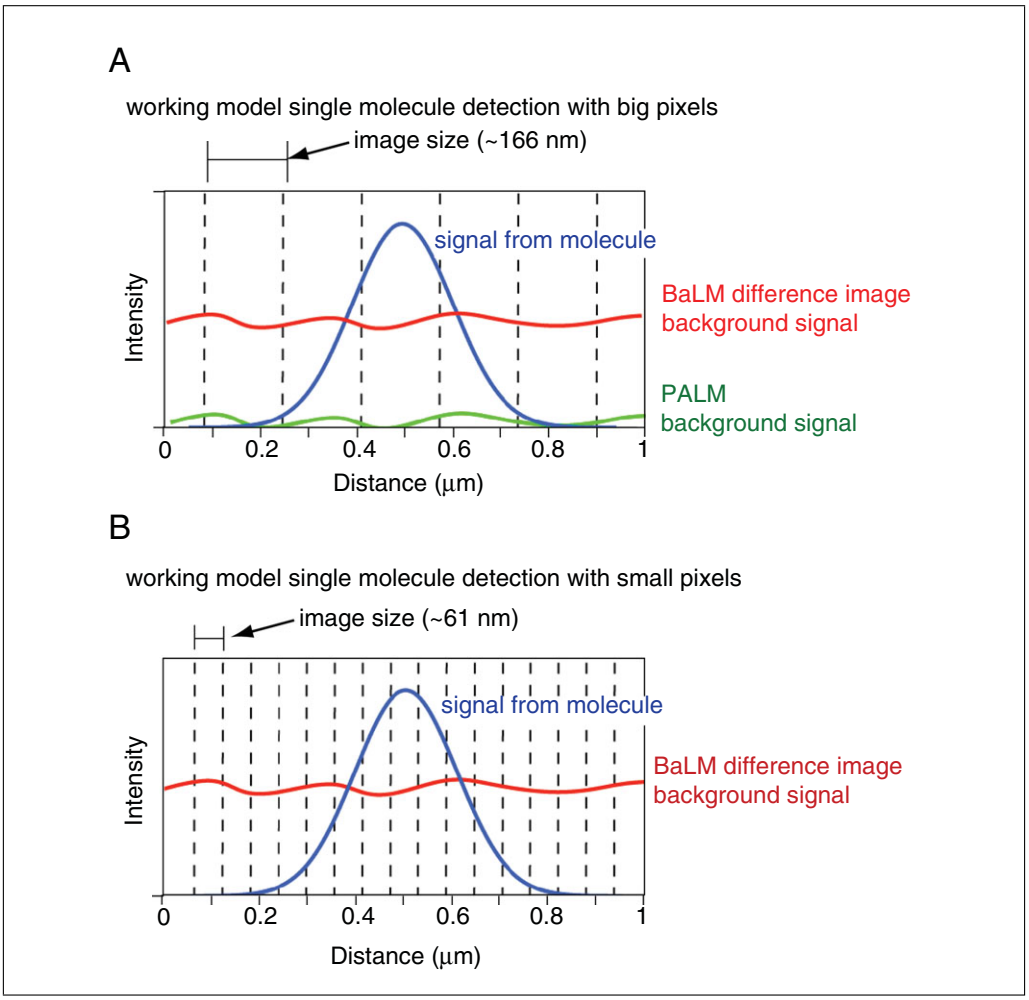

Figure 21.8.2 Sampling frequency in relation to background fluorescence levels of PALM and BaLM datasets. Theoretical model to illustrate the sampling frequency of a proposed point spread function (blue) in the context of both PALM and BaLM representative backgrounds (green and red, respectively) with (**A**) large image pixel size (~166 nm) typically used for many TIRF microscope configurations and (**B**) smaller image pixel size (~61 nm) representative of a sampling frequency used for BaLM. In the case where single molecules are stochastically activated in low number (as in PALM), the background is typically low due to the inactivity of the majority of fluorophores. In this case, the larger pixel size is sufficient, as the signal is sampled (blue) several times before the signal decays to background. On the other hand, in the case of BaLM (red), where virtually all of the fluorescent molecules in a specimen are activated, the post-subtraction dataset yields a higher background (red), and the larger pixels cannot sample the signal (blue) before the signal-to-noise ratio depletes (as in A). However, with smaller pixels (higher spatial sampling frequency), the signal may be sampled a sufficient number of times before the signal decays (as in B). Image adapted from Burnette et al. (2011b). For the color version of this figure, go to *http://www.currentprotocols.com/protocol/cb2108*.

the acquisition parameters and post-acquisition analysis (discussed in in Basic Protocols 3 and 4).

Materials

General microscope hardware

Vibration isolation table (e.g., Technical Manufacturing Corporation, Kinetic Systems Inc., Newport Corporation)

Motorized inverted fluorescence microscope (e.g., Nikon Ti-E, Olympus IX-81/83, Zeiss Axio Observer, Leica DMI6000) with motorized and encoded *Z*-axis motor and switchable tube lens element. For reference, the authors use a Nikon Ti-E.

Motorized and encoded specimen stage with appropriate insert(s) for desired imaging chamber(s). As an alternative, a basic non-moving stage bolted to the stand offers stability of the sample without the cost of a motorized stage, but is not recommended due to the difficulties in navigating the sample.

Epi-fluorescence illuminator

Filter cube turret

Appropriate wide-field epi-fluorescence filter cubes for visual examination of sample

Wide-field excitation source (e.g., mercury arc lamp, EXFO Excite 120)

Automated focus assist (e.g., Nikon PFS, Olympus ZDC, Zeiss Definite Focus, Leica Adaptive Focus Control)

Appropriate laser lines and launch for desired probes (e.g., 405 nm, 440 nm, 488 nm, 561 nm, 640 nm, 647 nm). For stability and lifetime, the authors recommend diode lasers capable of producing at least 25 mW (out of the fiber), which is suitable for initiation of most bleach/blink events in a reasonable time frame. As a reference, the authors use 405 nm, 488 nm, 561 nm, and 640 nm diode lasers with respective powers out of the fiber of 19.1 mW, 38.9 mW, 44.7 mW, and 21.9 mW.

Appropriate laser safety mechanisms (interlocks to prevent laser light directed to eyepieces, scope-mounted laser safety modules to prevent stray laser light emission, appropriate personal protective equipment, etc.)

High-performance workstation ($\geq$2.0 GHz processor, $\geq$8 gigabytes of RAM) and monitor

TIRF-specific microscope hardware

High-numerical-aperture objective lens (NA $\geq$1.45)

Motorized TIRF illuminator for variable angle illumination (can also incorporate an epi-fluorescence illuminator)

EMCCD (electron multiplying charge coupled device) camera

An Andor DU-897 as well as a liquid-cooled Photometrics Evolve have both been used successfully by the authors for BaLM imaging

4$\times$ or 2.5$\times$ magnification optics (see protocol below for details)

Emission filter wheel (e.g., Sutter Instrument, Prior Scientific, Ludl Electronic Products)

Appropriate laser clean up filter and dichroic mirror for desired laser lines (mounted in fluorescence cube turret). For reference, the authors used a quad TIRF cube purchased through Nikon, which housed Chroma filters and dichroic mirror corresponding to: Zet405/488/561/635$\times$ (quad laser clean up filter), and Zt405/488/561/640rpc polychroic mirror.

High-quality emission filters (mounted in the emission filter wheel) appropriate for the desired imaging probes. For reference, the authors used Chroma ET emission filters which passed 447 nm (60 nm band-pass), 525 nm (50 nm band-pass), 600 nm (50 nm band-pass), and 700 nm (75 nm band-pass). When these emission filters were paired with the above mentioned dichroic mirror, clean-up filter, and lasers, it enabled proper excitation and emission of Alexa Fluor 405, 488, 568, and 640.

Optional: Additional laser blocking notch filters may be added to the emission light path (mounted with the emission filter) to ensure no bleed-through of laser emission

Setup of microscope hardware

1. Select a suitable location for high-resolution light microscopy.

   Choice of location of the microscope system before beginning is critical to avoiding potential pitfalls in imaging after the system is set up. Once together, the system is extremely heavy and cannot be easily moved without significant disassembly or possible damage to the microscope while moving. First, the system should be located in a space that can

be shielded from external light. In addition, it is highly recommended to keep the system protected from sources of vibration, keeping in mind that at high magnification, vibrations can be introduced from otherwise unnoticed sources such as air ducts and computer fans. Another extremely important consideration is dust, and every precaution should be taken to avoid introduction of dust and dirt to the area surrounding the microscope. Make sure that the microscope space contains adequate power resources and number of circuits to support the proposed system. While newer microscope systems generally do not draw nearly the amount of power that older systems required (predominantly because of the switch from gas to diode lasers), it is important to make sure that you do not share a circuit with other devices in the lab that require a large amounts of power or cycle constantly. Ideally, the system should be on its own circuit(s), with conditioned lines. Lastly, seeing that the microscope employs lasers as an excitation source, it is important that one comply with institutional regulations concerning laser-containing devices.

2. Assembly of the microscope stand and peripherals.

   Assembly of the bulk of the microscope can usually be done by experienced technicians per standard procedure. If you are experienced with microscope design, you may choose to do some of the installation; however, each manufacturer has specific hardware components that are unique to their systems, so it is encouraged that you consult the proper resources before attempting installation on your own. In either case, it is imperative that the stand itself be bolted to the vibration isolation table to minimize additional sources of movement and vibration in the system. This is helpful to know before starting the installation, as some vendors have plates that mount under the microscope stand that serve to secure it to the table. Additionally, where possible, it is recommended that any accessories that extend from the microscope stand (cameras, confocal scan heads, etc.) also be secured to the vibration isolation table if and where possible. Peripherals such as hardware controllers, computer mice, keyboards, monitors, wide-field excitation sources, etc., should be kept off of the vibration isolation table and on separate desks/stands.

3. Select appropriate secondary magnification.

   Although the setup and installation of a system used for BaLM may be accomplished by traditional methods for setting up TIRF (or spinning disk confocal) microscopes, magnification and detector choice may be atypical. As previously discussed, BaLM uses additional magnification (oversampling) to offset the large dynamic range inherent in pre-subtraction datasets and effectively spread signal across more sensor pixels. Both a liquid-cooled Photometrics Evolve EMCCD, as well as an Andor DU-897 EMCCD, were used to sample the image at 40 nm or 60 nm and were shown to be suitable for BaLM. Both cameras have a physical pixel dimension of 16 $\mu m \times 16$ μm. To achieve an image sampling frequency of 40 nm, a magnification optic of 4$\times$ is required in addition to the 100$\times$ objective lens. To achieve a sampling frequency of 60 nm, a magnification optic of 2.5$\times$ is required in addition to the 100$\times$ objective lens. While sampling at $\sim$60 nm is sufficient for many samples, the researcher is encouraged to try a range of secondary magnifications per the requirements of their specimen. For TIRF microscope systems, the secondary magnification optic should be placed just before the camera and can be included as a relay optic.

SAMPLE PREPARATION FOR BaLM: IMMUNOFLUORESCENCE LABELING OF α-TUBULIN IN COS-7 CELLS

BASIC PROTOCOL 2

The benefit of the BaLM approach is the ability to use standard fluorescent probes for imaging. Therefore, considerations for sample preparation are not substantially different than have been described previously for standard diffraction-limited fluorescence microscopy (Burnette et al., 2011a). It should be noted that in addition to immunofluorescence techniques as described here, it has also been shown that expression of fluorescent proteins can also be suitable for BaLM. One inherent challenge that must be considered, not only with BaLM but all superresolution techniques, is the problem of probe density. When trying to garner high spatial frequency information on the order of tens of nanometers, many researchers are surprised to find out that the labeling density of their sample

is unsatisfactory, especially if the structures being imaged are not known and cannot be interpolated by eye. For example, if the probe density of an actin fiber is not adequate enough to highlight it in a continuous fashion, the structure is naturally interpolated by the eye to understand its linear nature based on the viewer's prior knowledge of its arrangement. However, if the structure (or position) is not known, as may be the case with many experiments, the labeling density may not be enough to answer the question of a structure's position or arrangement. Therefore, the researcher must identify methods to increase the probe density of a particular sample. In many cases, this is a question of concentration and/or length of incubation of antibodies (in the case of immunofluorescence), or expression level (in the case of genetically encoded fluorescent chimeras). The following protocol will describe how to prepare COS-7 cells by immunofluorescence for successful BaLM imaging of microtubules.

Materials

Fibronectin from human plasma (Sigma)
Phosphate-buffered saline (PBS; Life Technologies, cat. no. 10010-015)
COS-7 (African Green monkey kidney) cells (ATCC)
Cytoskeletal stabilization buffer (see recipe)
Wash buffer (see recipe)
4% (w/v) paraformaldehyde/0.2% (v/v) glutaraldehyde prepared by diluting stock solutions of 16% paraformaldehyde (Electron Microscopy Sciences, cat. no. 15710) and 25% glutaraldehyde (Electron Microscopy Sciences, cat. no. 16220), respectively
Blocking buffer: 5% bovine serum albumin (fraction V) powder in PBS
Primary antibody: anti-α-tubulin antibody (Sigma)
Anti–mouse IgG conjugated to Alexa Fluor 488 (Molecular Probes)
Four-well chamber slides (Lab-Tek)

Fix cultured COS-7 cells and prep for staining

1. Choose an imaging chamber

   For these experiments, the authors have utilized four well Lab-Tek chambers. However, cells for BaLM can be cultured in any appropriately sized tissue culture format that will suit the size of the insert on the microscope specimen stage. For example, when purchasing a stage from a vendor, there will be options for many types of formats for specimen holding inserts (slide, chamber slide, 35-mm dish, well plate, etc.). While format does not necessarily matter, it is imperative that the particular vessel have a coverslip bottom. Furthermore, the thickness of the coverslip must be matched to the objective lens in use. For example, many modern optics require a no. 1.5 (170 μm) thickness coverslip. Lastly, the size of the vessel is directly related to the amount of reagents required to prepare the sample. For immunofluorescence preparations, antibodies can be quite costly, and for this reason, smaller formats are often utilized. For the purposes of protocol, the following steps will use volumes relative to a four well chamber slide.

2. Dilute fibronectin (from human plasma) in PBS to a final concentration of 10 μg/ml.
3. Coat coverslip-bottom chambers by aliquotting 200 μl of fibronectin (10 μg/ml) into each chamber of the four-well chamber slides and incubating at 37°C for 1 to 2 hr.
4. Wash chambers times, each time with 500 μl per well of PBS.
5. Remove PBS. Plate COS-7 cells at the manufacturer's recommended seeding density, and incubate overnight at 37°C or to desired confluence.
6. Begin live cell extraction by briefly washing cells with 500 μl per well of prewarmed (37°C) PBS.
7. Lyse membranes by adding 500 μl of cytoskeletal stabilization buffer for 5 min.

8. Aspirate cytoskeletal stabilization buffer, replace with 500 μl of wash buffer, and let incubate for 1 min.
9. Fix with 500 μl per well of 4% paraformaldehyde/0.2% glutaraldehyde for 20 min.
10. Wash three times, each time for 5 min with 500 μl PBS.
11. Add 500 μl of blocking buffer for 10 min.

    There is no need to wash after this step as the antibodies are diluted in the same blocking buffer.

12. Replace blocking buffer with 500 μl of anti α-tubulin (primary) antibody (1:100 dilution in blocking buffer) for 1 to 2 hr.
13. Wash three times, each time for 5 min with 500 μl blocking buffer.
14. Add 500 μl per well of secondary antibody (anti–mouse IgG conjugated to Alexa Fluor 488; 1:100 dilution in blocking buffer) for 1 hr.
15. Wash three times, each time for 5 min with 500 μl of blocking buffer per well.
16. Add 500 μl of PBS and keep at 4°C until samples can be imaged.

OPTIMIZATION OF BaLM DATASET ACQUISITION

BASIC PROTOCOL 3

As mentioned previously, acquisition of BaLM datasets can be accomplished from a variety of samples stained with a spectrum of standard fluorescent probes. As such, there is not a single protocol that is applicable to all possible preparations. For example, since this technique exploits properties of both beaching and blinking associated with the majority of fluorescent probes, the acquisition parameters (laser power, exposure time, TIRF angle, etc.) can vary, especially considering the combination of hardware (objective lens, secondary magnification, laser head power, etc.) in a particular lab. Therefore, the protocol below describes how the sample preparation described above was previously imaged in order to encourage these fluorophore events to occur on a time scale and at sufficient concentration (within a given frame) to be optimal for BaLM microscopy. As the step annotations will describe, slightly adjusting these parameters will allow for a range of similar samples to be imaged. In fact, as the researcher becomes more familiar with the occurrence of these events in their specific sample prep, it will be straightforward to modify this basic protocol for use in more samples. Since it can be difficult to appreciate single-molecule fluorescence events during acquisition of a BaLM dataset, it may be necessary for the researcher to apply the image subtractions (see Basic Protocol 4 below) periodically to several frames to make sure that there is an optimal concentration and reasonable quality of single molecule PSFs for BaLM analysis. It is helpful to note that the researcher will need to decide whether or not to add fluorescent beads to the sample as a fiducial at this point. This addition is highly recommended if the experimental parameters can tolerate this, due to the fact that, despite a researcher's best efforts to minimize vibration and introduction of movement, it may be necessary to correct for drift in the image (electronic stages have various specifications for drift and accuracy that may be lower than the desired resolution of the dataset). Fluorescent beads are commercially available for most of the common wavelengths used for fluorescent imaging. For further recommendations on whether or not to use microspheres for image registration purposes, the reader is highly encouraged to read the "Image Alignment" subsection under Basic Protocol 4.

Materials

Sample
100-nm TetraSpeck microspheres (Molecular Probes)

Multidimensional imaging software: imaging software capable of experiment management of *X*/*Y* location (for automated stages), *Z*-axis position, wavelength of excitation (laser), emission filter position, laser power, TIRF angle, camera settings, and timing of frame acquisition; this software is sold by a variety of microscope vendors and third-party companies, as well as in freeware format (while each have their benefits and limitations, the researcher is encouraged to ensure compatibility with their system, and a good understanding of this software is required before acquiring datasets for BaLM)

Defining the acquisition parameters for BaLM imaging

1. *Optional:* Add fluorescent beads as fiducial markers for later drift correction.

   Add microspheres at a concentration such that 3 to 5 beads are visible per the camera's field of view (determined empirically). This number seems adequate for alignment purposes while not obscuring sample information. However, it should be noted that more beads (>20 per field) will enhance the quality of the StackReg alignment (detailed below) should the experiment be able to accommodate this. If this is the case, the increased image information may be suitable for the "alignment using StackReg plugin" method described in the Basic Protocol 4.

2. Screen the sample by wide-field epifluorescence or TIRF microscopy at low excitation energies to find the desired field of view.

   Upon initial screening of the sample, it is recommended that the user find a suitable field of view with the appropriate technique (wide-field or TIRF in this case), keeping either the lamp or laser at the lowest possible power to avoid prematurely bleaching the sample. In order to compensate for the loss of signal at lower excitation energies, additional steps to increase sensitivity and avoid bleaching during screening are encouraged. Such techniques may be sensor binning, image scaling, and/or high gain modes.

3. Define the bleaching and blinking parameters suitable for BaLM.

   Once a field of view has been found, it is necessary to define the imaging conditions for BaLM. In general, the ideal conditions are those that allow for as many isolated fluorescent molecules to be visualized and fitted as possible after subtraction of a given frame from the one preceding it (in the case of bleaching or blink-off events) or the one following it (for blink-on events). For a more detailed description of image subtractions, see Basic Protocol 4. Bleaching and blinking are stochastic processes that are a function of many parameters including the probe itself, molecular oxygen concentration of the buffer, pH, excitation energy, etc. As such, rather than obtaining a calculated value for each condition, it is much more practical to determine these values empirically, by imaging under a range of conditions for a particular sample. More specifically, it is recommended to start imaging at low laser powers and short integration times and increase these parameters until sufficient events can be appreciated by visualizing fluctuations in intensity. For example, the authors used a range of laser powers from 10% to 50% (relative to each laser) with integration times from 200 msec to 1 sec. The bleaching and blinking events were spread out from 400 frames to 3000 frames depending on the sample to ensure, first, that a reasonable number of molecules could be detected per frame, and second, that there would be enough frames to integrate signal from the majority of the structure. This latter consideration can mostly be mitigated by taking more frames than necessary. The experienced user will be comfortable with examining these events by eye (via the monitor) in real time, as single-molecule blink and bleach events are revealed as subtle fluctuations in intensity during the diffraction-limited acquisition phase. Video 1 (http://www.currentprotocols.com/protocol/cb2108) illustrates what a typical BaLM dataset acquisition looks like before image subtractions have taken place. It is important to note, however, that this dataset was dynamically scaled to demonstrate fluctuations in intensity throughout the video. In actuality, bleaching throughout this time course was significant and should be expected in similar experiments. The novice may find it helpful to perform the image subtractions in the next section to evaluate the number and quality of the resultant single molecule images.

IMAGE ARITHMETIC AND LOCALIZATION ANALYSIS FOR BaLM

BASIC PROTOCOL 4

Before localization analysis can be applied, image subtractions need to be performed that will allow for the visualization of isolated fluorescent signal arising from single molecules. This is accomplished in two steps. First, bleaching events, where individual fluorophores are irreversibly turned off (in addition to blink-off events, where fluorophores undergo transient dark states), can be rendered as single-molecule images by the subtraction of a given frame from the preceding one. Since the majority of fluorescent molecules do not undergo bleaching or blink-off events in a given frame, much of the intensity of the image is subtracted away, leaving only intensities from two sources: signal from single-molecule fluorescence, and noise. Second, blink-on events, or events where fluorophores transition from a dark state to an excited state, can be revealed by subtraction of an image from the following one. Video 21.8.2 (see *http://www.currentprotocols.com/protocol/cb2108*) demonstrates a diffraction-limited TIRF dataset (left panel) juxtaposed with the PSFs generated from that dataset (right panel) after image subtractions have been applied. This protocol can be broken down into three parts. First, the image arithmetic (subtractions) required for PSF generation will be detailed by stepping the user through the necessary steps in the freely available software ImageJ. Second, once single molecule–derived signal has been uncovered throughout the dataset, the procedure for proper [sub-pixel] registration/alignment will be detailed. Finally, the user will be instructed on how to properly localize the dataset through one (of the many) methods for Gaussian fitting (through the use of QuickPALM). It should be mentioned that QuickPALM is an efficient method for being able to generate and visualize the localization information from the dataset. However, QuickPALM does not calculate the localization precision for each molecule. With the introduction of localization-based superresolution methods by many companies, there are numerous programs which have been developed commercially that provide localization precision measurements in reasonable time frames. In addition, there are many freely available programs and plugins (such as the GraspJ plugin for ImageJ; *http://code.google.com/p/graspj/*) developed by various labs which can be applied to the resultant dataset to calculate localization precision. The reader is encouraged to explore the excellent reviews and tutorials available for these plugins available both on the developer Web sites, as well as in the literature (Henriques et al., 2010; QuickPALM; GraspJ).

Materials

ImageJ software (*http://rsbweb.nih.gov/ij/download.html*)
Stacks T-functions ImageJ plugin (*http://rsbweb.nih.gov/ij/plugins/builder.html*)
TIFF stack of bleaching/blinking data
Stacks Building ImageJ plugin (*http://rsbweb.nih.gov/ij/plugins/builder.html*)
StackReg ImageJ plugin (*http://bigwww.epfl.ch/thevenaz/stackreg/*)
QuickPALM ImageJ plugin (*http://code.google.com/p/quickpalm/*)

Revealing image bleaching/blinking events by image subtraction

1. Download and install the "Stacks T-functions" ImageJ plugin.

   As releases for ImageJ come out periodically, the reader is referred to the ImageJ Web site for instructions on proper installation of the core program, plugin installation, and compatibility of plugins with various updates of ImageJ software.

2. Open a TIFF stack of bleaching/blinking data using ImageJ.

   It is important to note that various acquisition software packages have their own proprietary image/dataset formats. However, there is almost always an option to export the dataset to a series of TIFFs (as single files) or a TIFF stack (one file containing a series of TIFFs). Once in this format, ImageJ (as well as almost any imaging software) will be able to recognize and analyze the file(s).

3. Reveal disappearing fluorescent puncta (bleach or blink off events) by using the "Delta F down" function in "Stacks T-functions" plugin.

   Keep this file separate, but open it, as it will be needed for a following step.

4. Reveal appearing fluorescent puncta (blink on events) by using the "Delta F up" function in "Stacks T-functions" plugin.

   Keep this file separate, but open as it will be needed for a following step.

5. Use the "Stacks Building" plugin to combine the resultant up and down TIFF stacks into a single stack.

6. Save this file for the subsequent steps of image alignment.

Image alignment

Drift correction is essential for proper image reconstruction of any single molecule–based superresolution technique. Precise alignment must be achieved with a resolution below the diffraction limit. In the following protocol steps, there are two options for researchers in the context of dataset alignment. The first is to use signal from the sample over time as a marker of drift, and the second is to use fluorescent beads. In order to enable software to properly register datasets on a sub-pixel basis over time, fiducials are conveniently created by adding fluorescently labeled microspheres (which are themselves diffraction-limited) to samples just before imaging. These microspheres act as point sources that appear in every frame during the acquisition (due to their resistance to photobleaching), and thus can be used to correct for drift over the entire dataset. However, using fluorescent microspheres for BaLM analysis is not as straightforward because they do not appear in subtracted images. Therefore, we have devised two strategies to deal with this problem. First, the ImageJ plugin StackReg can be used to align the raw images before image subtraction. StackReg works well for images with a higher information content [e.g., before substantial sample bleaching or in fields with many ($>$20) fluorescent microspheres], but does not work well when fluorescent signals from the sample diminish in intensity or few spheres are present. Since bleaching of the sample is a basic feature of BaLM datasets, and adding many microspheres can obscure biological structures of interest, we have developed another technique that utilizes a few (3 to 5) fluorescent microspheres as fiducial markers. This technique is based on adding images of the microspheres from the original acquisition dataset to corresponding subtracted image data. This results in a stack that has the images of both the molecules revealed by subtraction and the corresponding fluorescent fiducials. Plugins such as QuickPALM or GraspJ, which have been developed to analyze PALM and STORM data, are able to use less image information (fiducials) for drift correction. In addition, these plugins can perform fitting, localization, and image reconstruction. In regard to the localization, we have found that QuickPALM is a practical way to localize the fluorescent molecules revealed by BaLM image subtractions and to reconstruct a final BaLM image through an easy-to-use graphical interface. With some basic user inputs into the QuickPALM plugin concerning the dataset (pixel size, SNR, and maximal full width half maximum), the user is able to generate localization data based on the subtraction-generated PSFs with relative ease. Of course, the quality of the output is dependent on the many factors previously discussed. Figure 21.8.3 demonstrates a diffraction-limited TIRF image of a COS-7 microtubule (A), along with its corresponding BaLM dataset (B), and overlay of the two (C). The inset in panel C demonstrates the relative intensity profiles of the diffraction-limited image vs. the post-BaLM rendered microtubule.

Image alignment using StackReg plugin

7. Download and install the "StackReg" plugin for ImageJ.

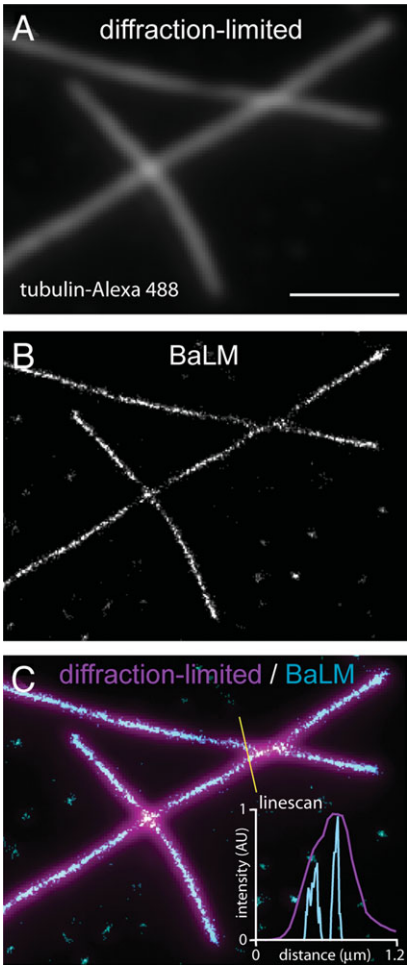

Figure 21.8.3 BaLM reconstructions using the QuickPALM plugin for ImageJ. Microtubules from COS-7 cells fixed and immunostained for α-tubulin and labeled with Alexa Fluor 488 were imaged using traditional diffraction-limited TIRF microscopy (**A**). Datasets from TIRF-acquired images under conditions ideal for BaLM were aligned, localized, and rendered using BaLM methods described (**B**). An overlay of the diffraction-limited TIRF dataset with the rendered BaLM data (**C**) conveys the respective spatial information differences between the two techniques. Intensity profile (C, inset) representing the line scan (yellow) in the overlay image, depicts the respective resolutions of both the diffraction-limited signal (purple) as well as the localized BaLM data (blue). Image adapted from Burnette et al. (2011b). For the color version of this figure, go to *http://www.currentprotocols.com/protocol/cb2108*.

8. Use the "Translation" setting in StackReg to align the original datasets (pre-subtraction).

   This works well to align data sets with larger amounts of image information. However, once samples become bleached, the registration and alignment quality degrade.

9. Subtract the aligned images with the T-Functions plugin.

   See protocol for image subtraction detailed above.

Alternative image alignment: Image alignment using fluorescent microspheres

10. Open an original dataset (pre-subtraction) in which fluorescent microspheres had been added and which at least 3 to 5 spheres are visible in the field of view.

11. Locate a fluorescent microsphere that is visible throughout the entire dataset for the desired stack (make sure that it does not drift out of the field of view during the acquisition).

12. Draw a region of interest around this sphere by using the box tool in ImageJ.
13. Create a stack of just the microsphere by using the "crop" function in ImageJ.
14. Save this TIFF stack separately.

    This is now a single fiducial that can be added to a subtracted dataset.

15. Repeat this step to produce 3 to 4 stacks of separate microspheres.
16. Open the corresponding subtracted dataset (per image subtraction sub-section above).
17. Using the "Stack Inserter" function of the "Stack Builder" plugin of ImageJ, insert the cropped microsphere stacks to a portion of the image (e.g., corners) that does not obstruct image information.

Localization of subtracted Images with QuickPALM plugin for ImageJ

18. Open a TIFF stack from the previously subtracted dataset containing the embedded microsphere stack information in ImageJ.
19. Navigate to the "Plugins" menu in ImageJ and highlight "QuickPALM" in the drop-down menu. Select "Analyze Particles."
20. A window will appear where the user can fill in some basic metrics from the image acquisition such as SNR, image plane pixel size, and maximum full width half maximum.
21. Additional settings the authors recommend are to enable "Smart SNR," "Online rendering," and "Stream particle info directly into file." These steps allow for visualization of the rendered localization data as well as outputs the localization values as the computation proceeds.
22. After the analysis is complete, the user will have the rendered image file as well as the list of localization data in a separate table that can be saved independently.
23. At this point, if the user wishes to correct for drift, highlight QuickPALM again (under the plugin's drop-down) and then navigate to and click "Correct Particles Drift."
24. Follow the on-screen prompts which will guide you through the creation of ROIs, which should be placed around the microsphere stack data embedded into the image.
25. Once ROIs have been created, navigate back to the "Correct Particles Drift" submenu, which will now correct for drift in the image.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Cytoskeletal stabilization buffer

100 mM PIPES
4% (w/v) 30,000 MW PEG
10 μM phalloidin (Sigma, cat. no. P1951)
10 μM Taxol (Sigma, cat. no. T7402)
5 mM EGTA
5 mM $MgCl_2$
Prepare fresh just before use

Wash buffer

100 mM PIPES
10 μM phalloidin (Sigma, cat. no. P1951)
10 μM Taxol (Sigma, cat. no. T7402)
5 mM EGTA
5 mM $MgCl_2$
Prepare fresh just before use

COMMENTARY

Background Information

BaLM microscopy was developed out of a necessity to garner high-resolution information from samples stained with standard fluorescent probes and labeling methodologies ubiquitous among cell biology laboratories. As mentioned in the introduction, prior to development of BaLM, significant work had already been accomplished in order to localize single molecules from the PSFs of stochastically activated fluorophores. In fact, these complex operations had already been integrated into nicely packaged plugins for freely available software like ImageJ. The challenge in the development of the BaLM technique was the extraction of single-molecule information from a large number of simultaneously fluorescent proteins, unlike the stochastic activation of a small subset of single molecules which is the case with other single-molecule techniques like PALM and STORM. However, the bleaching and blinking inherent in fluorescent proteins, previously considered a burden for various imaging and biochemical studies (Ha and Tinnefeld, 2012), could be exploited as a means to reveal single-molecule fluorescence given that they all would undergo this process stochastically. While all probes differ in their rates of either bleaching or blinking, the acquisition parameters can be modified to encourage these events to occur and further adapt to changing sample types.

Theoretically, one should be able to subtract a given image from the one immediately before it to reveal bleach or blink-off events in a given diffraction-limited dataset. Likewise, for blink-on events, one should be able to subtract an image from the subsequent one. If this is done by using traditional TIRF datasets, for example, most of what is seen is noise due to the fact that such datasets have inherently higher background as a consequence of imaging thousands of simultaneously fluorescing molecules at one time. More importantly, in the previous scenario, the signal from individual molecules is averaged out by comparatively large pixels. Through the use of additional magnification not aimed at imaging beyond the diffraction limit, BaLM was able to spread out the dynamic range to more pixels and mitigate this obscurity to single-molecule events. Combined with simple image arithmetic and powerful localization software that had been previously developed by others, BaLM became a reasonable way to access high-resolution information from quite common sample preps.

Critical Parameters

As mentioned previously, there are a few considerations for BaLM that are critical to success, beyond the actual microscope itself. First is the careful preparation of the sample, which must have enough fluorescent molecules integrated into the desired target to be able to image at higher resolutions. This applies to either the concentration and length of incubation of antibodies for immunostaining procedures or to the time of expression of a fluorescent construct inside a transfected cell. The second critical parameter is the optimization of the acquisition of BaLM datasets. This is difficult to translate in the scope of a single protocol, given that this technique applies to so many probes and conditions. However, keeping signal-to-noise ratio always at the forefront when considering imaging conditions is critical. Specific solutions involving identification and mitigation of as many noise sources as possible, such as background fluorescence, cross-reactivity of antibodies, or interference from out-of-focus fluorescence, are imperative. Lastly, hardware configurations and settings during the acquisition are extremely important parts of the experimental process, as these will constantly change between samples, even of the same type and in the same dish. The researcher is highly encouraged to take the time to try a range of hardware settings such as laser power, TIRF angle, and integration time to adjust the bleaching and blinking of the sample across a reasonable time frame. In our hands, many of our datasets were on the

order of 10 min with respect to total time of acquisition.

Troubleshooting

The user is strongly encouraged to spread the time of acquisition out to avoid overlapping single-molecule events that reduce the quality of the PSFs generated after image subtraction. Since the timing of hardware components is critical during BaLM acquisition, a significant number of problems can be avoided by making sure that the communication between the software and hardware elements is as efficient as possible. Common strategies include ensuring that only necessary hardware devices are active within the acquisition parameters, as well as keeping background processing to a minimum. The largest delay that should occur during data acquisition is the exposure (and frame readout) itself, and potentially the emission-filter change (if using multi-color BaLM). Keeping this communication clean will help in the total time of acquisition in addition to preventing exposure of the sample that is not linked to integration.

Anticipated Results

Many researchers see the benefit of imaging beyond the diffraction limit and are immediately drawn to superresolution techniques in order to answer pressing biological questions. However, one should take note of the applicability of these techniques, including BaLM, as there are several limitations that may make superresolution, especially point localization–based methods, ill-suited for a particular question. Cellular dynamics poses one of the most common limitations to many current superresolution techniques. While there are certain configurations that are getting closer to imaging live-cell environments, the point-localization–based superresolution techniques have long had limitations in this area due to their iterative nature and in some cases toxic buffers. Advanced sensor-laser triggering paradigms have improved these acquisition conditions; however, they are far from the temporal resolution achievements of diffraction-limited microscopy.

Lastly, it is beneficial for the researcher entering the superresolution field to consider some misconceptions with regard to superresolution imaging. The term "superresolution imaging" itself is a bit misleading. First, it does not describe one particular technique, or even group of techniques with the same principle, but only that a given dataset achieves a resolution that surpasses the classical diffraction limit, described by Abbe's formula for the resolving power of a lens:

$$d = 0.61 * \frac{\lambda}{\mathrm{NA}}$$

where d represents the minimally resolved distance in nm, λ is the wavelength of light in nm, and NA is the numerical aperture of the objective (Murphy and Davidson, 2012). Second, superresolution imaging does not mean that the imaging itself is not diffraction-limited. For the most part, superresolution imaging still employs much of the same diffraction-limited hardware cell biologists use for more traditional forms of fluorescence microscopy such as wide-field, confocal, or TIRF. However, clever ways of illuminating the sample and post-acquisition computational analysis confer the ability to access superresolution information. Finally, while there are some exceptions, the majority of superresolution images are not images per se, but rather datasets generated by such computation.

Time Considerations

The time commitment for BaLM microscopy may or may not be significant, depending on several parameters. First, if one is not interested in the localization precision, which can require significantly more computation time, then the majority of the time is spent on sample preparation and the dataset-registration process (depending on the method). More specifically, analysis of datasets not requiring precision measurement are on the order of minutes, with the majority of the time being spent on file management (analysis itself takes seconds). However, depending on the program utilized and the size of the dataset, the localization process for determining the precision of localization can take on the order of a few hours.

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