Superresolution Microscopy in Immunology Research

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Abstract

During the last decade many different superresolution (SR) microscopy techniques have been developed, offering high resolution imaging in conventional light microscopy settings. SR techniques can roughly be subdivided in techniques applying structured illumination and single molecule localization techniques. Although SR microscopy has been implemented in developmental biology and neuroscience, it is not as commonly used in immunology research. This review aims to evaluate the current application and future potential of SR microscopy in different immunology research niches. Furthermore, the current innovations in SR microscopy are discussed.

Current literature available on application of SR microscopy in immunology research confirms that this is a comparatively novel technique: results obtained using these techniques are in some cases contradictory, which is most probably due to a lack of standardized protocols. Evaluation of the different niches shows that implementation of the different SR techniques could potentially lead to a significant gain in knowledge. Undertakings to standardize imaging protocols and the design of more efficient and broadly applicable fluorophores will assist in the maturation of SR microscopy into a widely applied technology, which might resolves some of the still prevailing mysteries in immunology.

Introduction

In order to fully understand cellular processes, visualization is important. Only then can protein complex formation be fully appreciated in time and space. If this is not the case, visual input is still a requirement for mathematical models that aim to dissect these processes.

Fluorescence microscopy is one of the methods which has been and still is of great importance in cellular research. It offers the possibility to image live cells and follow processes through time, thus allowing the studies in a physiological context, albeit modified to allow imaging. One of the biggest advantages of this technique is high sensitivity imaging of single structures or molecules independent of nearby structures, due to the high contrast of fluorophores to a dark background. A drawback of this approach is that there is a lack of context visualization. Furthermore, the sensitivity of fluorescence microscopy is limited in its capacity to discriminate two objects that are in close vicinity, which is also described as the resolution.

The maximum resolution is defined by Abbe's law as approximately half the wavelength of the electromagnetic wave used to visualize the object. This limits the resolution of conventional fluorescence microscopy to a maximum of 200nm laterally and about 600nm axially, with wavelengths ranging from 400 to 650 nm (Figure 1). Using electron microscopy (EM), far lower resolutions (about 0.5 nm) can be reached on a regular imaging setup due to the smaller wavelength of electrons. The drawback of this technique is that samples have to be fixed for preparation purposes, and this method is therefore not compatible with live cell imaging. Furthermore, labelling of samples is nowhere near as efficient as in confocal microscopy as mainly the epitopes on the surface of sections are available for binding. In immunology research, both fluorescence microscopy and electron microscopy do not fulfil the needs of researchers, as many the processes and molecules that are studied are often smaller than can be imaged using conventional fluorescence microscopy (Figure 1), but are also very dynamic, and therefore impossible to image using EM. In addition, it is often the case that sporadic events have great impacts on cellular processes, and capturing such an event requires voluminous datasets. Combinations of high resolution with live cell analyses, as in correlative light and electron microscopy (CLEM) could solve some of these issues[1]. Yet, the generation efficiency of these datasets limits general application in immunological research.

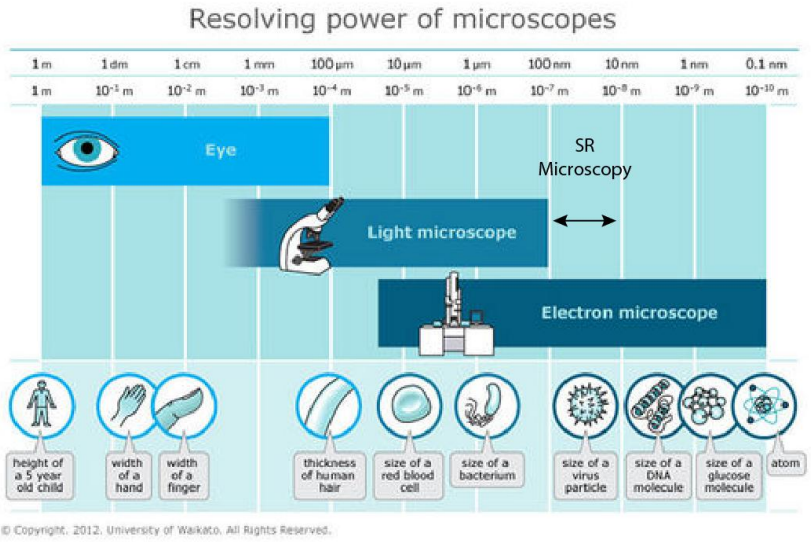


Figure 1: The resolving power of microscopes. The black arrows indicate the resolution area that can be visualized using SR microscopy on top of conventional light/fluorescence microscopy. Adapted from[2].

A relatively new method filling the gap left by EM and conventional fluorescence microscopy is superresolution (SR) microscopy, or nanoscopy, which is based on visible light imaging systems. Multiple different SR techniques have been developed independently of each other, and most of them are based on fluorescence. The two main approaches within which there are variations, are spatially patterned excitation of fluorescent molecules and single fluorescent molecule imaging. The first SR microscopy technique was implemented in 1993[3], but since then many more techniques have been which have significantly increased the resolutions up to 20 nm laterally and 50nm axially[4,5].

Interestingly, although application of SR microscopy has led to interesting results in developmental biology and neurology[6], this method is only sparsely used in immunology research. The aim of this review is to introduce the guiding principles of SR microscopy, the different methods with their respective benefits and drawbacks, and their current use and potential in immunology research.

Chapter 1: The different SR microscopy techniques

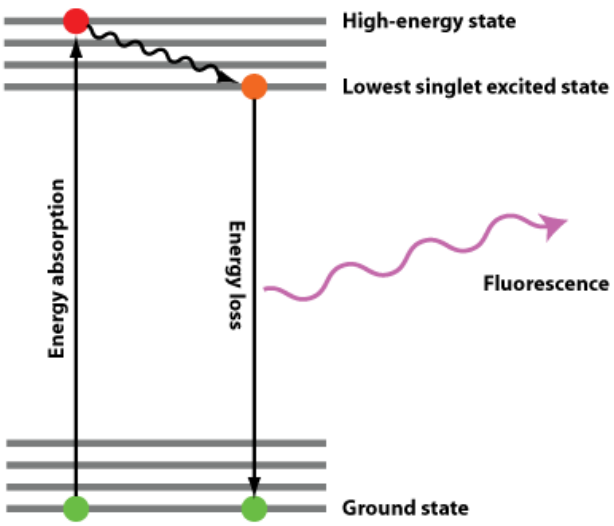
In this literature review the most important and influential SR techniques will be discussed. They are subdivided in the following two categories: techniques using hardware shaping of the illumination source (STED/RESOLFT, (S)SIM, NSOM), and techniques based on single molecule localization, also known as pointillism (PALM, STORM, PAINT, GSDIM, SOFI).

The theoretical underpinnings of SR microscopy will be explained based on the techniques that exploit these mechanisms. Some specific characteristics of fluorophores for instance, will be discussed in the pointillism section.

The applicability of the most important innovative SR techniques in immunology research will be evaluated based on several considerations, such as imaging speed and multicolour imaging, and this compared and summarized in Table 1.

As SR microscopy techniques rely on fluorescent molecules, some basic features of fluorophores will be introduced first.

A fluorescent signal is emitted when a fluorescent molecule returns to its ground state after being excited to a high-energy state by energy absorption. In fluorescence microscopy the source of energy for the fluorophores is laserlight with specific wavelengths. This process is illustrated in a Jablonski diagram (Figure 2). The period of time during which the fluorophore stays in the high energy state before falling back to the ground state, while emitting a photon, is described as the fluorophore life time. The stability of the fluorophore is a measure for how long it takes before the fluorophore is photochemically damaged, and thus bleached, by this laserlight. Photobleaching during an experiment leads to an underestimation of the total number of labelled molecules. Photostability is especially important for deterministic SR microscopy approaches, such as SIM and STED[7]. The quantum yield of fluorophores describes the ratio between the number of photons that are absorbed and the number of photons that are emitted by the fluorophore.



Figuur 1 Jablonski diagram[77]

The fluorophores that are currently used in SR microscopy can be roughly divided into two groups, namely: fluorescent proteins and organic dyes. Fluorescent proteins can be genetically engineered to be expressed as part of the protein of interest, whereas staining with organic dyes often relies on interactions with antibodies recognizing the protein of interest, which requests cell permeabilization[8]. Quantum dots, potentially interesting fluorophores, are semi-conducting photostable nanoparticles which have been used in pointillism methods, although they are prone to photoblinking activity[9,10], which is discussed into more detail below.

When selecting fluorophores for SR microscopy purposes, one should consider the sizes of the different probes. Fluorescent proteins are typically 4nm, whereas the antibodies used to link organic dyes (1-2 nm) to the molecules of interest are typically 10nm[7,11]. Although fluorescent proteins seem ideal for live cell imaging, they might disturb cellular processes when overexpressed and can cause phototoxicity[12]. Furthermore, fluorescent proteins have lower photon yields than most organic

dyes[8,13]. Current research is aimed at the development of smaller, membrane permeable organic probes, to provide better alternatives for fluorescent proteins[7,8,14].

Chromatic aberration can occur upon simultaneous imaging of multiple fluorophores with different wavelengths. Chromatic aberration describes the inability of a lens to focus different colours to the same focus point. The resulting imaging artefacts can be corrected by software, but only when the right controls, such as fiducial markers, are taken along[8].

Methods using hardware-based shaping of the illumination source

The techniques in this category use hardware-based illumination patterns to access sub-diffraction information. This is either done by positive or negative patterning: depending on which part of the sample is illuminated and what information is recorded.

Resolution is determined by the point spread function (PSF) of the microscope, which can also be described as a measure of 'blurryness' induced by the diffraction of light due to the experimental set-up. The resolution is limited by the width of the PSF, which is determined by the photon wavelength, the size of the opening of the objective (numeric aperture (NA)), and the refraction index of the media through which the light travels[11,15]. The resolution can be increased by deconvolution of the acquired data, as applied in SIM. In deconvolution software, the PSF is used to recalculate blurred intensity information in axial image series to original object positions.

(S)SIM

Positive patterning is employed by spatially structured illuminated (SIM), which was first described by Gustafsson in 2000[16]. By illuminating the sample with spatially structured light (e.g. stripe-shaped sinusoidal interference) Moiré fringes are created due to the mixing of different frequencies: photons emitted by the local fluorescent dye and the excitation light. High resolution information is obtained by scanning the sample with this pattern from different angles. However, as the resolution of the illumination pattern itself is also limited by the diffraction of light, the resolution can only be increased twofold using this technique.

SIM can also be applied in 3D imaging, using multiple beams with structured light at a time, which together create 3D illumination pattern[17].

In 2005, a variation on SIM, called saturated structured illumination microscopy was first described (SSIM)[18]. In SSIM, different states of fluorophores, namely the dark and light states, are exploited. The sample is illuminated with patterns with a high intensity which saturate fluorophores, causing them to enter a dark state and thus creating sharp dark regions in the sample. These excitations patterns have a higher spatial frequency, and thus result in a higher resolution, as it is no longer limited by the diffraction of light.

SIM is a relatively inexpensive technique, as it uses regular lasers and can be applied on conventional light microscopes using standard dyes and protocols, whereas SSIM is more complex[16,19]. SIM has the fastest imaging speed of all SR techniques, although it relies on scanning, and both SSIM and SIM can be used for (multicolour) live cell imaging. However, the illumination intensity used in SSIM can induce phototoxicity. Furthermore, the gain in resolution compared to conventional fluorescence microscopy when using SIM is limited. In conclusion, due to the relative ease of application and inexpensiveness of these techniques, they user-friendly methods of SR microscopy. However, application in immunology research will be limited to certain fields of interest,

such as cytoskeleton organization due to the limited resolution of SIM and the phototoxicity associated with SSIM.

STED

Stimulated emission depletion microscopy (STED) was first described in 1994 by Hell *et al.*[20], and uses negative patterning. This method focuses the excitation beam through a pinhole on the sample, thus exciting only the fluorophores in a limited area of the sample. The gain in resolution is obtained using a second laser beam, which is applied shortly after the first, before fluorescence has taken place. The STED beam inhibits fluorescence in the outer regions of the area illuminated by the first excitation beam, creating a doughnut shaped area where fluorescence is depleted. This dark area surrounds a central area where the fluorophores are able to emit photons. The resolution that is obtained depends on the intensity of the STED beam, as a stronger STED beam increases the fluorescence depletion area. When using the STED technology, it is very important to choose the right fluorophores, as the wavelength of the depletion laser should not overlap with the excitation range of the dye[19]. For the same reason it has proven to be difficult to image multiple colours in one sample, the maximum is currently set at two[19].

Conventional STED is stretched to the third dimension using isotropic STED (isoSTED)[21]. The 3D-PSF of an illuminated area usually has a cigar-shape due to asymmetric convergence, which is detrimental for the axial resolution. By simultaneously applying two different STED patterns (STEDxy and STEDz) isoSTED increases the axial resolution by creating a round, or isotropic, focal point, which is uniform in all orientations[21]. Using this approach, a 40nm resolution can be reached[21].

High resolution information can be obtained using STED, but this requires high illumination intensity of the STED beam, which might in turn cause phototoxicity. Furthermore, as STED is a scanning technology, the time resolution for larger samples is low. Therefore, live cell imaging using STED is limited to smaller, or less dynamic processes. Disadvantages of STED are the high costs of the system, and the complications in multicolour imaging as the depletion laser wavelength should not overlap with the excitation range of the fluorescent dye[19]However, when the data have been obtained, there is no need for post-processing, whereas this is the case for most other SR technologies. STED has been used in immunology research, and the high resolutions that can be reached will be valuable for future research.

RESOLFT

Reversible saturable optically linear fluorescence transitions (RESOLFT) microscopy is a technique developed by Hoffman *et al.* and was published in 2005[22]. This technique exploits the photoswitching capabilities of fluorophores at low illumination intensities, while scanning the sample with one or multiple “zero’s”[22]. Zero’s are small, sub-diffraction sized areas in which the fluorophores are permitted to emit photons, while the surrounding fluorophores are kept in a dark state. Essentially, STED is such a technique, as its zero is the illuminated area in the dark doughnut, but STED uses up to eightfold higher illumination intensities[22]. It is important to note that this technique exploits the basic transition of standard fluorophores, whereas techniques based on stochastic photoswitching, such as PALM and STORM, rely on photoactivatable fluorophores[23,24]. GSDIM, which is in essence a single molecule detection method, is also a RESOLFT technique as it does not rely on photoactivatable fluorophores[23,24].

Due to the low laser intensity, RESOLFT is associated with less phototoxicity than STED. Furthermore, the image acquisition time is shorter due to the scanning with multiple zero’s.

However, it takes longer to reach appropriate levels of fluorescence in the sample due to the lower excitation light intensity[22]. This problem could be solved using fluorophores with shorter pixel dwell times, which would enable live cell imaging[22]. This method is not widely used, and future results should demonstrate whether it has potential in immunology research.

NSOM

Near-field scanning optometry (NSOM) was the first single molecule detection microscopy method imaging below the diffraction limit to be implemented. It was first described by Betzig *et al.* in 1993, and is based on the funnelling of light through a small aperture (e.g. 100nm) in a probe that is positioned very close to the sample in order to limit the illuminated area[3]. The probe scans the surface of the sample, while collecting sub-diffraction limit data, as the resolution is not dependent on the diffraction limit due to the application of near-field illumination through a very small aperture[3]. NSOM is not compatible with live cell imaging as the scanning movement is guided by sample-tip forces, and therefore, cells have to be fixed[25] . Due to its limited imaging depth, NSOM has mainly been applied in research on membrane structures such as lipid rafts and receptor complexes[25]. In combination with FRET (Förster/fluorescence resonance energy transfer) imaging of 10nm the axial direction has been achieved[25].

Although high resolution information can be obtained using NSOM, and the instrument is not very complex, the demands of sample preparation are restrictive and the imaging depth is limited. Furthermore, as this is a scanning method using a very small aperture, image acquisition times are long. Currently, this technique is by not compatible with live cell imaging and due to the limited imaging depth, few immunological processes can be studied using this technique. Therefore, large-scale implementation of this method in immunology research is deemed to be unfeasible.

Techniques based on single molecule localization (pointillism)

Single molecule localization SR microscopy techniques are based on *in vitro* single molecule detection using beads, described in the 1980's[26]. This work provided methods to determine the centroids of single fluorophores by diffracted photons. This method was refined further by manipulation of the fluorophores during imaging in such a way that only few fluorophores were visible at any time in the imaging field. Such spacing of fluorophores allowed diffraction limited imaging of individual molecules. Using this approach, different subsets of the fluorophores present in the sample are imaged over time. Combination of all fluorophore centroids, which are calculated using the PSF, results in an image representing the 2D scatter plot of the calculated XY positions of the individual fluorophores. This approach is also referred to as pointillism[9], as the resulting image is built up out of single, defined, dots.

Pointillism microscopy techniques are often based on total internal reflection fluorescence (TIRF) microscopy. In TIRF microscopy as the background signal is very low due to the use of the evanescence wave illumination[12]. Therefore, single fluorescent molecules will stand out, and can be localized with high precision. Depth of imaging is limited (to about 100nm) but can be increased by aiming the laser in a somewhat tilted angle, this is referred to as the HILO (highly inclined laminated optical sheet) modus of TIRF imaging[11]. Using the HILO modus, the imaging depth can be increased to several microns, but the tilted angle will increase the background scatter and thus decrease the signal to noise (S/N) ratio[11].

The first three most important single molecule localization techniques (PALM, FPALM, and STORM) were simultaneously developed by three independent groups and published in 2006[27–29]. Pointillism methods highly rely on specific characteristics of fluorophores, such as photoactivation or switching. Single molecule localization and resolution of dense structures can be achieved as individual fluorescent molecules stochastically return from a dark state to a light state upon low intensity illumination of specific wavelengths. The resolution that is reached using these methods relies on the fluorophore characteristics, as the number of photons that is emitted determines the strength of the signal. This is also dependent on the quantum efficiency of fluorophores, which is described as the percentage of photons that produces electrons, and is thus recorded, when hitting an (electron-multiplying) charge-coupled device ((EM)CCD).

Acquisition times of pointillism images are specified as the time it takes to localize enough fluorophores to provide the needed resolution to resolve the structure of interest. Depending on the sample size and structure density, the acquisition time can be up to several hours[27]. Image acquisition times can be minimized using fluorophores with high photon yields. Furthermore, specific buffers containing oxidizing and reducing reagents can be used to increase the photon yield, reduce photoblinking, and elongate the dark state of organic fluorophores, thus increasing the resolution[7,8,30].

Photoblinking is fast switching between the dark and light stage, and is also called fluorescence intermittency. This can occur when fluorophores transition from an excited state to the ground state via an intermediary triplet state[7]. In pointillism methods this phenomenon can lead to multiple observations of the same fluorescent molecule. The algorithms only partially correct for this phenomenon, thus limiting the quantitative interpretation of pointillism data[30].

PALM

Photoactivated localization microscopy (PALM) is based on stochastic serial photoactivation and subsequent imaging until bleaching of single photoactivatable or photoconvertible fluorescent proteins (PA-FP)[31,32]. This technique was first demonstrated with lysosomal and mitochondrial proteins and the HIV-1 Gag protein by Betzig et al. in 2006, using cryosections of cells that had been transfected to overexpress PA-FPs[27]. Fluorescence photoactivation localization microscopy (FPALM) is a similar technique, which was also first described in 2006[29].

Conventional PALM can be extended to the third dimension with interferometry PALM (iPALM), or biplane FPALM (BP FPALM)[5,33]. iPALM is a combination of PALM with interferometry developed in 2008, is used to perform quantitative high-precision positional measurements[5]. Biplane FPALM enables 3D imaging of thick samples via a modified fluorescent signal detection path[33]. In this technique, images of activated fluorophores are fit with a 3D PSF to determine their spatial. Lateral positioning stays the same, is independent of the axial position[33].

Single particle tracking PALM (sptPALM) is a modification of conventional PALM that enables imaging of dynamic processes[34]. Using this technique the formation of virus like particles (VLPs) by HIV-1 Gag and concentration of VSV-G proteins has been be imaged over time[34].

Using PALM and FPALM, high resolutions can be reached at low laser intensity, and single particle tracking in living cells has been demonstrated. Multicolour imaging is essential to obtain information on the context of cellular processes when using pointillism methods, as the data that are obtained are represented as pointillism clouds. However, the number of colours that can be imaged is limited due to the broad spectra of photoswitchable fluorescent proteins[8]. Another disadvantage of PALM is

the extended data acquisition time due to the stochastic fluorescence, which limits the opportunity to visualize dynamic processes.

STORM

Stochastic optial reconstruction microscopy (STORM) is another technique that localizes the exact position of single fluorophores, and was also first described in 2006[28]. In STORM, all fluorophores are first converted to a dark state from which they are stochastically activated, enabling localization of single fluorophores[28,35]. The fluorophores are typically reversibly photoswitchable. In the early experiments cyanine dye pairs were used, in which one of the dyes, the activator, is activated by light of a specific wavelength, which in turn activates the reporter dye that is also part of the probe[28,35]. Different activator-reporter dye combinations enable multicolour imaging of up to three colours[28,35]. More recently it has been shown that this technique can also be implement without the need for an activator dye, this is referred to as direct STORM (dSTORM)[36]. Using this technique, multicolour imaging with minimal cross-talk using four different fluorophores has been demonstrated[30].

Conventional STORM can be extended to the third dimension (3D STORM) using a cylindrical lens that creates different focal planes in the x-y axis[37]. The image of the fluorophore then becomes a measure of its distance from the focal plane while determination of its lateral position stays the same.

Using STORM high resolutions have been reached, also in 3D, and data acquisition times are shorter than in PALM. However, illumination intensity is higher that is needed for organic fluorophores is higher, which leads to more phototoxicity. Furthermore, live cell imaging is complicated without endogenous expression of the fluorophore, and dataprocessing is elaborate and not fool-proof. The restrictions of both PALM and STORM techniques can be dissolved by designing a wider variety of fluorophores, which is described in more detail in the last chapter.

Since the development of the first single molecule localization techniques, several other techniques using similar, but slightly different approaches have been developed. Among these methods are some which use a new approach for single molecule localization: a reversible binding interaction between a specific cellular target and a biomolecule carrying a fluorescent moiety[7].

PAINT

Points accumulation for imaging in nanoscale topography (PAINT) is based on diffusion of fluorophores[38]. The fluorophores used in this technique only emit high numbers of photons when they are immobilized by binding to the target molecule, which occurs via electrostatic coupling or hydrophobic interactions[38]. These interactions are of a transient nature, and the fluorescent signal disappears when the probe dissociates or is bleached. To ensure that the spacing is sufficient to localize individual fluorophores, the ratio of probes and target molecules has to be determined up front[38]. This technique is considered especially useful for lipid structures due to its interaction nature[38].

GSDIM

Ground state depletion microscopy followed by individual molecule return (GSDIM) was first described in 2008[24]. GSDIM is based on far-field fluorescence microscopy, and combines continuous wide-field illumination with a continuously operating camera[24]. The fluorophores are first switched to

a dark stage, from which they will stochastically return, enabling visualization of single fluorophores[24]. This technique differs from STORM and PALM in that it does not need photoactivatable fluorophores, but rather uses the basic transitions of standard fluorophores[24]. For this reason, this technique is also coined as a RESOLFT technique[23,24]. Imaging of densely labelled structures is enabled by bleaching or 'shelving' of fluorophores in a stable dark state before image acquisition[24].

SOFI

Superresolution optical fluctuation imaging (SOFI) was first described in 2009, and uses fluorescence signal fluctuations of single emitters to determine the position of single fluorescent molecules[39]. This technique was developed using a conventional wide-field microscope, and does not specifically depend on photoactivation[39]. By evaluating each individual pixel that is recorded over time, the fluorescence from emitters near that pixel can be filtered out. In this way, only the highly correlated fluctuations are analyzed, which increases the resolution, which is dependent on the pixel size[39]. This technique can be extended to 3D by scanning through the sample, but the axial resolution is limited to approximately 200 nm[40]. As photoblinking is not problematic in this technique, quantum dots could be readily used[7].

Principle	Wide-field and confocal	Structured illumination: Moiré Effect		Structured illumination: Saturated emission depletion	Pointillism by photoswitching	Near-Field scanning through small aperture
Acronym		SIM	SSIM	STED/ RESOLFT	PALM/FPALM/ STORM	NSOM
XY-resolution (nm)	200-250	100	50	20-80	20-50	20-120
Z -resolution (nm) (3D)	500-700	200	-	45-125	20-30 (STORM) 75 (BFPALM)	10 (near field)
Speed (512x512 image)	Ms	ms	s-min	ms-min (dependent on size)	s-min (dependent on size)	s-min (dependent on size)
Scanning	Confocal	no	no	yes	No	yes
Intensity	Moderate	moderate	high	moderate (RESOLFT: low)	moderate-high	low
Live-cell imaging	Yes	yes	yes	yes	Yes	no
Dyes	Any	photo-stable	switchable	switchable	switchable	any
Multicolor	up to 5	3	no	2	up to 4	2
Data processing	No	yes	yes	no	yes, elaborate	no
Instrument complexity	Low	medium	high	high	low-high	low

Table 1: Comparison of microscopy methods[11,19]

The table above illustrates the scope of SR microscopy techniques that have been introduced. Only those techniques that are relevant for and broadly applicable in immunology research will be discussed below.

Chapter 2: Application of SR microscopy in immunology research

Having introduced the different SR microscopy techniques, the focus in this chapter will be on how the different SR techniques are implemented in immunology research, and the research areas within immunology research in which these techniques might prove useful in the near future. To this date, SR microscopy has been used in only a limited set of immunology studies, of which the scope is illustrated below.

Receptor organization and clustering

The spatial organization of different cell-surface expressed receptors on immune cells has been studied intensively using conventional fluorescence microscopy. However, single receptors are sub diffraction-sized, and therefore there remained some controversy on the clustering dynamics of single receptor subunits. Using SR single molecule microscopy techniques combined with mathematic data analysis, the dynamics of receptor clustering could be studied and modelled[41–43]. There is some variation in the study set ups, as is to be expected with comparatively new techniques. The simplest study models are based on artificial lipid bilayers, whereas more advanced studies image single (live) cells which are activated by a ligand-coated coverslip. An advantage of studies on proteins expressed on the plasma membrane is that these proteins can be stained with superior organic dyes without the need for permeabilization.

Studies on the T-cell receptor (TCR) have lead to contradictory results, which is probably due to the varying experimental procedures as described above[44,45]. However, the prevailing consensus is that CD3 and linker of activated T-cells (LAT) are preclustered in distinct protein islands in resting T-cells, as was determined by Lillemeier *et al.* using high speed PALM[46]. Clustering of these proteins has been found to be maintained after T-cell activation, but the smaller protein islands were found to concatenate into microclusters, which were more prevalent in the immunological synapse[46]. Furthermore, the preclustering of CD3 has been found to be dependent on the immunereceptor tyrosine-based activation motif (ITAM)[47].

Experiments dissecting the activation of the B-cell receptor (BCR) using confocal microscopy have led to contradictory hypotheses, an issue that could be addressed using SR microscopy[44]. The first SR technique used to visualize the organization of individual BCRs was dSTORM[48]. Using dSTORM, the size and density of the BCR nanoclusters could be quantified by combining the strong fluorescent signal of the organic dye with statistical data analysis[48]. Based on these results, it has been proposed that IgM and IgD were preclustered apart from each other in primary B-cells in protein islands with different receptor densities. These islands were shown to be influenced by cytoskeleton disturbance and tetraspanin-mediated CD19 immobilization[48]. STORM has also been used to measure single molecule BCR diffusion in live cells, which was shown to be dependent on proteins linking the membrane and the cytoskeleton[49].

Pathogen-binding C-type lectins (DC Sign) are highly expressed on dendritic cells and macrophages and have been studied using STED and NSOM[50]. Nanocluster formation of DC Sign has been shown to be mediated via the neck region of these receptors using these SR techniques[50]. Furthermore, the ability to form clusters was shown to influence the degree to which pathogens dock to the receptor[50].

The inhibitory receptor KIR2DL1, an MHC-I-binding immunoglobulin-like receptor which is expressed on natural killer cells (NK cells), has been imaged with PALM and GSDIM[42]. PALM was used to characterize the spatial patterning of the receptor, which was found to be clustered in circular nanoclusters, that increased in density upon activation of the receptor with antibodies[42]. Activation by the activating receptor NKG2D resulted in smaller, more numerous microclusters of KIR2DL1. Using GSDIM, it was determined that stimulation of KIR2DL1 with NKG2D has no influence on the density of MHC-I clusters and the diameter and density of the individual clusters[42]. It was found that results obtained in live cell imaging of KIR2DL1 cluster formation using GSDIM technology were comparable to the results obtained in fixed cells using PALM[42].

The immunological synapse

The site of interaction between an antigen presenting cell (APC) and effector T-cells and NK cells is called the immunological synapse (IS). The synapse is formed upon interaction of the two cells via different receptors and antigens. The outcome and dynamics of the IS are dependent on poorly understood signal integration, and therefore the functions and dynamics of all the proteins that are involved are subject to ongoing research. As this concerns the dynamics of single proteins, SR techniques are ideal to dissect the dynamics. Most studies described below used “artificial synapses” to study the individual molecules. These synapse systems exist of single APCs or lymphocytes which interact with coverslips that are coated with specific molecules or proteins. Recently, using 3D-STED, an immunologic synapse between two cells (an NK cell and a T-lymphocyte) has been imaged for the first time[51]. Although the ideal method to study the IS, a combination of multicolour, 3D, SR microscopy[44], has not been implemented (yet), SR techniques have led to a gain in knowledge and even paradigm shifts[52,53].

Synaptic vesicle release of lytic granules in the IS and the role of syntaxin 7 (Stx7), a protein involved in vesicle fusion, therein has been studied using SIM[54]. Stx7 was shown to mostly colocalize with Rab7, a maker for late endosomes. Knock down of Stx7 resulted in an increased colocalization of Rab7 and CD3, indicating that Stx7 plays an important role in the trafficking of recycling TCRs back towards the plasma membrane or immunologic synapse[54].

The implementation of SR microscopy techniques has led to a paradigm shift regarding the lytic granule secretion in the IS. Using confocal microscopy, an actin ring was visualized at the IS, and it was hypothesized that the lytic granules were secreted through the hole in this ring and did not interact with the actin cytoskeleton. Using dual colour STED lytic granules were found to be closely associated with the actin skeleton and to be secreted through small holes in an actin mesh[52]. 3D-SIM revealed the presence of an F-actin ring at the IS, but also visualized a mesh of actin fibres inside this ring, with a less dense mesh than the actin fibres outside the ring-structure[53]. Using a mathematical approach, it was shown that the holes in the mesh were large enough to permit passage of lytic granules, which was validated using EM[53]. Localization of the lytic granules to the less dense actin mesh was determined to be dependent on the microtubule organization centre (MTOC) using dual-colour 3D SIM[53].

Cellular protein trafficking

Transport vesicles have a size of approximately 60nm and are therefore not visualized using conventional fluorescence microscopy. SR microscopy could thus play an important role in the elucidation of the trafficking and modification pathways of antigen presenting proteins such as MHC-I and -II and glycolipid presenting proteins such as the CD1 family. Other processes of interest are e.g. the detailed clustering of membrane cargo on the cell surface, intra-Golgi vesicle budding sites, and ER exit assemblies. A first step towards a better understanding of these processes would be the possibility to follow single vesicles throughout the cell.

Cellular vesicle trafficking has been studied in yeast using PALM[43]. As fixed cells were imaged the vesicles could not be followed through time, but he number of accessible PI3P molecules and vesicle diameter enabled determination of the origin of the vesicle, as 'younger' vesicles had a smaller diameter and less accessible PI3P binding sites[43].

Using STED the trafficking of small cargo within and between Golgi stacks of fused cells was studied[55]. SR microscopy enabled determination of the size of inter-Golgi transport vesicles and staining of the coatomer[55]. These experiments were performed on fixed cells, but live cell imaging using SR techniques would provide even more information on Golgi trafficking and vesicle organization of especially the intra-organelle clustering present in endosomal structures.

Exosomes

The term exosomes is used for small membrane vesicles with a diameter of 40 to 100nm which derive from multivesicular bodies (MVBs) and are secreted extracellularly. These vesicles serve different purposes, depending on the load they carry[56]. The generation of exosomes does not occur randomly, but they are found to carry specific proteins, that derive from different cellular compartments[56] . Exosomes are involved in many immunologic processes, such as viral infections and activation of the immune system[57,58]. Up to this date there have been no reports of studies use SR microscopy to image exosomes, but STED has been suggested as a useful method, as it can be used to determine the size, morphology and protein distribution of vesicles[59].

Nanotubes and cytonemes

Upon cell-cell contact, long membranous structures, called nanotubes can arise. Nanotubes form bridges between cells and can play a role in immunological processes using the membranous structure for cell-cell transport[60]. Viruses, such as HIV-1, have been reported to use nanotubes for cell-cell spread, but can also induce membranous bridges between cells on their own[61,62]. These virus induced membranous bridges are called cytonemes and are much shorter than nanotubes (typically 6µm compared to 25µm)[61]. Viral spread via cytonemes occurs via the outside of the membrane structure[61] . Nanotubes and cytonemes have only been imaged using EM techniques[60], but SR techniques might prove very useful in dissecting the immunological implications of these specific cell-cell contacts.

Microbiology

Viruses, bacteria , and parasites hold close relations with their hosts. Much information has already been obtained using conventional microscopy techniques, but in particular some of the smaller, more dense and/or dynamic structures and mechanisms remain unresolved. SR microscopy techniques provide the possibility to study these structures in more detail and resolve controversies surrounding these subjects. Of all viruses, HIV-1 has been studied intensively using SR microscopy, whereas other viruses are studied less. Research focused on bacteria and parasites sparsely implements SR technologies, especially the micro-organisms in the context of their host remains underexposed.

An interesting study from a mechanistic point of view is the visualization of viral DNA (vDNA) at a single molecule level using gSTED[63]. After incorporation of modified nucleosides, the vDNA can be tagged with specific antibodies using CLICK chemistry. Using this method, the vDNA of HSV-1, vacciniavirus, and adenovirus has been visualized using both confocal and STED microscopy[63]. Using STED, vDNA could be studied in the context of the viral capsule[63].

As mentioned above, HIV-1 and its interactions with the host have been studied in more detail using different SR microscopy techniques (SIM, STORM, PALM, STED) and focusing on different proteins such as cellular restriction factors[64,65], capsid antigen[66], and Env and Gag[62,67].

The distribution of the Influenza HA protein in the host cell membrane has been studied at a nanoscale in live cells using PALM already in 2007[68]. This study was recently followed up, reporting that the HA distribution is correlated with actin density[69].

The bacterial structure outside of both *Bacillus Subtilis* and *Escherichia Coli* has been studied using STED and SIM[70]. Previous studies using TIRF microscopy to visualize MreB, an actin ortholog, were inconclusive or had contradictory outcomes due to the low imaging depth and resolution. As MreB filaments have a length of typically 125nm, SR microscopy is needed to visualize these structures in detail. Combining the results of SIM and STED experiments, MreB filament growth and structure was elucidated and it could be concluded that MreB is not only an important part of the cytoskeleton but is also likely to be involved in cytomotive functions[70]. Another study on *E.Coli* used PALM to visualize the clustering and positioning of the chemotaxis receptors through tracking of three relevant proteins[71]. An example of the study of bacteria in the context of the host has been performed with *Shigella Flexneri.* Upon disruption of the vacuolar membrane by this intracellular pathogen, subresolution-sized membrane remnants are created, which are hypothesized to be involved in signalling cascades. These membrane remnants have been visualized using PALM[72].

The parasite *Toxoplasma Gondii* has been studied using dual colour-STED to visualize the endocytic system-derived secretory organelles (micronemes) which enable the parasites to survive intracellularly in more detail[73]. Two different microneme-associated proteins were visualized and found to colocalize, this was not possible using conventional microscopy, as the density of microneme structures was too high[73].

In conclusion, SR microscopy methods have filled quite some knowledge gaps and resolved some controversies in cellular structure or processes already, despite its relative recent introduction. It is encouraging that in the cases that there were EM data available, these overlapped with the data obtained using SR microscopy[68]. This reinforces the notion that SR microscopy might be the perfect combination between confocal and EM microscopy; uniting high resolution with live samples, higher staining efficiency, and (3D) multi-colour imaging. Unfortunately imaging of dynamic processes has proven to be difficult using SR techniques. However, using pointillism methods large amounts of quantifiable data, such as the exact coordinates of proteins of interest, are obtained that can be used for more statistical approaches in cell biology.

Chapter 3: Conclusion and future perspectives

After evaluating application and potential of SR techniques in immunology research, it can be concluded that more implementation of SR microscopy has the potential to result in more detailed knowledge on immunologic processes. Although SR microscopy has applied for several years, there is still room for improvement, due to the large number of different techniques and the imaging capacities demanded by immunology research. Concerning the experimental procedures, such as choice of fluorophore or fixation and incubation methods, there is a lack of consensus about the best imaging conditions and subsequent analyses, which in turn results in contradictory or conflicting experimental outcomes[44]. It could be a matter of time before consensus is reached regarding these subjects, but initiatives to describe standardized imaging procedures and characterize fluorophores speeds up the process of implementation universal methods[8,30].

The design of proper controls to allow for correction focus drift, chromatic aberration and other imaging artefacts is another topic that needs attention. Standardized control experiments have been developed, but there is room for improvement[74]. Especially pointillism methods require sophisticated software extensive data analysis to compile all fluorescence data into one picture, while correcting for the imaging artefacts described above.

Current improvements on the SR technology are largely focused on the fluorophores, as these determine the resolution in single molecule imaging techniques and are the limiting factor in time resolution. Recent developments are aimed at the design of small peptide tags that can be used to link permeable organic dyes to the molecule of interest. This allows staining of live cells, whereas fixation procedures are needed for antibody-mediated staining[7,8]. Some examples of recent developments in staining using membrane permeable fluorophores are bio-orthogonal CLICK chemistry[74], sortagging[75], TMP[8,14], and SNAP[7,35]. As the tags used in these approaches are typically very small (several peptides), they enable high labelling densities.

A fluorophore-based approach used to enhance the resolution even further, aims to reduce photoblinking, which is intrinsic to the fluorophore, but can be limited using specific buffers[30], thus preventing imaging artefacts caused by multiple observations of the same molecule.

There are also undertakings to design a broad panel of fluorophores without overlapping excitation and emission spectra to permit multi-colour imaging. This in turn enables visualization of protein-protein interactions at a nanometer scale in live cells. Further improvements are aimed at the development of fluorophores with better photoswitching capabilities and higher photon yields per imaging cycle[30], which decreases imaging time and could thus provide opportunities for live cell imaging.

The immunologic processes that could be visualized using live cell imaging occur in seconds to minutes. Some processes will thus be easier to record than others. Loose micro-organisms diffuse rapidly for example, but travel at much slower once tethered and during internalization. It is worth to realize that imaging speeds are limited by the fluorophore yield, and when imaging speed increases, this comes at the cost of lower fluorescence signals. This in turn decreases the signal to noise ratio, up to the point that it becomes too low to enable SR image composition. Improvements on the hardware of SR techniques will increase imaging speed, but this might not be enough to resolve the most dynamic processes. These processes could be studied using combinations of techniques, such as visualizing the process in live cells using conventional fluorescence microscopy, followed by fixation of the cell on the moment the process of interest occurs, enables SR microscopy[43]. Fluorescence correlation spectroscopy (FCS) is a technique that is typically implemented on

fluorescence microscopes and is used to the concentration fluctuations of fluorescent molecules using the spatiotemporal correlation of single molecules[76]. The resolution of this method is limited when applied on conventional microscopes, but application of the technique on SR microscopes could enable visualization small vesicle trafficking[76].

In conclusion, after analyzing all pitfalls and drawbacks, there is still a long way to go for SR microscopy techniques before they will meet all requisites to widely implemented in immunology researhch. Currently, costs and skills that are needed to operate the microscopes are a hurdle, together with the lack of standardized procedures and controls. Over time, some of the techniques that have been introduced and discussed above will fail to become widely implemented, as other SR techniques, such as PALM and STORM have more potential. These SR techniques can become valuable tools to gain a better understanding of the dynamic field of immunology.

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List of abbreviations

APC = antigen presenting cell
BCR = B-cell receptor
BP PALM = Biplane PALM
DC Sign = Pathogen-binding C-type lectins
dSTORM = direct STORM
EM = electron microscopy
EMCCD = electron multiplying charge-coupled device
ER = endoplasmic reticulum
GSDIM = ground state depletion followed by individual molecule return
FCS = fluorescence correlation spectroscopy
F PALM = fluorescence photoactivactivation localization microscopy
FRET = Förster/fluorescence resonance energy transfer
HILO = highly inclined laminated optical sheet
HIV-1 = human immunodeficiency virus-1
iPALM = interferometry PALM
IS = immunological synapse
ITAM = immunereceptor tyrosine-based activation motif
LAT = linker of activated T-cells
MHC = major histocompatibility complex
MTOC = microtubule organization centre
MVB = multi-vesicular body
NA = numerical aperture
NK cells = natural killer cells
NSOM = near field scanning optometry
PAINT= points accumulation for imaging in nanoscale topography
PA-FP = photoactivable fluorescent protein
PALM = photoactivated localization microscopy
PSF = point spread function
RESOLFT = reversible saturable optically linear fluorescence transitions
SIM = structural illumination microscopy
SOFI = superresolution optical fluctuation imaging
sptPALM = single particle tracking PALM
SR = superresolution
SSIM= saturated structural illumination microscopy
STED = stimulated emission depletion
STORM = stochastic optical reconstruction microscopy
Stx = Syntaxin
TCR = T-cell receptor
TIRF = Total internal reflection fluorescence
VSV= vesicular stomatitis virus

Layman's summary

Microscopy provides scientists with a better understanding of cellular processes. Since the earliest forms of microscopy, many innovations have been implemented to enable visualization of the smallest molecules. These innovations increase the resolution, that is, the smallest distance between two dots with which the two dots are still visible as two separate dots instead of one. One of these innovations is fluorescence microscopy, which functions through labelling of the molecule(s) of interest with a light-emitting label, which makes those molecules stand out from the rest of the cellular environment. Due to features inherent to light, the diffraction limit, the resolution is limited to approximately 200nm using conventional fluorescence microscopy. Another innovation increasing the resolution was the use of electrons to visualize cellular structures. As electrons have other features than light, and a lower diffraction limit, they enable a resolution of 1-2nm, but sample preparation is thus demanding, that it is not compatible with live cells.

In the 1980's a theory for a new method enabling high resolution live cell microscopy, using conventional light microscopy, was developed. Due to high resolution information that could be obtained using this technique, it was called superresolution (SR) microscopy. Many different techniques based on this theme have been developed, and these techniques have been applied in neurology and developmental biology, but their application is limited in other fields of science. In this review, the current application and potential of SR microscopy in immunology research is discussed, as many immunological processes remain unresolved and research could potentially benefit from high resolution, live cell microscopy techniques.