Effective, non-invasive, high-resolution imaging of biological tissues

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New advances in imaging technology now allow for thorough high-resolution investigation of biological tissues that were previously impossible. Whereas traditional confocal light microscopy offers lower resolution images of cells prepared on a sample slide, newer techniques are capable of high resolution three-dimensional imaging, tracking movement within a cell, and even non-invasive imaging of tissues in vivo. These techniques have been successfully applied to challenges such as imaging scaffolds embedded in soft tissues, human chromosomes, and biopolymers. However, careful evaluation of new technologies is still necessary as not all techniques are appropriate for each sample. Researchers must consider desired resolution, sample size, and acceptable sample preparation in order to produce the best reproduction of the biological tissue. This review details the relative merits of several new innovations in fluorescence imaging, as well as their effective application to current research and possible areas of future exploration.

Keywords: Fluorescent imaging; SIM; PALM; Bessel Beam microscopy; Two photon excitation

1. Introduction

Recent innovations in imaging technology have made enormous progress with regards to biological tissues and live cells. In the past, imaging biological tissue and live cells was primarily possible using light and electron microscopy. However, these and other older techniques suffer from inadequacies, two of the most prominent among them being low resolution and excessive sample preparation. For example, transmission electron microscopy (TEM) provides high resolution images, but only of samples fixed in epoxy and sliced to a thickness of 50 nm or less to prevent scattering effects [1]. Nuclear magnetic resonance (NMR) spectroscopy delivers detailed structural information, but requires purified samples of exclusively smaller molecules [2]. Crystallography offers molecular maps of proteins with near atomic resolution; however, only a limited number of proteins will crystallize and often in non-native configurations. Membrane proteins serve as a good example of this as they are a critical part of drug delivery research but difficult to crystallize [3]. A non-native sample offers little context to explain its function and often may not demonstrate the same structure as the functional form. Drawbacks such as these all derive from preparing a sample outside of its native environment and have motivated the search for new ways to visualize cell interiors. Therefore non-invasive imaging of biological tissues and live cells is a primary goal for further research.

In addition to being non-invasive, newer imaging techniques also strive to increase resolution to study dynamic processes within live cells. Unlike atomic force microscopy (AFM), which can observe a native sample but only relay surface information, nanoscale labels which can be resolved in time as well as three-dimensional space will allow dynamic processes and interactions to be studied either in a tissue or individual cells. To an extent these efforts have succeeded; crucial mechanisms within the cell can now be more easily tracked, three-dimensional visualizations produced, and techniques continue to become less invasive. However, these considerations must still be weighed when choosing an imaging technique as no single technology has overall superiority.

Research into imaging biological tissue has proceeded by two main paths; x-ray diffraction and fluorescence imaging. Given the degree to which fluorescence has been adopted in biology, this chapter will cover the following fluorescence techniques: single molecule localization, stimulated emission depletion microscopy (STED), structured illumination microscopy (SIM) and saturated structured illumination microscopy (SSIM), photo-activation localization microscopy (PALM), stochastic optical reconstruction microscopy (STORM), selective plane illumination, and Bessel beam microscopy. Several of the most promising techniques will be discussed both theoretically and in the context of their contributions to recent experiments.

2. Considerations

Since no imaging technique can be applied perfectly to all samples and situations, a variety of factors need to be considered in order to choose the technique that will produce the best images. It should be noted that obtaining the “best” image is not necessarily as simple as producing one with the highest resolution possible since biological tissues present unique imaging challenges. Thus these optimization criteria are labeled “considerations”, rather than advantages and disadvantages, as what might be catastrophic for one sample might be a saving grace for another. These considerations are discussed here first in order to give sufficient background to facilitate the discussion of the experimental merits of each imaging technique.
2.1 Imaging Time

Imaging time is a practical consideration that arises in every experiment, and of the methods considered here total imaging time can range between a few seconds and several hours [4, 5]. In the case of three-dimensional reconstructions, it is also necessary to consider how many frames will be needed to form the final image. For example, a fluorescence image can achieve “super-resolution” by averaging multiple images together but this requires additional images of sparse fluorophore populations [6]. There may also be additional time built in by the switching speed of the fluorophore and the photon emission rate. Therefore the time to obtain each individual image may be multiplied many times to create the final image. Fluorescence imaging is challenging because exposures for a long time or at high intensity can result in photobleaching of the fluorophores and reduce the detectable signal.

2.2 Source

For fluorescence microscopy, there are source considerations rooted in the excitation wavelengths necessary for one or more types of fluorophore [7]. Fluorescence microscopy can also require a step to introduce fluorescence into the sample if none of the biological components are naturally so. For example, a sample cell can be genetically engineered to include a fluorescent tag; however, the same cannot be done to image something of concern in a living human being [7].

2.3 Detector

Complementary to the source consideration is the choice of detector. A CCD camera is normally used for fluorescence microscopy, often with quantum efficiencies as high as 90% [8]. Images then might need to be assembled by an algorithm which uses multiple fluorophores to achieve super-resolution images of a structure [9].

2.4 Depth of Field

Depth of field is the thickness in the image through which the microscope can be focused while still maintaining sufficient resolution. A technique’s depth of field must be considered because visualizing an organelle in a single cell is very different than tracking cells in a tissue or a scaffold in a limb. This is especially important for biological tissues because it is not always acceptable to slice a sample in order to image the interior, either because of the damage that it will cause the sample or due to the volume of material being imaged. As many researchers would like to apply these techniques to live cells, depth of field should also be considered as components could move in and out of the image at different time points.

2.5 Resolution

Lastly there is the issue of desired resolution. Image resolution is intrinsically linked to all of the above characteristics and more often than not the researcher will encounter a trade-off between higher resolution and other desirable characteristics. Resolution is defined as the ease by which adjacent features in an image can be distinguished and the experiments considered here generally quantify it in one of two ways [7]. The Fourier ring correlation resolution can be calculated by dividing a data set in half and forming two separate three-dimensional reconstructions; these are then compared to estimate the resolution [10]. There is also the Rayleigh criterion for calculating resolution which determines the smallest resolvable lateral separation of two high-contrast objects in the image [11]. However, there are cases where higher resolution is not paramount, such as when imaging a larger feature or in an experiment focused on temporal changes. In most other cases, new technologies aim to deliver higher resolutions in order to see details that might have escaped previous studies and track even smaller cellular components. It is important to remember that improvements in theoretical resolution are not necessarily coupled to improvements in practical resolution. This can be due to discrepancies in experimental setups or flawed parts that restrict the ability of the practical resolution to achieve model predictions [12]. There are also boundaries on the achievable theoretical resolution which derive from variables such as source quality, fluorophore size, and the detector’s ability to register a signal.

3. Applications

The above section on considerations has emphasized that every method will inherently involve a balancing act between various desirable image characteristics. However, that should not be taken to mean that all methodologies fall short of their intended goal; in fact, the following techniques have been applied successfully to imaging biological materials and have opened up new areas of research. Some select techniques will be explained both in theory and in practice to demonstrate the range of biological tissues that can now be imaged, as well as the situations for which each technique is best suited.

Techniques that use native or introduced fluorescence for imaging have existed for decades; however, more recently techniques classified to as “super-resolution fluorescence microscopy” have been developed to image processes on
much smaller length scales such that they could even look inside an individual cell. Improving fluorescence microscopy in particular is worth investing researchers’ time and effort because it offers superior time resolution, less invasion of tissue, the ability to track interactions between multiple agents, investigation of thick samples, and image resolution not limited by the diffraction of light. These are desirable when imaging biological tissue, and especially living samples, therefore super-resolution fluorescence microscopy techniques have been used in a variety of experiments designed to investigate dynamic processes.

Fluorescence microscopy is simple in theory; a fluorophore is induced to fluoresce and its location is captured by a CCD camera. As such, fluorescence microscopy techniques parse information in real time and can be used on live samples often with no additional preparation. These are also attractive methods due to the simplicity of collecting and processing the data produced. Early work on multifocal multiphoton microscopy by Straub et al. has demonstrated the potential of fluorescence microscopy to produce image slices that could be combined to yield three-dimensional reconstructions on the order of seconds for all but the most strongly scattering samples [13].

Today, a variety of photo-activatable and photo-switchable fluorophores are available to researchers, and can be specifically attached to different cellular components in order to track biological processes via multicolor imaging [7]. In the past, the diffraction of light has limited the resolution to about 200-300 nm which is larger than many biological structures [7]. Thus far one of the biggest challenges to the field has been improving the practical spatial and time resolutions of the methodologies such that they are relevant for the desired applications. This has been achieved by super-resolution techniques which are unlimited by the diffraction of light. These methods are particularly important to investigations of biological tissues because protein interactions within a cell occur on a much smaller scale than is observable with traditional light microscopy and often at high local densities [12].

While super-resolution fluorescence images are not limited by diffraction, there are other factors which limit the techniques’ precision. All in all, the resolution will only be as good as the probe used to define the important features and the detector’s ability to resolve those probes. Therefore fluorophore labeling density needs to be balanced such that there are enough probes to map the sample but not so many that individual probes cannot be resolved [7]. Often this is dealt with by manipulating the excitation volume such that wide-field illumination is used to image at low concentrations and the excitation volume is reduced as the density increases [14]. Part of the process also consists of choosing the correct probes for the application since they come in a variety of sizes and can be excited at different wavelengths. Researchers might also be restricted by the sample tissue itself as a human cannot be genetically engineered to include a simple GFP tag nor can they undergo damaging fixation techniques [7].

3.1 Single-Molecule Localization

One method used to produce super-resolution fluorescence images is single-molecule fluorescence imaging. This is done with a wide-field microscope that can manipulate the double-helix point spread function (PSF), where the PSF is the three-dimensional intensity distribution of the image of a point object [7]. Pavani et al. imaged sparse sets of photoactivatable fluorophores in order to get 10-20 nm resolution in a 2 µm thick polymer sample [6]. The group made their double helix PSF technique unique by relating the axial position of the fluorophore to the angle between the PSF lobes and also relied on super-localization techniques that are based on the center position of the PSF being determined with great certainty [6]. Their results can be seen in Fig. 1 which demonstrates that a double helix PSF method is capable of imaging many more fluorophores in close proximity in a single image than traditional PSF imaging.

![Fig. 1](image_url) 3D superlocalizations of a low concentration of DCDHF-P molecules in a thick PMMA sample. (A) Comparison of the standard PSF (i.e., Upper, SLM off) to the DH-PSF image of 2 molecules (Lower, SLM on). (Scale bar: 1 µm.) (B) Representative image of many single molecules at different x, y, and z positions. (Scale bar: 2 µm.) Reproduced from [6]. Copyright PNAS 2009.

However, since these maps require multiple images to be knit together to produce the final high-precision image, algorithms are needed to reconstruct the fluorophore maps. Two of the successful algorithms which are used in different situations to produce such maps are called radial symmetry and compressed sensing. Radial symmetry works by replacing the PSF with a point representing its center coordinates and has produced high resolution images of E. coli
[15]. Alternatively, compressed sensing uses a PSF model to estimate several molecules at once from populations of densely packed signals [9]. The radial symmetry technique is therefore better suited for noisy images for which PSF models do not exist and the compressed sensing technique can be used to reduce the number of necessary frames for well-characterized samples [9]. The compressed sensing method can handle activated fluorophore densities an order of magnitude higher than single-molecule fitting methods, and has been used to image microtubules in live cells with 60 nm spatial resolution and 3 second temporal resolution [16].

3.2 Stimulated Emission Depletion Microscopy

While most super-resolution fluorescence techniques follow the general procedures described in the introduction, many groups have developed variations best suited for their particular research which are worth considering. Stimulated emission depletion microscopy (STED) is a point-scanning technique that works by refining the excitation volume with another laser to suppress undesirable excitation around the excitation beam, therefore theoretically achieving unlimited resolution [7]. STED is often used to image samples labeled with multiple fluorophores which have different activation wavelengths to determine position [7]. However, in practice the required high power density for STED can cause increased photo-bleaching and photo-toxicity. Though this can be reduced in some cases, techniques that avoid this by only activating a fluorophore for a portion of the imaging process are becoming increasingly attractive [17].

3.3 Structured Illumination Microscopy

Structured illumination microscopy (SIM) can achieve twice the diffraction-limited resolution in three dimensions and decouples the acquisition speed from the size of the lateral field of view [18]. SIM does this by patterning the illumination field so that interference brings high-frequency features down to more detectable low frequencies and makes normally unobservable regions of frequency space visible [7]. Figure 2 shows SIM work by Fiolka et al. on cells with vesicles and the actin cytoskeleton labelled to demonstrate its superiority to conventional microscopy in that it can track these dynamic processes occurring within a cell.

![Fig. 2](image)

Saturated structured illumination microscopy (SSIM) also introduces interference into the illumination field to achieve resolutions beyond the diffraction limit, but it factors in the nonlinear behavior of saturation fluorescence emission as well [7, 19]. This means that the fluorophore emission is saturated to incorporate spatial features into the excitation pattern which are smaller than those achievable by diffraction [7]. SSIM therefore has potential to improve on current methods as well. For example, Elf et al. used mapped lac repressors in E. coli samples with genetically-encodable fluorescent proteins. While they achieved a time resolution high enough to measure the kinetics of binding in the cell, they faced difficulties due to photobleaching which could possibly be mitigated using SSIM techniques [20].

3.4 PALM/STORM

Other groups have taken a different approach to obtaining super-resolution, selectively activating only portions of the fluorophores during collection. Wang et al. used stochastic optical reconstruction microscopy (STORM) to study samples with dense fluorescence via a system that can image approximately 1000 molecules per cell per minute. This can be seen in Figure 3 which tracks the locations of proteins known to associate with DNA. Floris et al. discuss a new combination of photo-activation localization microscopy (PALM) and STORM, which both excite only a fraction of photo-switchable fluorophores within each imaging cycle, to investigate chromatic organization [21]. Chromatin in particular is challenging to image because it needs to be examined on a nanometer length scale and proteins of interest are densely packed. However, PALM and STORM have already been used successfully. This method has been used on directly labeled DNA structures in vitro to produce images with a resolution of 20 nm from photoswitchable cyanine dyes used for single-molecule localization [7].
3.5 Structured Plane Illumination

Additionally, one of the most interesting techniques that will continue to yield improvements is structured plane illumination, which also limits photobleaching. This is done by combining super-resolution structured illumination microscopy with ultrathin planar illumination to study thicker or more densely fluorescent samples [12]. These methods are especially useful for thicker specimens because they mitigate the problem of out-of-focus fluorescent excitation by creating a sheet of light parallel to the imaging plane [14]. This decreases the illuminated volume by exciting fluorophores only within the focal plane [14]. Gebhardt et al. apply this in a method called reflected light-sheet microscopy (RLSM) which involves the creation of excitation light sheets with a thickness of about 0.5 µm. This let them image glucocorticoid receptors bound to DNA using two-color single-molecule imaging of two different protein pairs for improved understanding of the action of transcription factors [14]. The improvement of RLSM compared to HILO (an existing technique) can be seen in Figure 4.

3.6 Bessel Beam Microscopy

Recently, many groups have moved toward a specific type of light-sheet microscopy called Bessel beam microscopy. This technique consists of a shaped scanning beam which can penetrate deeply in samples, without significantly sacrificing contrast or resolution [22]. The Bessel beam method also reduces photobleaching and has fewer scattering artifacts [23]. Like all light-sheet techniques, Bessel beam microscopy is fast and reduces the light absorbed by the sample by limiting the excitation volume to a single focal plane at a time [23]. But Bessel beams are often preferred to...
other selective plane illumination methods for two reasons. First, their self-reconstructing abilities allow for larger depths of focus since they reform after passing obstructions and second, Bessel beam images are nearly isotropic in three dimensions whereas conventional plane illumination images are not [12, 22].

Bessel beam imaging can be further subdivided into techniques that have been experimentally refined for specific applications. Two-photon excitation (TPE) light sheets can be used to reduce undesirable excitation by suppressing Bessel side lobes and produce sheets 0.5 µm thick [23]. There is also optical sectioning structured illumination microscopy (OS-SIM) which is patterned such that a computer can eliminate background fluorescence [12]. Thus far, TPE has been the more promising of the two as OS-SIM is slower and more phototoxic to cells [12]. TPE light sheet microscopy combines the large penetration depth of TPE point-scanning microscopy with the higher acquisition speed and reduced photodamage of light sheet microscopy to great effect [24]. Additionally, Fahrbach et al. have investigated sectioned Bessel beams (SBB) which have a portion of their angular spectrum obscured which allows them to decouple optical sectioning from the depth of field. While the group acknowledges that SBB has a slightly lower light efficiency, this is offset by the approximately 15% improvement in contrast which makes it desirable for their intended application [25].

Experimentally Bessel beam microscopy techniques have improved researchers’ ability to image biological tissues tremendously. Fahrbach et al. produced a system that is easily added to a standard lab microscope and claim that their sectioned Bessel beams combined with confocal-line detection improve optical sectioning by a factor of four compared to unsectioned Bessel beams with the same depth of field. Gao et al. tested their own super-resolution Bessel beam structured plane illumination on C. elegans embryos up to 10 mm in thickness with up to two times higher axial resolution compared to confocal microscopy [12]. They have also shown that their technique outperforms OS-SIM in Fig. 5.

Also promising are results from Planchon et al. who imaged live cells with a three-dimensional resolution of 0.3 µm with a speed of almost 200 image planes per second to get images such as Figure 6. Moving forward, this could be tested on large ensembles of cells with the eventual goal of imaging entire tissues.
4. Conclusion

While the techniques covered here span a variety of different operating concepts and instrumentation requirements, they share the common goal of imaging of biological tissues under native conditions in order to increase understanding of how those tissues operate in a live organism. Many of the experiments aim to increase resolution without sacrificing important experimental considerations such as depth of field and photobleaching. In order to image tissues within living cells and tissues, fluorescence imaging has developed to take images quickly with spatial resolutions approaching that of x-ray diffraction technologies. Not only can multicolor imaging methods track dynamic processes, they can do so in living cells or tissues with no sample preparation beyond the introduction of the fluorescing elements. While a single one of these methodologies could be sufficient to image a biological tissue and produce novel information, these technologies should be taken together for the most comprehensive sample imaging suitable for a variety of experimentation conditions and samples. In the future, there is potential for researchers using biological tissue to not only see improvement in resolution but also to procure bench-top diagnostic systems that are fast, non-invasive, and safe for in vivo imaging.

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References