Published in final edited form as:

Biochem Soc Trans. 2009 October ; 37(Pt 5): 1042–1044. doi:10.1042/BST0371042.

The “Super-resolution” revolution

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Abstract

We are currently in the midst of an exciting revolution in microscopy. In many ways, this has been happening for several decades, but it is the rate of development of new methods that has increased recently. The past two years have seen an impressive proliferation of new instruments for imaging at higher resolution, imaging single molecules and faster and more sensitive multidimensional live cell imaging. These include Light sheet microscopy, Stimulated Emission Depletion, Structured Illumination and live cell imaging on the OMX platform. However, new probes and image analysis methods have also been crucial for the development of these revolutionary methods.

Introduction

The 2008 Nobel prize in Chemistry was awarded to Tsien, Chalfie and Shimomura for their discovery and development of the green fluorescent protein (GFP). GFP and numerous other fluorescent proteins have been applied very widely, leading to an indisputable impact on biological and biomedical imaging and research. However, they represent the flagship of a fleet of technological developments in the last few decades that involve using fluorescence to visualize molecules inside living cells. These include new ways to prepare and mount specimens, a breathtaking variety of new probes and labels in addition to fluorescent proteins, a variety of exciting new instruments and new methods of post-acquisition image analysis. Perhaps the most headline grabbing of the most recent developments has been the emergence of the so called “super-resolution” instruments and analysis methods, which overcome the long standing resolution barrier due to the diffraction limits of light. While these “super-resolution” methods are quite clearly a step change in our ability to visualize molecules within cells, in most cases, the increase in “resolution” is more strictly and correctly an improvement in the precision of determination of the position of molecules. Ever since the first powerful compound microscopes were built, the resolution of light microscopy was essentially limited by the diffraction of light, through well understood phenomena in optics, to about half the wavelength of light used to illuminate the sample. For example if green light is used, the resolution limit of the highest quality objective is approximately 250nm in the plane of focus (X,Y) and 500nm in the perpendicular direction, or optical axis (Z). Remarkably, in the last few years this “fundamental resolution limit” of light microscopy has been overcome in a number of independent ways. These collectively have been referred to as “super-resolution” methods, although they depend on a variety of principles that have been around in the physical sciences for many years and in general depend on improvements in precision of imaging rather than resolution per se. This increased precision is gained through a combination of illumination regimes coupled to the subsequent processing of the imaging. The term “super-resolution” has caught on and is a useful shorthand for some very clever tricks that are not always easy to explain in a simple intuitive manner. The basis for each of these techniques is rather different and it is very difficult to predict which if any will emerge as the predominant method of choice. In this

report, I describe several of these new instruments and discuss their likely contribution to our understanding of molecular mechanisms in biology. Since most of these methods are complementary in nature, it seems most likely that each will find its specific niche and specialized set of applications. None are likely to be a panacea that will cover all applications and outcompete the rival methods.

STED

In widefield microscopes the entire specimen is illuminated evenly and an image created by an objective lens is captured onto a CCD camera. Even the most perfect and sophisticated objective lenses do not produce a perfect image of the specimen onto the camera. In fact, an inherent part of the way lenses work is that they produce out of focus light that blurs the image by a process equivalent to the mathematical function of convolution. In the early 1980s computational methods known as deconvolution were borrowed from other branches of science and applied to biological imaging in order to reassign the out of focus light to its point of origin [1]. A little later, an alternative physical method of removing the out of focus light emerged with the development of the laser scanning confocal, whose history of development is reviewed elsewhere [2]. Confocal microscopes illuminate the specimen with a spot and scan the spot across the field of illumination to produce an image. It includes pin holes in the light path to form a sharp spot on the specimen and remove out of focus emitted fluorescent light. The resolution of the confocal is limited, amongst other things, by the size of the confocal spot, which in turn is limited by the diffraction limits of light. Making the pinhole smaller and smaller cannot overcome this limitation. However, an important super-resolution method has been developed by Prof. Stephan Hell at Max Plank, Göttingen. It depends on a trick that inhibits the fluorescent emission by molecules that reside in the outer region of a laser spot that excites the molecules. This is achieved using a ring-shaped second laser that causes a phenomenon known as Stimulated Emission Depletion (STED) [3]. A STED instrument can now be purchased commercially from Leica, but it has the drawbacks of not being applicable to all fluorescent dyes, requiring specialized lasers and losing a lot of light in the process of STED. The “resolution” improvement of STED is truly spectacular in XY but can also be hugely improved in Z using two opposing objectives, known as 4Pi technique [4]. However, the 4Pi system is quite difficult to implement, because of the light path has to be equivalent in both directions, requiring relatively thin specimens mounted between two cover slips and extremely precise alignment of a complex light path. An equivalent widefield method using two objectives, known as I⁵M has also been developed, by Profs. Mats Gustafsson, John Sedat and David Agard at UCSF [5]. However, I⁵M, suffers from similar limitations to the 4Pi microscope and has not been commercialized, remaining a prototype instrument on a “physicist set up”.

Structured Illumination

A widefield method for achieving super-resolution was developed by Profs. Mats Gustafsson, Dave Agard and John Sedat. Instead of scanning a small laser spot across the specimen, structured illumination (SI) involves shining a fine striped pattern of light on the specimen. Images are acquired at multiple angles and phases of the stripes in order to build up a composite image that has twice the resolution in XY and Z, after processing. SI works because small features in the specimen, that are below the diffraction limit of a conventional microscope, create interference patterns that are larger than the diffraction limit and can be used to calculate the appearance of the original feature by processing the data after acquisition [6,7]. SI will be available later this year as a commercial product as part of the OMX microscope, developed by John Sedat at UCSF. Although more modest than STED in its increased resolution, SI can be used with most fluorescent dyes and conventional light sources, although it is quite a slow method and does require a bright specimen that is relatively resistant to bleaching. Future developments offer the promise of fast live SI and a

non-linear variant of SI, with even better resolution. Non-linear SI involves the use of saturated illumination and has been shown in a proof of concept paper to be capable of a four fold and greater resolution improvement [8]. However, this non-linear SI has not yet been applied to biological specimens and will probably require specialized fluorochromes or illumination regimes. Non-linear SI will probably only be initially used with specimens containing very bright and abundant signals that are not easily bleached. Interestingly, the OMX microscope is also capable of conventional fast multidimensional widefield imaging and has excellent sensitivity in four simultaneous wavelengths of light in its non-super-resolution mode of operation.

FIONA, PALM, STORM

These methods have been developed by a number of investigators in parallel and given various exotic acronyms, but all depend on the same simple principle. Using the distribution of photons imaged in a group of pixels surrounding the centre of a discrete focus of fluorescence, the centre of intensity can be calculated by centroid analysis to a very high degree of precision, limited only by the availability of photons. Fluorescence Imaging with One-Nanometer Accuracy (FIONA) is the simplest of these methods, involving a group of molecules that are colocalized to a single point [9]. The position of the population of molecules can be calculated to approximately 5nm precision, about the size of a GFP molecule. The same principle has been applied to imaging single molecules. The best known of these single molecule methods is Photo-activation Light Microscopy (PALM), developed by two physicists Profs. Eric Betzig and Harald Hess in collaboration with a Cell Biologist, Prof. Jennifer Lippincott-Schwartz [10]. Using a photo-activatable GFP, PALM relies on activating only a small number of non-overlapping molecules in a dense field and then capturing their fluorescence and calculating their position with a precision of approximately 10nm. Through repeated rounds of further activation and capturing, an image of all the molecules in the field can be assembled with such pin point precision. PALM uses relatively simple hardware, so will have a wide applicability. A more recent variation of this method, Interferometric PALM (iPALM) was developed by Prof. Harald Hess, in which interferometric methods are used to image individual molecules at 5nm precision in the Z axis [11]. Like 4Pi, iPALM involves the use of photoactivatable mCherry and two apposing objectives, so is technically very difficult to build and use. It is currently not clear whether iPALM will become a turnkey commercial instrument.

Light sheet microscopy

A serious disadvantage of both widefield and confocal microscopy is that the specimen is illuminated with a cone of light, so that parts of the specimen that are not in the focal plane are also illuminated. This has several undesirable consequences, the most important of which is the bleaching of signal in regions of the specimen that are not being imaged. Multiphoton imaging is one way to overcome this problem, as it involves exciting fluorochromes with two or more photons simultaneously, which only occurs at the plane of focus where the illumination is sufficiently intense. However, multiphotons require a large amount of energy from pulsing lasers, which in themselves are quite damaging to the specimen. An alternative approach developed by Prof. Ernst Stelzer at EMBL, is to illuminate the specimen from the side by a sheet of light, so that only the plane of focus is actually illuminated. This method is known as Selective Plane Illumination Microscopy (SPIM) and more recently, a scanning version known as Digital scanned Light Sheet Microscopy (DLSM) has been developed [12]. Both of these designs involve mounting the specimen in a rotatable holder that allows the capturing of the entire 3D volume by carrying out tomography. Therefore SPIM and DLSM are very well suited for live cell imaging of large structures such as Zebra fish embryos [13]. In addition the use of light sheets provides substantial improvement in resolution and depth of penetration into thick specimens. The

advantages of light sheet illumination are likely to lead to an expansion of the range of uses of this technique by combining it with other microscopy methods.

Concluding remarks

While it is very easy in hindsight to appreciate why the seminal work on GFP deserves a Nobel prize, it is much harder to predict which if any of the current emerging “super-resolution” methodologies will lead to the most important breakthroughs in the future. Nevertheless, it is clear that the pace of development of new microscopy methods and instruments has been truly remarkable in the past few years and our ability to visualize molecules in cells is currently being revolutionized.

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