Advances in super-resolution imaging: applications in biology and medicine

M. Baztán1, P. Fernández-Robredo1,2,3, S. Recalde1,2,3, A. García-Layana1,2,3 and M. Hernández1,2,3*

1Experimental Ophthalmology Laboratory, School of Medicine, University of Navarra, Irunlarrea Street 1, Los Castaños Building, 31008 Pamplona, Spain.
2Department of Ophthalmology. Clínica Universidad de Navarra, University of Navarra, Avda Pio XII, 36, 31008 Pamplona, Spain.
3IdiSNA, Navarra Institute for Health Research, Irunlarrea Street 3, 31008 Pamplona, Spain.
*Address correspondence to: Dr. María Hernández. Department of Ophthalmology. Clínica Universidad de Navarra, University of Navarra, Spain. e-mail: mahersan@unav.es

Keywords: medicine; biology; super-resolution; 3D.

1. Definition

Science has made impressive advances over the last century; this would not have been possible without the simultaneous development of the necessary technology. More specifically, optical microscopy enables the observation of cell morphology, but scientific advances have made necessary the investigation of much smaller structures on the nanoscale, such as protein complexes or organelles [1]. This is not possible with conventional microscopes, mainly owing to the diffraction of light. The order of diffraction increases when intricate details of the object become finer. Electromagnetic wave diffraction is a physical characteristic of light that was first described by Ernst Abbe in 1873. It is an obstacle that critically affects the resolution of images, and constitutes a barrier that was, until recently, unsurmountable.

The resolution of a microscope is determined by the width of the Point Spread Function (PSF). Two fluorophores separated by a distance less than the PSF cannot be distinguished as separate images. The maximum resolution of an optical system is the minimum distance between two sources at which they can be distinguished as separate, therefore offering more details [2].

Moreover, the resolution of fluorescence microscopes is usually limited to ~250 nm, which makes the observation of very small structures difficult. Nonetheless, among other advantages, fluorescence microscopy allows micro-molecules to be visualised and located within the cell. In contrast, electron microscopy (EM), which is not affected by the diffraction of light, provides high resolution, although it is not the most commonly used technique [3]. To overcome these problems, super-resolution microscopes are not limited by the diffraction of light and allow images to be taken with a higher resolution than the diffraction limit. They provide much clearer pictures, which facilitates a better understanding of the biological processes that are the focus of biomedical studies. It is important to note that certain techniques that are not yet included as routine methods are under continuous development [4].

2. History: how has technology evolved?

In 1873, Ernst Abbe stated that the diffraction of light limits the resolution of light microscopes, which under optimal conditions can achieve a maximum resolution of ~200 nm [5]. Attempts were made to improve the resolution of microscopes, and the confocal microscope was developed in 1957 [6]. The problem caused by the diffraction limit of light was not resolved until a century after Abbe’s observation. Prior to this, EM (Hertz, 1857–94) was selected as the alternative option for viewing nanoscale structures. The concept of using electrons instead of light to observe tiny structures and organelles presented challenges. Although the technique allows very high resolution, the processing of the samples entails severe damage [3].

The first technique that solved the problem of light diffraction was stimulated emission depletion (STED) microscopy developed by Stefan Hell in 1994 [7]. This was followed by reversible saturable optical linear fluorescence transitions (RESOLFT), structured illumination microscopy (SIM) [8], stochastic optical reconstruction microscopy (STORM) [9], photo-activated localization microscopy (PALM) [10], and fluorescence photo-activated localization microscopy (FPALM) [11]. Some of these strategies have made possible the live-cell super-resolution imaging of dynamic structures [4].

The development of STED microscopy earned Stefan W. Hell the Nobel Prize in Chemistry in 2014, awarded for the development of super-resolution techniques. Independently of Hell, Eric Betzig and William E. Moerner laid the foundations for the super-resolved microscopy single fluorophore. Figure 1 summarises the principal events in the evolution of microscopy, including the birth of super-resolution. It is worth emphasizing that our main area of expertise is the investigative ophthalmology and the application of super-resolution techniques.
3. Super-resolution techniques

The current super-resolution techniques can be divided into two groups. Techniques that obtain the imaging an ensemble of molecules by illumination patterns, among which are the STED and SIM microscopies, and on the other hand, super-resolution techniques that image single molecules, such as STORM and PALM.

3.1 Stimulated emission depletion (STED)

The first technique to overcome the challenge presented by the diffraction of light was STED microscopy, developed by Stephan Hell in 1994 [7]. To achieve this landmark, a new pattern of illumination was used to visualize nanoscale structures. This illumination pattern reduces the region of fluorescence detection to a smaller area than the diffraction-limited spot.

STED microscopy uses two lasers with very different functions. Typically, the fluorophores in the sample are excited by a conventional laser (the excitation laser). A second depletion laser (the STED laser) with a donut-shaped intensity profile is also used. The STED laser deactivates the fluorescence in the illuminated field and the central area remains unaffected. As the intensity of the STED beam increases, the area of spontaneous emission is reduced, thereby extending the stimulated field. As a result, the PSF is reduced, consequently improving the quality of the ensuing image. The use of two lasers significantly reduces the area taken up by the excited fluorophores.

3.2 Structured illumination microscopy (SIM)

Similar to STED, SIM microscopy is a super-resolution technique based on the patterned illumination of the sample. A grid pattern is used to illuminate the sample. The grid interferes with the object behind it, generating more information than by the use of lenses alone. By superposing the two patterns, a characteristic distribution of fluorescent emission is produced, known as “moire fringes”, that can be resolved even if both patterns are too fine. The grid of illumination used in SIM is defined by a foreknown spatial frequency and orientation. Therefore, by using computer-generated algorithms, the information contained within the moiré fringes can be recovered, enabling the reconstruction of the image with high precision, regardless of the blurring effect and the data lost during image processing [12]. The application of this super-resolution microscopy technique can improve the resolution of the image by up to 100 nm on the XY axis, and by 250 nm on the Z axis, similar to STED technology [13]. A finer illumination grid creates a higher resolution.

3.3 Stochastic optical reconstruction microscopy (STORM)

STORM was developed by Michael J Rust in 2006 [9]. It is a single-molecule localization-based super-resolution imaging technique, and has achieved a resolution of 20 nm. The image is constructed from the highly accurate localization of individual fluorescent molecules that are switched on and off using light of different wavelengths.

The principle on which STORM it is based is that a fluorescence-emitting molecule can be localized with an accuracy of 1 nm if it meets two conditions: it emits enough photons to achieve the visualization of that molecule, and no other molecules are within ~200 nm [14].
This technique uses photoswitchable probes (capable of being switched by an optical signal), which can be both dyes and fluorescent proteins [2], and are turned on or off by different wavelengths of light. The light that activates them is low in intensity so most of the fluorophores to remain dark, but a few turn on stochastically, the image is obtained, and the other light turns them off. A super-resolution image is obtained by repeating this cycle several times. One of the main advantages of fluorescence microscopy is its capacity for multicolour imaging, which is essential for the study of the interactions between different biological structures or molecules.

3.4 Photoactivated localization microscopy (PALM)

PALM and FPALM were developed in 2006 by Eric Betzig and are based on the same principle as that used in the STORM technique [10]. The fluorophores are stochastically activated, located, and the super-resolution image is obtained by repeating this cycle several times. Fluorescent photoactivatable proteins are used and they are activated by a laser pulse. Once they are on, they are illuminated with different wavelengths of light for long enough to become bleached. Thus, these fluorophores cannot be reactivated in subsequent cycles [2]. Recently, PALM and FPALM have been extended to live-cell imaging. The difference between them is that PALM uses a TIRF (total internal reflection fluorescence) microscope, whereas FPALM uses a more traditional confocal microscope.

3.5 Reversible linear optically saturable transitions fluorescence (RESOLFT)

RESOLFT microscopy is based on the same principle used in STED, which uses a laser to switch off the fluorophores from the periphery of the excitation to achieve super-resolution. In RESOLFT an optical transition is used enabling super-resolution but at a much lower intensity than the depletion laser used in STED [1].

The advantages and disadvantages of the different super-resolution techniques are summarised in Table 1.

### Table 1. Advantages and disadvantages of the super-resolution techniques.

<table>
<thead>
<tr>
<th>Method</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
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<tbody>
<tr>
<td>STED</td>
<td>Several-fold resolution improvement (~0-70 nm).</td>
<td>- Effective image resolution is limited by the intrinsic optical resolution and by the density and size of the fluorescent labeling.</td>
</tr>
<tr>
<td>SIM</td>
<td>Two-fold resolution improvement (~100 nm).</td>
<td>- Low speed of image acquisition.</td>
</tr>
<tr>
<td>STORM</td>
<td>Improves resolution one order of magnitude (~10-55 nm).</td>
<td>- Expensive equipment.</td>
</tr>
<tr>
<td>PALM</td>
<td>Improves resolution one order of magnitude (~10-55 nm), allowing single molecules to be tracked within a cell.</td>
<td>- Complicated devices in the STED technology.</td>
</tr>
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3.6 Combination of super-resolution techniques

A combination of super-resolution techniques can be applied to obtain much more information about the structure of the analysed molecules. LIMON (light microscopical nanosizing) microscopy combines SPDM (spectral precision distance/position determination microscopy) and SMI (spatially modulated illumination) microscopy. Using this technology, it is possible to obtain additional information by a dual colour reconstruction of the 3D spatial arrangement of molecules and cellular structures [15].

Other microscopy that usually is used in combination with other techniques is the LSFM (lightsheet fluorescence microscopy). This is performed to increase speed of the imaging process and to reduce the photodamage and the bleaching of the fluorophores in living cells and plant [16]. The resolution of these combinations will be lower than other imaging methods, but you can achieve significant improvements in axial resolution incorporating SIM or STED modes to LSFM platforms.

4. Associated technology

The great advances made in the development of super-resolution microscopy would not have been possible without the parallel evolution of other essential tools. Most of these new technologies improve the labelling of nanoscale structures.

There are many different types of fluorophores with different properties, and it is essential to base the choice of fluorophore on the technique being used, the features of the sample, and the structures to be visualized.

First, it is important to check that the microscope system being used is compatible with the range of excitation and emission of the fluorophore. For multicolour techniques, it is imperative to confirm that the spectra do not overlap. Moreover, it is important to take into consideration the type of sample because some tissues can be damaged under certain conditions. For example, living cells may be damaged when exposed to ultraviolet light for extended periods. To avoid this, a fluorophore that operates in another range of emission must be selected. In addition, the emission must be...
very bright to locate the molecule and avoid background noise. This is of special importance in SIM techniques that detect only one fluorophore.

Another very important feature is photostability, meaning that a constant flow of photons must be maintained. This depends mainly on the chemical characteristics of the molecule. This is one of the most important properties for STED microscopy [4].

4.1 Fluorescent proteins (FPs)

Fluorescent proteins (FPs) have been used extensively in live imaging since the original green fluorescent protein (GFP) was first isolated from the jellyfish *Aequorea victoria* in the early 1960s. Scientists Roger Y. Tsien, Osamu Shimomura, and Martin Chalfie were awarded the 2008 Nobel Prize in Chemistry for the discovery and development of GFP. There are different types of FPs with particular properties that can be applied to diverse techniques. Although some techniques such as STED do not need special fluorophores and thus can use simple FPs, others such as PALM have special requirements and need FPs with the ability to change their spectral properties on irradiation. FPs can be classified into the following three families:

- Irreversible photoactivatable FPs (PA-FPs) are turned on by light of a given wavelength. Examples include GFP and its derivatives. Temporal resolution is limited by the photobleaching rate. Irreversible probes should be very bright but not necessarily photostable.
- Irreversible photoshiftable FPs (PS-PFs). The fluorescence excitation and emission spectra of PS-PFs change according to illumination. The emission of most PS-FPs changes from green to red. EosFP is the most commonly used red PS-FP for super-resolution imaging, and was used in one of the first demonstrations of PALM imaging [10]. The main disadvantage of monomeric EosFP, however, is that chromophore formation only occurs at temperatures below 30°C, which limits its use in mammalian cells [17].
- Reversible photoactivatable FPs (PA-FPs) can be turned on or off reversibly. PA-FPs are useful for super-resolution imaging because the same fluorophore can be imaged multiple times. For example, Dronpa, a photoswitchable fluorescent protein, only loses 25% of its original emitted fluorescence after 100 activation-quenching cycles. Reversible fluorophores such as cyanine (Cy) dyes can be turned off and therefore do not have to be photobleached. Reversible photoswitching is mandatory in RESOLFT imaging.

4.2 Quantum dots

Quantum dots are inorganic nanocrystals that are usually formed of cadmium selenide (CdSe) and zinc sulphide (ZnS). They are widely used for single-molecule imaging because they are very photostable and produce extreme brightness, with a high quantum yield. In biological applications, they are coated with a passivating layer to improve solubility, and they are conjugated with antibodies or streptavidin to visualize the structures that they are being used to label.

4.3 Nanobodies

Nanobodies are single-domain antibodies obtained from the camelid family. They have only one antigen-binding domain and are very small; with a diameter of 4 nm they are 10 times smaller than normal antibodies, which make them suitable for super-resolution techniques. They are very specific, which makes them very useful for detecting and locating proteins when combined with a fluorophore to be detected by high-resolution immunofluorescence. Nanobodies are used in super-resolution as cellular structural markers. For example, they can be generated against the vertebrate nuclear pore complex (NPC) and used in STORM imaging to locate individual NPC proteins with < 2 nm epitope-label displacement [18].

5. Applications of the super-resolution in cell biology

5.1. Cytoskeleton

The cytoskeleton is a structure that is involved in many cellular processes and is of huge interest to many biological researchers. It is a complex network formed of filaments, and it is important to differentiate and observe them clearly. For that reason, sufficient resolution to observe nanometre-thick filaments is required. Fluorescence microscopy is a very useful tool for investigating cytoskeleton structure. Labelling only the filaments and studying their 3D distribution is an excellent method for observing their configuration, function, and dynamism, and for detecting changes in the cytoskeleton under different situations [19].

The retinal pigment epithelium (RPE) is a monolayer of cells that performs many functions vital for retinal preservation. To prevent the toxic effects of accumulated photo-oxidative products, photoreceptor cells undergo a daily renewal process wherein about 10% of their volume is shed and subsequently phagocytosed by adjacent RPE cells [20]. The degeneration of the phagocytic function in RPE cells contributes to the development of age-related macular degeneration (AMD), the most common cause of blindness in the western world [21].
To gain insight into the phagocytosis of the RPE, we used a super-resolution confocal microscope (Airyscan, LSM 800 Zeiss, Germany) to visualize the regions of phagocytosis in the cytoplasm of ARPE-19 cells. For this purpose, we first incubated the cells with fluorescent beads. We then investigated the organization of F-actin cytoskeleton filaments, the most prominent component of the cytoskeleton, using a fluorescent phalloidin marker (rhodamine-phalloidin, Sigma, MO, USA) that regulates phagocytosis. The super-resolution technique was a useful tool for investigating cytoskeleton organization in these experiments, as demonstrated in Figure 2A-D.

Fig. 2. Fluorescent staining images of ARPE-19 cell line. A-D images show the cells were labelled with rhodamine-phalloidin marker (red) used for staining the actin cytoskeleton. Previously, the cells were incubated with fluorescent GFP-beads (green) to evaluate the phagocytosis. E-H images show the cells were immunolabelled for GRP78bip antibody (green), a chaperone involved in the signalling regulator of endoplasmic reticulum stress. B, D, F and H correspond to the zoom areas studied. Nuclei were stained with DAPI (blue) marker. Figures A, B, E and F were captured by confocal microscopy conventional technique while figures C, D, G and H were obtained by super-resolution technology (LSM 800, Airyscan, Zeiss, Germany). Scale bar: 20µm.

5.2 Endoplasmic reticulum

Until recently, it was generally accepted that the rough endoplasmic reticulum (RER) was formed of flat sheets. However, super-resolution techniques, specifically SIM microscopy, have shown that the majority of the RER is formed of very small and flexible contractile tubules, which endow it with the ability to move inside the cell cytoplasm [22]. In our studies on phagocytosis in RPE cells, we investigated endoplasmic reticulum stress using a chaperone, GRP78bip. Thanks to the super-resolution technique, the identification of regions of cytoplasm stress was more precise and detailed (Figure 2E-H).

6. Applications in science areas

6.1 Neuroscience

Super-resolution is an extremely useful tool in the field of neuroscience. Synapses are essential for neuronal function. The structures that make up synapses, dendritic spines, are very small and dynamic. Fluorescence labelling allows the visualisation of vesicle movements or changes to dendritic spines in living cells with good resolution. One of the most important objectives of studies in this area is an understanding of the dynamics of synapses in different situations. As mentioned above, these techniques are not used routinely, so the published information is still insufficient [23]. Dendritic spines comprise a thin neck and head of 0.5–1 µm [24], which varies according to the neurological disease concerned [25]. Owing to their small size, super-resolution techniques are essential for the study of dendritic spines in living cells. Before such techniques had been developed, EM was used, but it required processing that does not permit live cell imaging. Super-resolution has provided a more precise means of investigating the morphology and component parts of dendritic spines [26]. Changes in structures related to actin in the necks of dendritic spines have been observed using time-lapse STED microscopy. The quantification by super-resolution of morphological parameters, such as the
neck width and the curvature of the head of spines, improves our understanding of the function and plasticity of synaptic connections [27] (Figure 3).

![Fig. 3. A, B images show the resolution by using confocal microscopy (A) versus STED (B) of dendritic spines in living hippocampal slices. Scale bar: 1µm. C, Three-dimensional reconstruction of dendritic spines obtained by live-cell STED imaging in organotypic hippocampal slices from thy1-YFP transgenic animals (originally published in Nägerl et al., 2008, [27]).](image)

Synaptic vesicles and processes related to their exocytosis can also be displayed and monitored using these techniques. STED has been used to record by video synaptic vesicles of ~40 nm diameter within the axons of neurons to investigate their movement within and between synaptic buttons [28]. After the exocytosis of the vesicle, the membrane is recycled by endocytosis. Many details of this process are still unknown and it is unclear what happens to the components of the vesicles. Synaptotagmin 1 is a calcium sensor protein with an essential role in the exocytosis of these vesicles. STED has revealed that after exocytosis, synaptic vesicles remains in clusters in isolated areas. These results show that some components remain together and do not diffuse in the cytoplasm [29].

In neuroscience, resolution below the diffraction barrier is important because it facilitates the labelling of small structures. However, the combination of super-resolution techniques with quantitative biochemistry is a good option, especially in the case of protein quantification [30].

6.2 Genetic

Before super-resolution techniques, chromatin (a complex of DNA, protein, and RNA) was studied by EM and conventional optical microscopy. However, even though EM allows the nanoscale study of chromatin fibres, it is not very reliable for some studies because the sample processing can damage the specimen, and optical microscopy only allows the observation of structures above 200 nm. Super-resolution fluorescence microscopy, which is non-invasive and highly sensitive, permits the study of a wide spectrum of structures (10–200 nm).

These techniques allow the study of the organization of the different stages of condensation of chromatin and the localization of DNA-associated proteins. One of the biggest problems is that during mitosis, the chromosomes have a high packing density, and it can be difficult to isolate the structures for observation. Moreover, interpretation of the images of chromatin during the interphase can be difficult [31].

6.3 Embryology

Embryonic development is a very complex and dynamic process that involves highly regulated processes such as proliferation, migration, apoptosis, and differentiation. Live imaging techniques are very useful for investigating the morphology and location of cells in living embryos, and for monitoring how they change over time. Three-dimensional super-resolution techniques are an excellent means of accomplishing these objectives [32]. Vital dyes, quantum dots, and genetically encoded FPs have been used in the labelling process to visualize embryonic structures. One example of the use of super-resolution techniques in embryology is the study of the blastomeres of mammalian embryos. They occur in non-adherent, round, and large forms. In this context, 3D-SIM technology is a highly detailed and practical technique for studying mammalian embryos and spermatozoa [33].

7. Applications in clinical areas
7.1 Ophthalmology

Super-resolution techniques such as STED or SIM are useful for biomolecular studies, but the use of high-powered lasers and the necessity of immobilizing the sample make it unsuitable for living animals or humans. Super-resolution has also been used in the field of diagnostics. The combination of different techniques has increased resolution in biomedical diagnostics.

A good example is the combination of optical coherence tomography (OCT) and adaptive optics in ophthalmology [34]. OCT is a non-invasive diagnostic technique that allows cross-sectional imaging for visualisation of the back of the eye [35], and is used for AMD, glaucoma, and diabetic retinopathy diagnosis [34]. Visualization of microscopic changes in the retina allows early detection of pathologies, leading to earlier treatment that can stop or slow the development of the disease, improving the prognosis and avoiding severe loss of vision [36, 37].

![Fig. 4. A, B represents a OCT cross-section slide of a retinal human microstructural image across the fovea. Different layers can be observed (e.g., choroids, retinal pigment epithelium and nerve fiber layer). C, by combining several cross-section images, a three-dimensional volume and a projection view image of the vessels can be extracted (modified from Reif and Wang, 2012 [37]). D represent a SD-OCT image from a mouse (C57Bl6/J) retina, (Biop tigen Envisu 2200). On the right, the layers of the retina are represented. Abbreviations: GCL: ganglion cell layer; INL: inner nuclear layer; IPL: inner plexiform layer; NFL: nerve fiber layer; OPL: outer plexiform layer; ONL: outer nuclear layer; RPE: retinal pigment epithelium.](image)

Adaptive optics was originally used to overcome the effects of enlargement and blurring caused by the turbulent atmosphere of the earth in the images provided by telescopes [38]. In the field of ophthalmology, OCT was firstly introduced in 1991 by using the technique with a fundus camera [39]. The combination of OCT and angiography (OCTA) reduces ocular aberrations, and corrects for natural imperfections of the cornea, lens, and vitreous humour in real-time.

This tool has allowed improvements to the quality of \textit{in vivo} observations of the human retina (Figure 4A, B, C) [40]. Moreover, OCTA improves lateral resolution and increases sensitivity to weak reflections, allowing its use for 3D images of the retina, and facilitating the study of ocular architecture for the diagnosis of many diseases.

Fluorescein angiography (FA) is the classical method for observing the fine details of the retinal vasculature and early changes in ocular pathologies such as AMD and glaucoma. FA is effective but it requires an injection of a toxic fluorescent dye. However, FA cannot detect significant pathological capillaries. OCTA represents a potentially attractive alternative for this purpose because it is a non-invasive technique and provides 3D resolution for the investigation of the microvasculature of patients. Another benefit of the OCTA application is that it greatly reduces the lateral size of granular artifacts and speckles, and provides a detailed image of the studied area [34].

We used spectral domain OCT (SD-OCT) for the non-invasive, \textit{in vivo}, 3D imaging of the mouse retina (Figure 4D). \textit{In vivo} visualization of retinal structures in mice experimental models can provide a precise, quantitative description of retinal layers and permit the visualization of retinal microvasculature and pulsatile flow dynamics, parameters that have not been easy to measure until now.

7.2 Mammalian pathology

Mammograms are used for the early detection of breast cancer. The technique detects calcifications and masses, but the density and size of the pathological structures can vary in the same area of the tissue. For this reason, maximum quality and resolution are required for diagnosis. A new algorithm has been developed to improve mammography images and to highlight masses and calcifications.
In this learning-based algorithm, super-resolution forms a high-resolution image from a set of low-resolution images, without modifying the original image. First, a non-subsampled contourlet transform (NSCT) improves the quality of the images after determining the regions of interest. Then, the super-resolution algorithm is applied to improve resolution, predicting high-frequency components and deleting distortions. Finally, a high pass filter is applied to sharpen and highlight the region of interest (Figure 5) [41].

Fig. 5. Improvement of the quality and resolution of a mammography. A image represent a mammography after the NSCT. B image represent a mammography after applying the super-resolution algorithm. C image represent a mammography after highlighting the regions of interest (originally published in Pak et al., 2015 [41]).

8. Concluding remarks

Fluorescence super-resolution microscopy techniques are very novel tools that are still in the early stages of development. Different super-resolution applications can be employed depending on the type of sample under scrutiny, and they all have advantages and disadvantages. Such techniques still require improvements. Despite this, they have already proved effective and useful in many biological studies, and have facilitated the study of biology from a completely new perspective, allowing us to recover nanometric information that was previously unattainable. Although they are useful, they are still evolving and are not yet routinely used because many researchers do not have access to them, among other factors. However, their use is gradually spreading. Because diagnoses are required for a wide range of diseases, super-resolution platforms have been developed in recent years, and have had a significant impact on global health by facilitating effective and reasonable diagnostics. It is probable that in the future improvements to all these techniques will be aimed at overcoming their major disadvantages, improving temporal resolution, developing more photostable or brighter fluorescent probes, and enhancing 3D and multi-coloured imaging. Moreover, combinations of techniques that are in the early stages of development will be improved.

Acknowledgements: The support by Figure 3 to Dr. Marcus Fruttiger Laboratory, University College London (UCL) () is gratefully acknowledged.

References


