

AR INSIGHTS

Technical Review: Types of Imaging—Direct STORM

ABSTRACT

In the 1990s, new concepts of microscopy revolutionized the imaging field by breaking the lateral resolution diffraction limit for the first time, even with propagating light and regular lenses (i.e., far-field). In 2006, several research groups independently showed super-resolution microscopy using high-precision localization of single fluorophores. These new developments in single-molecule spectroscopy enabled a different approach to achieving nanometer-scale optical microscopy. Direct stochastic optical reconstruction microscopy (dSTORM) is a technique of single-molecule super-resolution imaging that does not require an activator fluorophore. This technique is used to visualize cellular structures with a resolution of approximately 20 nm. dSTORM is compatible with many conventionally used fluorophores. This article provides an overview of the principles and uses of dSTORM. Advantages and disadvantages of dSTORM are also discussed. *Anat Rec*, 297:2227–2231, 2014. © 2014 Wiley Periodicals, Inc.

BACKGROUND

Microscopy plays an important role in understanding cellular functions. Developments in technology and improved manufacturing have resulted in a large improvement in image quality. However, researchers were originally faced with a limit in optical resolution. Visible light in the far field has a spatial resolution of approximately 200–300 nm in the imaging plane. Consequently, this does not allow investigation of cellular structures and protein complexes that are only tens of nanometers in size. Recently, several new types of technology, collectively called super-resolution microscopy (also known as nanoscopy), have been developed that break or bypass the classical diffraction limit, and improve the optical resolution to the macromolecular or molecular level. Super-resolution microscopy can be categorized into two types, termed near-field and far-field (Schermelleh et al., 2010). Near-field super-resolution imaging overcomes the diffraction limit by removing the lenses, thereby abolishing the need to focus. Near-field super-resolution imaging has been used to study the organization of several membrane proteins, but this type of imaging is technically difficult. Additionally, the near-field approach cannot be used for intracellular imaging. The far-field approach uses lenses,

which are placed at a distance from the sample. Many of these super-resolution technologies have become commercially available, making them an attractive option for researchers.

Detection of single molecules provides new possibilities for achieving subdiffraction-limit spatial resolution. The location of a single emitter can be established with high accuracy if there are a sufficient number of photons collected. Stochastic optical reconstruction microscopy (STORM) is a recent far-field super-resolution imaging technique in which a fluorescence image is constructed from highly accurate localization of individual fluorescent molecules, which are switched on and off by different-colored lasers. STORM imaging consists of a series of imaging cycles. The STORM technique was first presented by Rust et al. (2006) using Cy3–Cy5 dye pairs as the optical switch to activate a subset of fluorophores. These authors showed a resolution of 20 nm for RecA-coated circular plasmid DNA. Heilemann et al. (2008) introduced a variation of STORM called direct STORM (dSTORM) in 2008. This technique uses conventional photoswitchable fluorescent probes that are able to be reversibly cycled between a fluorescent and dark condition by irradiation, using light with different wavelengths, without requiring an activator fluorophore. This article focuses on the super-resolution microscopy technique, dSTORM.



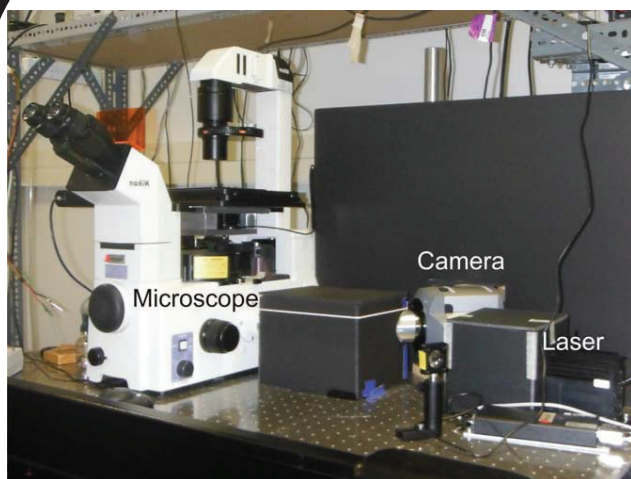


Fig. 1. Photograph of a custom built dSTORM microscope developed at the University of Auckland. The system consists of a Nikon TE2000 inverted microscope with a 1.49 NA oil immersion TIRF objective, an electron multiplying charged-coupled device camera, and a solid state 671-nm laser. For further details, refer to Baddeley et al. (2011).

BASIC PRINCIPLES OF DIRECT STORM Theory of Localization Microscopy

Direct STORM is a form of localization microscopy that uses conventional fluorescent dyes. The key concept in localization microscopy is that the position of a single fluorescent molecule can be determined to an accuracy of an order of magnitude below the diffraction limit of the same molecule. Inherent in this method is the requirement to image fluorescence molecules individually in time, allowing the position of thousands of molecules to be determined, thereby creating a high resolution image (van de Linde et al., 2011a).

Initially, single molecule localization was achieved with photoactivated light microscopy (photoactivatable localization microscopy [PALM] or fPALM) (Betzig et al., 2006; Hess et al., 2006) and STORM (Rust et al., 2006). PALM uses genetically engineered fluorescent proteins, such as photoactivatable green fluorescent protein (PA-GFP) to label structures of interest. PA-GFP is initially nonfluorescent, but can be converted to the fluorescence state by a brief pulse of 405-nm laser light. By controlling the laser intensity, only subsets of PA-GFP molecules (that are spatially resolved) become activated. The activated PA-GFP is then imaged and bleached by exciting with a 561-nm laser. This cycle of activation, imaging, and bleaching is then repeated many thousands of times, enabling construction of a super-resolution image from the positions of single-molecule events (Betzig et al., 2006; Hess et al., 2006).

STORM achieves single-molecule localization by using a Cy3–Cy5 dye pair as a switchable fluorophore attached to DNA or protein. With this technique, a 633-nm red laser is used initially at high intensity to push all of the Cy5 dye molecules into a dark state. A second 532-nm green laser pulse is then used to excite Cy3, which in turn reactivates Cy5, albeit at a much lower density. These Cy5 molecules can then be excited and localized with a red laser. Repeating this procedure many thousands of times allows construction of super-resolution images in a similar manner to PALM. However, an advantage of the STORM method is that the fluorescence is brighter and each fluorescent molecule can be cycled hundreds of times before irreversible bleaching (Rust et al., 2006). Subsequently, conventional fluorophores, including Cy5, were found to be photoswitched without the need of an activator fluorophore (Heilemann et al., 2008; Baddeley et al., 2009a). This ability to use standard fluorophores has facilitated the application of existing antibody and nucleic acid labeling procedures for super-resolution imaging.

Control of Photoswitching

The ability to control photoswitching between bright and dark states is important for successful dSTORM imaging. Fluorophores that can be photoswitched include the widely available carbocyanine dyes (e.g., Alexa Fluor 647, Cy5) and rhodamine class dyes (e.g., Alexa Fluor 488, Atto 532). The conversion to a long-lived dark state is thought to involve photo reduction of the excited triplet state into energetically stabilized radical anions. In addition to laser intensity, the formation of a dark state is facilitated by the addition of a thiol, such as β -mercaptoethylamine, to slide mountant. Conversion back to a bright state involves oxygenation of the reduced radical back to the singlet ground state (van de Linde et al., 2011b). This can occur spontaneously (Baddeley et al., 2009a) or can be promoted by use of an activation laser (Heilemann et al., 2008) or oxidizing agent (Vogelsang et al., 2008). Cyanine dyes are prone to irreversible oxidation and photocycling is substantially enhanced by removal of oxygen (van de Linde et al., 2011a). This is usually achieved by addition of enzymatic and chemical oxygen scavenging systems to the sample mountant (Vogelsang et al., 2008; Rasnik et al., 2006). By varying the laser intensity, thiol, and oxygen concentrations, the number of active fluorophores is optimized so that they are spatially separated at distances greater than the diffraction limit.

Constructing a Localization Image

To construct a super-resolution image, the position of single-molecule events needs to be accurately determined. To obtain these positions, a movie or image stack is recorded at a sufficient frame rate (20–100 Hz) to

catch the flashes from single fluorescent molecules switching on and off (van de Linde et al., 2011a). The shape of these events is described by point spread function (PSF) of the microscope, which is governed by diffraction of light and properties of the optical path. The center of the PSF can be accurately determined by the fitting of a Gaussian function. The image is then created by fitting a Gaussian function to every single-molecule event and rendering an image based on their positions (Rust et al., 2006). Fortunately, the fitting is achieved programmatically and in real time because many thousands of positions are required for each image (Wolter et al., 2010). Initially, localization images were based on using a symbol to denote the position of each single-molecule in a scatter plot. However, these visualizations obscure fine detail when the plot density exceeds symbol size. To overcome this, various visualization algorithms have been developed to convey the density of events as intensity levels in a manner that is intuitively similar to conventional fluorescent images. The resolution of the image will not only be affected by fitting accuracy, but also by the overall density of the single-molecule events (Baddeley, 2010). This is an important consideration when selecting structures to image because the concentration of these events will be dependent on the target density and the efficiency in which it can be labeled.

Microscope Setup

A major advantage of the dSTORM method is the inexpensive equipment and ease of optical alignment compared with super-resolution microscopes based on structured illumination and stimulated emission depletion (Soeller and Baddeley, 2013). Setups typically use an inverted fluorescence microscope with a high numerical aperture oil-immersion, total internal reflection fluorescent (TIRF) lens (Fig. 1). The TIRF lens is used to restrict laser illumination to a thin sheet, which provides a high signal-to-noise ratio required for detection of single-molecule fluorescence. This light sheet can be implemented using TIRF to illuminate structures within 200 nm of the coverslip or by an inclined plane to image deeper (Tokunaga et al., 2008). The imaging laser will depend on the fluorescent probe (e.g., 641 nm for Alexa Fluor 647), but needs to have sufficient power (e.g., 5–30 kW cm⁻²) to push the label into the reduced dark state. An electron-multiplying charged-coupled device is used to capture the fluorescence signal from single-molecule events over imaging areas of approximately 20 × 20 μm² at approximately 100 nm per pixel (van de Linde et al., 2011a). To work efficiently, the computing setup needs sufficient processing power to capture the data, but this is easily met by most modern desktops (Wolter et al., 2010). Because the system is based on a standard microscope, it is a simple matter to capture both wide-field and

super-resolution images of the same structures. This allows nanometer detail to be placed into an overall cellular and tissue context.

ADVANTAGES AND DISADVANTAGES OF DIRECT STORM

Advantages of dSTORM

Advantages of dSTORM include the following. dSTORM is a simple setup and relatively cheap. dSTORM can be used on tissue sections. This technique does not need special fluorophore pairs used in STORM. dSTORM can be used to visualize cellular structures with a resolution of approximately 20 nm without requiring an activator molecule (Leung and Chou, 2011). Other advantages of dSTORM are that small organic fluorophores can be used, which survive conditions of moderate excitation for a long time (i.e., high photostability), they emit thousands of photons, and external means can be used to control their rate of photoswitching (Heilemann et al., 2008, 2009). In addition, many commercially available fluorescent probes, which span the complete visible spectral range, are able to be applied as photoswitches in aqueous solvents by addition of reducing thiols. Conventional dyes can be used for dSTORM, thus not being reliant on genetic engineered fluorescent proteins. Conventional dyes are brighter than fluorescent proteins, and can be cycled hundreds of times, whereas fluorescent proteins are only used once. Antibodies can be used for dSTORM, and therefore, thousands of targets can be labeled. Two or more imaging channels are possible with dSTORM. Because of appropriate reducing conditions inside living cells, dSTORM can be combined with PALM to perform multicolor super-resolution imaging studies in live cells. Molecules are able to be localized more than once using dSTORM in contrast to PALM, where molecules are typically imaged once. dSTORM can also be performed in conjunction with confocal/widefield imaging to give a broad overview. This can be achieved because the sample is fluorescent before labeling. Finally, dSTORM is 3D capable.

Disadvantages of dSTORM

Disadvantages of dSTORM include the following. One disadvantage is that the microscope setup for dSTORM is affected by vibration. Another disadvantage is that localization is reliant on label density. Label density is affected by actual antigen density, and in dSTORM, this affects the quality of labeling. This is in contrast to PALM, which uses fluorescence proteins genetically attached to the target, and therefore, there is 100% efficiency of labeling. A potential disadvantage of dSTORM is that it uses chemistry to induce photoswitching rather than specialized fluorescence molecules. Therefore, this could affect living cells, which is a concern. In addition,



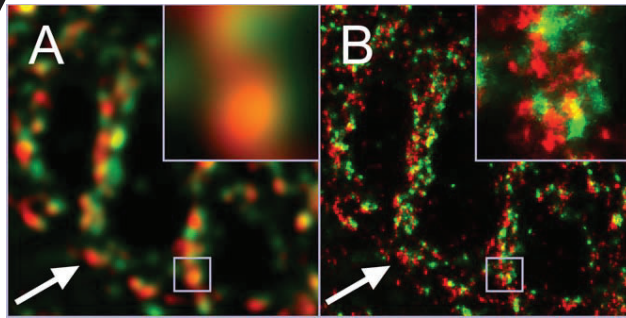


Fig. 2. Direct-STORM image of ryanodine receptor (red) and calsequestrin (green) in a transverse section of a human cardiac myocyte. (A) Diffraction limited rendering of the data is shown and is equivalent to what would be expected from confocal microscopy. (B) Super resolution image demonstrating a considerable increase in resolution over diffraction limiting imaging. The region of interest (insert) is $800 \times 800 \text{ nm}^2$. The white arrow indicates the surface of the myocyte.

for certain dyes (e.g., cyanine), oxygen is removed to control photoswitching, and this could affect live-cell imaging (Endesfelder et al., 2011). Finally, similar to other super-resolution technologies, this complex method requires a lot of attention from the researcher for sample preparation, and during imaging (Soeller and Baddeley, 2013), and the computer analysis is time-consuming.

USES OF DIRECT STORM

The many commercially available fluorescent probes, which can be applied as photoswitches, have enabled dSTORM to become successful for investigating the quantity, distribution, and density of cellular or membrane proteins in fixed cells. Particular applications include investigating the distribution and size of ryanodine receptor clusters in cardiac myocytes to improve the understanding of the biophysical properties of excitation-contraction coupling (Soeller and Baddeley, 2013; Baddeley et al., 2009b) (Fig. 2). Such intricate near-molecular scale detail is not able to be visualized using conventional fluorescence microscopy techniques. dSTORM has also been used to image microtubules and actin filaments in mammalian cells with a resolution of 21 nm (Heilemann et al., 2008).

Appropriate reducing conditions inside live cells (all cells contain reducing agents) allow live-cell imaging using dSTORM. dSTORM has been used to examine the dynamics and distribution of core histone proteins. Use of an organic fluorophore with high photon flux, as well as a rapid photoswitching ability in living cells, have enabled imaging of histone H2B protein dynamics in living human cells at an approximate resolution of 20 nm

(Wombacher et al., 2010). In addition, live-cell imaging using dSTORM of core histone H2B proteins in various eukaryotic cell lines can be performed with SNAP tags, which are commercially available (Klein et al., 2011). Imaging of DNA in living cells has been achieved with dSTORM using direct labeling with the commercially available cyanine-based Picogreen dye (Benke and Manley, 2012). dSTORM has also been used to characterize viral proteins and genomic RNA in a single virion in living cells (Alonas et al., 2014).

dSTORM can be combined with other super-resolution techniques, such as PALM. PALM and dSTORM can be combined for two-color super-resolution imaging in fixed cells (Endesfelder et al., 2011) and for three-color imaging of live cells to examine the spatiotemporal organization of membrane receptors (Wilmes et al., 2012).

Ellen Jensen

35 Southern Cross Rd.

Kohimarama, Auckland, New Zealand, 1701

David Crossman, PhD

Biophysics & Biophotonics Research Group

Department of Physiology

Faculty of Medical and Health Sciences

The University of Auckland

Private Bag 92019

Auckland 1142, New Zealand

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*Correspondence to: Ellen Jensen, 35 Southern Cross Rd. Kohimarama, Auckland, New Zealand, 1701.
E-mail: ellen_knapp2004@yahoo.com.au

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