Current optical sectioning systems in fluorescence microscopy

Pavel Křížek¹ and Guy M. Hagen*¹

¹Institute of Cellular Biology and Pathology, First Faculty of Medicine, Charles University in Prague. Albertov 4, 12800 Prague, Czech Republic

Optical sectioning in fluorescence microscopy can be achieved by a variety of approaches. Here we give an overview of the state-of-the-art optical sectioning systems currently available in fluorescence microscopy. Our review covers pinhole-based systems, structured illumination microscopy, and light sheet microscopy.

Keywords optical sectioning; confocal microscopy; structured illumination microscopy; spatial light modulators; super-resolution microscopy; computational microscopy; light sheet microscopy

1. Introduction

Thanks to the commercial availability of high performance confocal laser scanning microscopes (CLSMs), optical sectioning microscopy has become the central method for observing fluorescently-labeled samples with diffraction limited resolution. Indeed, along with the humble western blot, CLSM has become the central method in much of modern cell biology.

One of the main driving factors in the development of optically sectioning microscopes has been the limited imaging rates available in CLSM. The root cause of this limitation is photophysical [1]. The "dwell time", i.e., the time the laser spends at each pixel location, and thus, the time the photomultiplier tube (PMT) detectors have to integrate the signal must be approximately 5-10 μs to generate enough fluorescent photons to result in sufficient signal to noise ratios (SNR) in the final image. To generate an image of 512 x 512 pixels with a dwell time of 10 μs per pixel implies an imaging rate of about 2.6 seconds per image. What is really needed to speed up imaging rates is a higher sensitivity and lower noise detector, or a parallelization of the scanning process.

2. Pinhole systems

2.1 Confocal laser scanning microscopy

Confocal laser scanning microscopy (CLSM), even when not strictly required, is often chosen over widefield fluorescence microscopy due to the ease of use and high image quality attainable with modern instruments. For recent reviews see references [2-5]. The newest generation of CLSMs combine several lasers for excitation, a variety of scanning modes, and multiple detectors operating in parallel with advanced software for image acquisition, visualization, and analysis. The CLSM platform can be extended to accommodate other imaging modes, such as spectrally resolved imaging [6], fluorescence lifetime imaging (FLIM) [7], and fluorescence correlation spectroscopy (FCS) [8].

Beginning as early as 1993 with a commercial offering from Nikon [9], the imaging rates of CLSMs improved dramatically using resonance scanners [10, 11], an approach available today in the SP5 (now SP8) microscope from Leica [12], and the A1R+ from Nikon [13]. However, rapid scanning achieved by reduced pixel dwell times for a given image size results in noisier images. The solution for this is usually line-based signal averaging ¹. However, when combined with the newest high sensitivity detectors [1], resonance scanners have become a much more viable option.

Slit scanning confocal microscopes have also been well developed. Here, rather than a single scanning spot (CLSM), or multiple scanning spots (spinning disk microscopy, see Section 2.2), the sample is scanned with a single line of illumination. The fluorescence is then descanned by a slit rather than by a pinhole [14]. This technology is currently available from Zeiss as the LSM 7 Live [15, 16]. Even though a line is scanned, and the fluorescence is “descanned” by a slit, the resulting optically sectioned image does not exhibit confocality in only one direction as might be expected [15]. Further, the system has much better optical sectioning ability than spinning disk systems based on the Yokagawa disk units [15]. The Zeiss line scanning system has been used for imaging rapid biological processes such as embryogenesis [17], and for imaging of extremely fast cellular signaling events such as calcium sparks [18].

¹ The laser scans each line of the image several times and averages the result.
2.2 Spinning disk microscopy

In addition to CLSMs, several other approaches to achieve optical sectioning are available, including the broad category of tandem scanning microscopes, introduced in 1968 by Petráš and coworkers [19]. In these systems, multiple points of light created by multiple pinholes scan the sample. To achieve optical sectioning, the fluorescent light is focused back through the pinhole array (descanning). This is usually accomplished with rotating masks that contain the “pinhole pattern.” The original design has been considerably refined and is now considered a standard method which is embodied as spinning disk microscopy [20, 21]. When combined with electron multiplying charge-coupled device (EMCCD) cameras, these systems are able to achieve extremely fast multidimensional imaging [22].

Spinning disk microscopy is not limited to scanning with point arrays. A multi-slit scanning system is available from Olympus (the disk scanning unit, or DSU), which includes multiple disks to be used with different objectives [23]. Also, a new system based on aperture correlation microscopy [24-26] has recently been introduced and is offered by Zeiss as the VivaTome [16] and by Andor as the differential spinning disk (DSD) [27]. The DSU, VivaTome, and DSD are unique in that they use white light sources rather than lasers, and thus, offer a more economical alternative.

In another variation of tandem scanning known as swept field microscopy, a multifocal set of points is created by a microlens array and scanned across the sample using a galvanometer mirror. Fluorescence is directed through a pinhole array by the reverse side of the galvanometer mirror, and then proceeds to a camera which integrates the scanned image [28, 29]. Microscopes of this type are available from Visitech (VT Infinity) [30] and from Prairie Technologies (Swept Field Confocal) [31].

2.3 Programmable array microscopy

Programmable array microscopes (PAMs) use spatial light modulators (SLMs), e.g., liquid crystal on silicon (LCOS) microdisplays or digital micromirror devices (DMDs). A single SLM is used for creating the pinhole illumination pattern and also for descanning the image with the same pinhole pattern present on the display (there is one exception [32] which uses two displays). This introduces much more flexibility into the system because it allows the creation of arbitrary patterns of light for optical sectioning and allows easy exploration of illumination patterns, as well as applications such as fluorescence recovery after photobleaching (FRAP) [33-36]. An optically sectioned image is obtained by integrating the fluorescence signal in the camera during one exposure for one complete pattern scan, i.e., one complete set of illumination patterns which, when summed, result in homogeneous illumination.

The PAMs reported so far have not achieved lateral resolution beyond the diffraction limit, although optical sectioning performance can be better than in CLSM [33, 35]. Another limitation is in the range of possible SLM-to-sample-to-LSM magnifications which are possible when using a single SLM.

We reviewed PAMs more fully in the previous edition of this book series [37].

3. Structured illumination microscopy

Another broad category of methods for obtaining optical sectioning is structured illumination microscopy (SIM) [38]. Structured illumination microscopes all share a common feature – patterned illumination of the sample and widefield detection of the fluorescence signal using a camera with no mask in the detection path, i.e., non-descanned detection.

The illumination patterns are usually dots or stripes of light which may be formed by physical gratings or masks, or by coherent illumination effects such as laser interference or speckle, or by SLMs of different types. The optically sectioned image must then be obtained computationally [38-40]. Note that the final sectioned image may require a large number of images, depending on the spacing of the pinholes or slits in the illumination mask.

3.1 Grid confocal

The most familiar implementation of the method was introduced by Neil and coworkers [41]. Here, the illumination pattern consists of an array of stripes or fringes. Three images are acquired, with the position of the illumination pattern shifted by 1/3 of the pattern period in between each image. An optically sectioned image can then be obtained as

\[ I_2 = \left[ (I_1 - I_2)^2 + (I_2 - I_3)^2 + (I_3 - I_1)^2 \right]^{1/2}, \]

where \( I_2 \) is an optically sectioned image, and \( I_1, I_2, \) and \( I_3 \) are the three images acquired with different pattern positions. A widefield image can be obtained by averaging the three raw images. This method is sometimes called grid projection, fringe projection, or simply "grid confocal." Microscope add-on modules following this general design are available from Zeiss [16] (Apotome.2), and from Qioptic [42] (Optigrid). The Zeiss Apotome.2 now includes a set of three different grids, each with a different spatial frequency. Each grid is designed to be used with objectives of different magnification and NA. This allows the microscope to be used more effectively with a wide variety of samples, especially larger, thicker samples such as embryos of model organisms (Drosophila, C. elegans).
The general strategy described here has been extended in many ways. As with CLSM, increasing imaging speed has been one of the primary goals. In the original design, a physical grating or ruling is moved up and down by a piezo element. The position of the grid must be stable before image acquisition to avoid stripe artifacts in the reconstructed image. In the Zeiss Apotome, a glass plate is rotated about its pitch axis such that the image of the grating formed moves up and down in the image plane. Attempts to increase imaging speeds have been implemented using laser interference, with the phase of the pattern modulated by a piezo element [43]. More recently, an approach based on acquiring three simultaneous polarized images (using a three-way image splitter) has been demonstrated such that optically sectioned images can be reconstructed from a single camera exposure [44].

Other extensions of this approach have included, e.g., time-gated fluorescence lifetime imaging (FLIM) [45], endomicroscopy [46], and novel light sources such as LED arrays with individually addressable emitter stripes [47]. DMD spatial light modulators have also been used to help speed up image acquisition [37, 48]. Even the use of a standard, unmodified multimedia projector as both the source of light and of the illumination patterns has been demonstrated [49].

### 3.2 Virtual scanning microscopy

The earliest implementation of virtual scanning microscopy (VSM) was probably “electronic multiconfocal-points microscopy,” by Benedetti, et al. in 1995 [50]. This method used a physical mask consisting of a 2D pinhole array which was translated across the sample in the x and y directions in discrete steps. An image was acquired at each position of the pinhole array with a digital camera and the optically sectioned image was computed by simply taking a maximum intensity projection (MAX), or by taking MAX and subtracting a minimum intensity projection (MIN), i.e., MAX – MIN. Here it is assumed that the MAX term contains mainly contributions from parts of the sample that are in focus and the MIN term mainly contributions from out of focus regions.

Another possible processing method, scaled subtraction, works by composing in-focus and out-of-focus image segments using a virtual (i.e., digital) mask [38, 50, 51]. To use scaled subtraction, one needs to know, with great accuracy, the position of the illumination pattern in the camera image. By using a virtual mask corresponding to pre-established pinhole positions, an optically sectioned image can be computed as

$$I_c = \sum_{n=1}^{N} I_n \cdot \text{Mask}_n^{on} - \frac{1}{N} \sum_{n=1}^{N} I_n \cdot \text{Mask}_n^{off}$$

where $I_n$ are intensity values of the camera image captured at a given frame $n$ in the sequence of $N$ illumination patterns, $\text{Mask}_n^{on} \in [0, 1]$ defines the intensity of the virtual illumination pattern in the camera for a given frame $n$, and $\text{Mask}_n^{off} = 1 - \text{Mask}_n^{on}$.

The MAX – MIN processing method does not require any knowledge of the position of the illumination points in the camera image, simplifying the analysis considerably. However, compared to the scaled subtraction method, the reconstructed optically sectioned images are noisier and prone to artifacts resembling the illumination pattern [39].

The pattern positions for the scaled subtraction method might be determined from the raw data, but this is both inaccurate and difficult, particularly in sparsely labeled samples. It is possible to use Fourier analysis to determine the positions of a point lattice or stripe array based on the points or stripes that are visible in the raw data [52]. It is also possible to use image calibration methods [51].

The illumination patterns used in VSM need not be limited to points of light. A line scanning microscope using a virtual slit mask has also been developed, and was called virtual slit scanning [53]. This microscope used a laser beam focused into a line of illumination. The line scanned the sample, and the fluorescence was recorded with a CCD line detector. The VSM concept was also discussed for processing the data acquired using an LED-stripe array for slit scanning [47].

In other reports, the illumination pattern was produced by DMD [54] or LCOS [51] spatial light modulators (SLMs) rather than with a physical mask. In such a way, more flexibility is introduced into the system because it allows easy exploration of different patterns. This is extremely similar to programmable array microscopy (PAM) [33-37]. On the other hand, PAMs are much faster than SIM or VSM, which both require multiple images to be acquired to calculate an optically sectioned image.

### 3.3 Super-resolution with structured illumination

Originally conceived as an alternative to CLSM and intended to achieve optical sectioning, resolution enhancement is quickly becoming the main application of structured illumination microscopy [55-57]. Pushing the resolution of optical microscopes beyond the diffraction limit has become a field unto itself [58].

Super-resolution SIM (SR-SIM) uses the Moiré effect to sample high resolution information into the frequency passband of the microscope. In linear SR-SIM, the resolution achieved is at best a factor of two better than the...
diffraction limit \[55, 59\]. The limit of doubled resolution is lifted in nonlinear SR-SIM, where the resolution is theoretically unlimited \[60\].

SR-SIM has several advantages over other super-resolution techniques\(^2\). In SR-SIM, any fluorescent dye or any fluorescent protein can be used to label the sample. SR-SIM can also be used for 3D imaging (3D SR-SIM) \[56, 65\]. 3D SR-SIM is now commercially available from Zeiss (ELYRA S.1) \[16\], Nikon (N-SIM) \[13\], and API (DeltaVision OMX) \[66\], and is quickly becoming a standard technique. One very recent and exceptional study of fluorescence in-situ hybridization (FISH) approaches for studying the cell nucleus has made the broad utility and rapid adoption of 3D SR-SIM evident \[67\].

SR-SIM has also been used for live cell imaging, taking advantage of spatial light modulators for high-speed definition of the pattern \[68-71\]. The report by Fiolka and co-workers represents a new level of performance in 3D SR-SIM of live cells \[68\]. This, along with the Bessel-beam light sheet microscope (see Section 4) reported by Planchon and co-workers \[72\], represents the “2012 state-of-the-art” in live-cell super-resolution microscopy.

Nonlinear SR-SIM was first demonstrated with fluorescent beads, using fluorophore saturation as the nonlinear optical effect \[73\]. It was subsequently demonstrated that an alternative mechanism, the saturation of photoswitching in fluorescent proteins, could also be used to achieve nonlinear SR-SIM \[74\]. This has proved to be an important step, and much more convincing nonlinear SR-SIM images have recently been published based on nonlinear transitions in the fluorescent protein Dronpa \[75\].

Finally, it has been shown very recently (2012) that lateral resolution enhancement beyond the Abbe limit is possible also in VSM \[76\]. Calling the technique “multifocal SIM” (MSIM), the authors used a DMD for pattern definition coupled with widefield detection using an SCMOS camera. These cameras have extremely low readout noise (about 1 electron) and very fast readout (up to 100 images per second with a resolution of 2048 x 2048 or even 2560 x 2160) \[77\]. To achieve the resolution enhancement, the authors used image scanning microscopy (ISM) as described by Müller and Enderlein \[78\]. In ISM, the PMT detector in a standard confocal microscope is replaced by a digital camera. The fluorescence signal is descanned by a pinhole, and an image is acquired at each pixel position of the scanning laser beam. This limits the technique to small fields of view because the number of images required quickly becomes very large as the imaging area increases.

The resolution enhancement in image scanning microscopy comes from resampling the raw images (in the spatial domain) by a factor of two, resulting in resolution enhancement by a factor of \(\sqrt{2}\). This is the same effect that can be achieved in a CLSM operated with the pinhole all the way closed. Naturally, if the pinhole is all the way closed, no light can reach the detector. However in ISM, the effect can be reproduced digitally, becoming a viable method.

In the latest implementation \[76\], the resolution is further enhanced using a conventional deconvolution algorithm, ultimately to a factor of approximately two. The authors of this study imaged cells, as well as embryos of worms, flies, and fish. One of the main results of the paper was that ISM can image thick samples with enhanced resolution more effectively than conventional SR-SIM \[56\]. This is due to the sparse illumination pattern used. In conventional SR-SIM, a fringe pattern with high spatial frequency is used. The pattern contrast is quickly degraded when attempting to focus deeply into scattering tissues. This problem can be somewhat alleviated using a sparse pattern (see also \[79\]).

### 4. Light sheet microscopy

Another category of optical sectioning microscopes are systems which employ thin sheets of light for illumination of a sample with detection at an angle of 90 degrees to the light sheet \[80-82\]. This gives an optical sectioning effect without the use of pinholes (as in CSLM or spinning disk microscopes) or the use of computational methods in combination with structured illumination. This method can achieve extremely high sensitivity since all the light emitted by the sample is detected. Because only a single plane of the sample is illuminated, photobleaching and phototoxicity are vastly reduced. This has allowed imaging of embryogenesis in fish \[83-86\], \textit{C. elegans} \[87\], and \textit{Drosophila} \[88\] with unprecedented levels of spatio-temporal detail. The thickness of the light sheets used in these studies has been limited to about 3 μm or more, limiting optical sectioning thickness to this value. This also limited light sheet microscopy to imaging of larger samples, such as the above mentioned embryos. Recently, achievable axial resolution in light sheet microscopy has been reduced to about 0.3 μm using Bessel beam illumination \[89, 90\]. This, combined with a novel illumination and detection setup and the use of structured illumination, has allowed amazingly detailed imaging of live cells \[72\].

In some light sheet microscopy setups, a chamber with ports is used, allowing long working distance water immersion objectives to be introduced. The sample (e.g., a fish embryo) is then embedded in a small agarose plug which is suspended between the illumination and detection objectives \[84\]. The sample can then be rotated to offer multiple viewing angles, improving 3D reconstruction of thick samples \[91\]. Other light sheet imaging systems have

\(^2\) Photoactivated localization microscopy (PALM) \[61\] and stochastic optical reconstruction microscopy (STORM) \[62\] can achieve resolution in the 20 nm range, however they rely on photoswitchable fluorophores. Similarly, stimulated emission depletion microscopy (STED) \[63\] can easily achieve resolution below 100 nm in the devices which are commercially available, but not every dye or GFP variant can be used \[64\].
been integrated with standard inverted microscopes [87, 92, 93]. When used with a special imaging chamber, this allows the use of a high NA water immersion detection objective for single molecule tracking [93]. Light sheet microscopy has also been combined with fluorescence correlation spectroscopy (FCS), enabling the creation of "diffusion maps" which show the mobility of structures and molecules in the image [92].

5. Conclusions

Even though optical sectioning is well established in the form of confocal laser scanning microscopy, it continues to evolve. New technologies, such as more sensitive, lower noise detectors, allow the achievement of faster imaging rates with higher signal to noise ratios. Additionally, scanning the sample with an array of pinholes instead of using just one pinhole allows acquisition of images much more rapidly and is therefore more suited for live cell imaging.

The latest developments in the field have been focused on both structured illumination microscopy and light sheet microscopy. Today, structured illumination microscopy is used mainly for super-resolution imaging, where resolution beyond the diffraction limit of widefield microscopes can be achieved. We believe the most amazing results obtained so far in live-cell imaging with very high spatio-temporal resolution were those acquired by light sheet microscopy in combination with structured illumination [72]. As optically sectioning microscopes continue to advance, we can look forward to future breakthroughs in our understanding of biology at the sub-cellular level.

Acknowledgements  This work was supported by projects 304/09/1047, P205/12/P392, and P302/12/G157 from the Grant Agency of the Czech Republic, by project OP VK CZ.1.07/2.3.00/30.0030, and by the projects UNCE 204022 and Prvouk/1LF/1 from the Charles University in Prague.

References

Heintzmann R and Cremer C. Laterally modulated excitation microscopy: improvement of resolution by using a diffraction limited excitation microscopy.

Stemmer A, Beck M, and Fiolka R. Widefield fluorescence microscopy with extended resolution.

Schermelleh L, et al. Subdiffraction multicolor imaging of the nuclear periphery with 3D structured illumination microscopy.


Neil MAA, Juškaitis R, and Wilson T. Real time 3D fluorescence microscopy by two beam interference illumination.

Delić S and Blanca CM. Wide-field depth-sectioning fluorescence microscopy using projector-generated patterned illumination.


Křížek P, Raška I, and Hagen GM. Flexible structured illumination microscope with a programmable illumination array.


Neil MAA, Juškaitis R, and Wilson T. Real time 3D fluorescence microscopy by two beam interference illumination.

Gustafsson MGL, et al. Three-dimensional super-resolution imaging by stochastic optical reconstruction microscopy.


Gustafsson MGL, et al. Three-dimensional resolution doubling in widefield fluorescence microscopy by structured illumination.


