GSD Super-Resolution Microscopy

Background. GSD (or GSDIM – ground state depletion followed by individual molecule return) microscopy is a super-resolution imaging approach based on random sequential activation and localization of individual fluorescent molecules. A super-resolution image is then reconstructed from the positions of the many activated molecules [5]. An increase in resolution is achieved by detection of single fluorescence spots temporally separated from each other. This technique is also known as direct stochastic optical reconstruction microscopy (dSTORM) [6]. GSD/dSTORM microscopy uses conventional fluorescent probes such as labeled antibodies, fluorescent proteins, or chemical tags for achieving super-resolution at ~20 nm, which is more than ten times better than in conventional light microscopy. GSD technology is based on the property of many conventional fluorescent dyes to be photoswitched between fluorescent and dark states (a phenomenon known as "blinking"). At the beginning of an experiment, the majority of fluorochromes are transferred into a stable and reversible dark (non-fluorescent) state (OFF state) by applying a high intensity laser light. The OFF state has a lifetime in the range of a hundred milliseconds to several seconds. Either spontaneously or upon photoinduction by a 405 nm backpumping laser, single molecules will return to the ON state, stochastically emitting fluorescence for a short time before switching back to the dark state. The fluorescence signals produced by these molecules cycling between dark and bright states are recorded over time. Finally, a super-resolution image is constructed from thousands of images through the use of an algorithm that calculates the exact positions of single fluorophores [13]. The detection precision of the molecule position depends on the signal intensity, i.e., the number of collected photons.

3D Imaging. The localization of single fluorophores in the z direction is achieved by introducing a cylindrical astigmatic lens into the imaging path of the microscope. The lens changes the shape of the fluorescent spot based on its position in the z dimension, thus enabling 3D reconstruction [12]. When the individual fluorophore molecule is in the focal plane, the shape of its image appears round. However, when the fluorophore is above or below the focal plane, the shape of the image spot becomes elongated in the horizontal (x) or vertical (y) direction, respectively. Thus, the axial (z) position of each molecule can be obtained from the shape (ellipticity) of the imaged spot. Using this approach, a range of 800 nm in the z (400 nm above and below the focal plane) can be imaged with a resolution up to 50 nm. To extend the z-range, one can acquire a set of 3D GSD images in 800-nm steps and stack them into a single super-resolution image that covers larger volume. Using this approach, whole-cell 3D super-resolution images were acquired with a z-range of ~3 μm [5].

Leica SR GSD 3D microscope. GSD super-resolution imaging can be performed with the Leica SR GSD 3D microscopy system available in the Molecular Cytology Core. The system has three powerful imaging lasers (488, 560 and 642 nm) and a backpumping 405 nm laser that allow acquisition of three-color super-resolution images. TIRF illumination limits the depths of excitation and improves localization precision by reducing background fluorescence. The special 160x GSD oil-immersion objective is optimized for high-power laser emission. The Suppressed Motion (SuMo) technology of the microscope stage minimizes lateral drift to less than 20 nm/10 min and ensures stability of the sample during acquisition. The system is equipped with the fast and highly sensitive Andor iXon Ultra 897 EMCCD camera. The system is controlled by the HP Z60 workstation, which stores and analyzes thousands of images in real time, computes the positions of individual molecules, and constructs 2- and 3D super-resolution images. The rendering of a super-resolution image can be observed in real time.

Samples. GSD super-resolution microscopy can be performed on cultured cells, bacteria, or tissue sections. Sections of fixed tissue have to be mounted on glass coverslips (optimal size = 18 x 18 mm; acceptable size = 22 x 22 mm), while cells need to be grown on round (Ø 18 mm) or square (optimal size = 18 x 18 mm; acceptable size = 22 x 22 mm) glass coverslips, or in 35-mm glass-bottom

imaging dishes with a well diameter of 12 mm. Bacterial cells have to be immobilized on fibronectin- or laminin-coated coverslips or on thin agar pads [3]. The cover glass and glass bottoms of imaging dishes should be 170 µm-thick (size 1.5) and preferably made of high-tolerance (± 5 µm) glass.

Sample preparation. Fixation of tissue and cells grown on glass coverslips or glass-bottom dishes should be done using freshly prepared fixative [e.g., 2-4 % paraformaldehyde (PFA) in 0.1 M PB pH 7.2]. It is convenient to use a commercial 4% aqueous solution of methanol-free PFA sealed in glass ampules under inert gas (#RT 157-4, Electron Microscopy Science). Tissue slices are usually cut from fixed tissue samples and placed in the center of glass coverslips. Cells and tissue sections are fluorescently labeled by immunostaining or other suitable techniques [1]. When using immunolabeling, it is advisable to work with somewhat higher primary antibody concentrations. However, excessively high labeling density of cell structures can also have a negative impact on resolution because multiple fluorescent molecules emitting at the same time within a diffraction-limited area produce overlapping events (blinks) that reduce the localization precision [16]. The labeling conditions (primary and secondary antibody concentrations and incubation times) and staining quality (brightness, background) should be verified and optimized using conventional microscopy prior to GSD imaging. Usually, immunofluorescence protocols established and optimized in labs will work in GSD microscopy with only minimal modifications. It is beneficial to start with single-color staining and, once it is optimized, proceed to multicolor experiments. It is especially important to add additional rinsing steps after the incubation with primary and secondary antibodies for removing unbound antibody and reducing background. The size of probes can limit the achievable localization precision of proteins. Commonly used indirect immunofluorescent labeling may introduce a 24-30-nm displacement between the label and the epitope [7,18]. To circumvent this problem, one can use recently developed nanobodies, which are recombinant, antigen-specific, single-domain, variable fragments of camelid heavy-chain antibodies (e.g., Nano-Boosters from Chromotec). Due to their smaller size, nanobodies can deliver fluorophores as close as 1-2 nm to their target proteins [17,18]. Of note, the frequently used nuclear stains DAPI and Hoechst may cause imaging artefacts and therefore should not be used in samples prepared for GSD microscopy. If a DNA marker is needed, Picogreen has been shown to be a good dye for GSD/dSTORM imaging in live and fixed cells [2].

Sample mounting. For GSD/dSTORM imaging, the samples have to be mounted with special imaging buffer (PBS or TRIS) containing a reducing agent (e.g., β-mercaptoethylamine, MEA) and oxygen scavenging system (e.g., glucose oxidase or OxyFluor). The imaging buffer must be freshly made. The Molecular Cytology Core has a list of the most common imaging buffers for GSD. Please contact the core staff if you want to obtain a copy. Leica Microsystems has excellent guides and an instructional video on sample preparation and mounting [8,10].

GSD microscopy in live cells. It is possible to study living cells with the GSD/dSTORM microscopy, but phototoxicity – a result of the high laser power applied – and the dynamics of the structure of interest during the observation period should be carefully considered since imaging takes up to several minutes and therefore requires the target to remain stationary. Living cells can tolerate high laser irradiation intensities if excitation is performed at wavelengths of 640 nm or longer [15]. The most straightforward method of labeling proteins in live cells is to use fluorescent proteins, which can be genetically fused to target proteins of interest. YFP/Venus performs especially well in GSD/dSTORM microscopy [10]. Good results were also demonstrated with eGFP, Dronpa, and rsCherry [1,7]. Target proteins can be also labeled with SNAP and Halo tags utilizing cell-permeable fluorescent substrate tetramethylrhodamine (TMR). Other fluorescent probes tested in live-cell super-resolution imaging include Picogreen (nuclear and mitochondrial DNA) [2], MitoTracker Orange, Red and Deep Red (mitochondria), ER-Tracker Red (endoplasmic reticulum), LysoTracker Red

(lysosomes), and DiI (plasma membrane) [11]. The use of organic fluorophores in the GSD imaging of live cells is facilitated by the fact that the cysteine-containing tripeptide GSH is the most abundant low-molecular weight protectant and antioxidant in mammalian cells. This tripeptide is present in animal and human cells in the reduced state at millimolar concentrations. Therefore GSD imaging of live cells does not require addition of exogenous reducing agents (e.g., MEA) to the imaging buffer [1].

One should keep in mind that unlike in fixed specimens, molecules and subcellular structures move in live cells. This motion can deteriorate the spatial resolution and distort observed structures. Two types of motions are particularly important to consider in live-cell super-resolution imaging: (I) the motion of the probe molecules within the camera frame (e.g., diffusion), and (II) the motion/change of the structure during the acquisition period [14]. Minor shifts due to the last type of motion can be corrected using fluorescent beads (fiducial markers) incorporated with the sample.

Choice of fluorescent probes. Cy5, Alexa 647, and Dyomics 654 are among the best probes for GSD/dSTORM microscopy, with each providing ~5,000 detected photons per switching cycle [4]. Fluorescent proteins are another popular type of fluorescent probe. However, one should be cautious about potential artifacts on protein localization and function induced by fluorescent protein tags, which are ~4 nm in size and tend to oligomerize. Overexpression of the fusion proteins may also cause artifacts.

Recommended dyes for single color GSD *(only dyes that match our lasers are listed)*

Laser	Dye	Imaging buffer		
		MEA in PBS	GLOX+MEA	OxEA
488	ATTO 488	+	++	
	Alexa Fluor 488	+	++	++
	Chromeo 488	+		
	Oregon Green 488	+		
	Picogreen	+		
	eGFP			
	Chromeo 505	+		
	YFP / Venus		++	
560	Alexa Fluor 555	+	+	++
	Alexa Fluor 568	+	+	
	ATTO 565	-	+	
642	Alexa Fluor 647	+	++	++
	ATTO 647N	+	+	
	Cy5	+	++	
	ATTO 655	+	++	
	Dyomics 654	+	++	
	Alexa Fluor 680	+	++	

"-"rapid bleaching; "+" moderated photoswitching; "++" robust photoswitching.

Multicolor GSD imaging is possible using a combination of 2 or 3 dyes. Good results were achieved by embedding multicolor samples with the OxEA imaging buffer [9].

Suggested dual-color dye combinations for GSD

Atto 488 or AlexaFluor 488 and AlexaFluor 647

AlexaFluor 555 and AlexaFluor 647

AlexaFluor 568 and AlexaFluor 647
Atto 488 or AlexaFluor 488 and AlexaFluor 568

Suggested triple-color dye combination for GSD
Alexa Fluor 488, Alexa Fluor 555 or 568 and Alexa Fluor 647

It is recommended that you contact MCC staff to discuss specific combinations of dyes and imaging buffers for your multicolor GSD super-resolution experiments.

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