A New Approach for a Two - Photon and Super- resolution Microscopy

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ABSTRACT

There are Several technological revolutions underway which apply to much of biology like Structural imaging, Functional imaging, Patterned photo-stimulation and Super-resolution techniques which is can use for recording activity from many parts of single neuron in vivo or many neurons in vivo. 2-Photon Microscopy and Linked Optical Methods for Imaging Neurons can give Imaging deep within non-transparent living tissue such as brain….Two-photon fluorescence microscopy allows three-dimensional imaging of biological specimens in vivo. Compared with confocal microscopy, it offers the advantages of deeper tissue diffusion and less photo damage but has the disadvantage of slightly lower resolution. Two-photon microscopy is expected to have an impact in areas such as physiology, neurobiology, embryology and
tissue engineering, for which imaging of extremely scattering tissue is required. I am trying strive to meet the challenge offered by the chance to ask questions about the workings of as cell that we never thought we could answer, we need to be aware that the new technologies are still evolving. The current limitations of each technique need to be measured when identical them to specific biological requests. In this review, we briefly describe the principles of super-resolution optical microscopy and focus on comparing the features of each technique that are significant for their use in learning nanosensing in the cellular microenvironment.

KEY WORDS: Molecular structures ,Fluorescence , Biophotonics, Photon, cerebral cortex

نهج جديد لمجهر ثنائي الفوتون فائق الوضوح

الملخص :

هناك العديد من الثورات التكنولوجية الجارية التي تطبق على الكثير من الأحياء مثل التصوير الهيكلي، والتصوير الوظيفي، نمط التصوير المحفز والتقنيات فائقة الوضوح التي يمكن استخدامها لتسجيل النشاط في أجزاء كثيرة من الخلايا العصبية المايكروسكوب ثنائي الفوتون وما يتصل به من طرق التصوير الضوئية تعطينا القدرة على التصوير في أعماق الأنسجة
الحية غير الشفافة مثل الدماغ.

المايكروسكوب ثنائي الفوتون الومضي (الفلورسنت) يسمح بالتصوير ثلاثي الابعاد للعينات البيولوجية في الجسم الحي بالمقارنة مع المجهر متحد البؤرة ، فإنه يوفر مزايا القابلية على اختراق الأنسجة بشكل أعمق وأقل ضرر على الصورة ولكن لديه عيب يتمثل في حصول انخفاض طفيف في الوضوح .ومن المتوقع ان يكون لمجهر ثنائي الفوتون مساحة تأثير في مجالات مثل علم وظائف الأعضاء، علم الأعصاب، علم الأجنة و هندسة الأنسجة، والتي تطلب مسح وتصوير عدد كبير من الانسجة المتناثرة . وانا اسعى جاهدا لمحاولة الاجابة على الاسئلة التي تطرح حول طريقة معرفة عمل الخلية والذي لا نزال نحتاج للمزيد لتحديد الاجابة ، حيث اننا بحاجة إلى أن ندرك أن التكنولوجيات الجديدة ما زالت تتطور و تحتاج لأخذ القيود الموجودة لكل تقنية لأخذها في الاعتبار عند

استعمالها للإجابة على أسئلة بيولوجية محددة .في هذا الاستعراض، وصفنا مبادئ عمل المجهر الضوئي فائق الوضوح والتركيز على المقارنة بينه خصائصه مع كل تقنية والتي تعتبر مهمة لاستخدامها في دراسة الاستشعار النانوية في بيئة الخلية الميكروية.

1. Introduction:

For hundreds of years, researchers have employed light microscopes in order to make visual structures that are very small and thus unable to be seen without any visual aid. The desire to look inside the nanoscopic details of living cells has meant that technical innovations have increased, as have strategies for molecular labelling [1]. Of all the methods available, fluorescent microscopy is the one that has had most success due to its capability to visualise intracellular targets which have been marked with spectrally distinct colours (See Jablonski diagram (**Fig.1**)) . Furthermore, it is now possible to do research on protein dynamics by visualising the interior of cells without physical invasion as a result of the arrival of genetically encoded fluorescent proteins (FPs) [2]

In spite of the significant effects of live-cell fluorescent microscopy, there are basic limits to fluorescence microscopy in terms of resolution by light diffracting through through the optic pathways. Scientists including Rayleigh have found that such a limit imposed a lower limit on necessary space between 2 light-source fixed-points in order to achieve resolution. The necessary space mentioned above is usually identified by the formula ‘$d = \lambda/(2n\sin\alpha)$,’ where ‘d’ is the lower space limit in terms of resolvability, ‘λ’ is wavelength , ‘n’ is refraction index for the medium being used in the experiment and α is the angle of aperture in terms of microscopic objective. Diffraction constraint shows ‘point-spread function’ (PSF) in terms of an individual spot gauged at entire width-half-maximum. In the case of most fluorescent probes, we can say that $d = \sim 200$ nm.

As such, any protein of lower than 200 nm’s size would, in fact, seem 200 nm, as well as any protein less than 200 nm apart from another being impossible to separately identify from the other. It is obvious that resolution which is more than diffraction’s maximum level will be required for the purposes of defining structures and functions for dynamic cellu-nanomachines by employing light microscopy. What is more, it is possible that the limits arising from diffraction will actually be a bigger drawback for fluorescence microscopy compared to bright field microscopy. This is due to the fact that individual molecules can be labelled with different colours, but it is not possible to optically separate them using standard microscopy.

However, there has recently been a significant breakthrough for ‘light microscopy’ that removes diffraction’s constraints in terms of resolution-limitation. Because of super-resolution light microscopy’s advent, it has become possible to visualise the finer points of cell and macro-molecule make-up which had been literally invisible in the past. This technique finally possesses resolution levels that are needed for matching the detail-depth available with molecule-taffing approaches – in fact, molecule-specific resolution around 10–20 nm is very common these days. It is now very much possible to have visualising nano-sensing possessing a 60 nm resolution-depth.

2. Super-resolution/photon microscopy:

Any technique that can improve resolution-depth by a mimimum of x2 compared to standard micro-scopy is called ‘super-resolution microscopy.’ The ways in which this is achieved are often seen as either hardware or software-based in terms of their ways of shrinking the PSF (**Point spread function :** which is the convolution of the excitation) (**Fig.2**) and adding more resolution. For the purposes of giving a sound rationale for pairing technologies with particular uses in the field of biology, the discussion here will be limited to a pair of hardware-focused/’ensemble’ approaches as well as a pair of software-focused/individual-molecule approaches but, due to the fact that developments and new

iterations of the technologies are arriving as quickly as the first stage in the evolution of the field, some of the more important improvements to the original techniques will also be considered [4].

The manner by which many super-resolution techniques achieve better resolution is through having control in terms of the time and place that a fluorescent molecule can be seen. This is true for many modern approaches, including: ground state-depleting (GSD) micro-scopy, stimulated emission-depleting (STED) micro-scopy, saturated-structured illuminating micro-scopy (SSIM), photo-active localised micro-scopy (PALM) and stochastic optic-reconstructive microscopy (STORM). One technique that can be used to 'switch' the molecules 'on' and 'off' is makes use of the benefit of non-linear connections with excitations/emissions. As an example, very high excitation-light concentrations are able to be utilised for the rapid transit of fluorophores to a transient dark state where they are able to stay for a variable amount of time prior to slowly either going back to condition-of-origin or permanently bleaching if extra light is used.

In another way, light is usable on a photo-activatible/photo-switchable fluoro-phore for the purposes of turning 'off-states' to 'on-states' or colur-changing. With either of the above ways, data-depth gained can be increased provided that these transitions meet two requirements: firstly, that the transitions can be no more than a subset of a fluoro-phore's temporal or spatial position. Secondly, that any changes of state are capable of being detected while the unwanted signals are capable of exclusion. The basic idea of these types of reversible light-induced transition is called 'reversible saturatable optical fluorescent transition' (RESOLFT) microscopy, and it is a critical factor in most super-resolution techniques.

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2.1 Biophotonics:

The field of biophotonics involves state-of-the-art bio-therapeutic advances which have very recently provided new potential in terms of wide-ranging transferability for appropriate cutting-edge methods emerging from fields like electro-optics, quantum electronics, laser-tech as well as bio-therapeutics in general. Currently, almost non-invasive, cheap and fast bio-photonic methods are under development with the idea of their being replacement options for standard medical techniques used for diagnostics, monitoring and treating a range of illnesses.

2.2 Colours:

Research on dynamic molecular interactions has been made possible by the increasing availability of fluorescent probes. We can now detect multiple species with no need for any major cross-talk between channels (**Fig.3**). In terms of all enhanced resolution approaches, 'SIM' seems most similar to standard fluorescence microscopy in terms of label-choice. There are no specialist photophysics used in modulating the excitation light, but the number of images (up to 15) neede to make the image by employing the patterned illumination can lead to a problem with photobleaching. As well as this, each excitation wavelength needs a uniquely-spaced grid. This is due to the fact that diffraction depends on the wavelength of light and, as such, multicolour SIM (Software Image Map) can be attained in the best way by employing two dyes possessing approximately equal excitation spectra-levels but with a sufficient 'Stokes-shift' for the separation of emissions.

Two-colour STED has been applied utilising, in one case, double-pairings of excitation/depleting laser and, alternatively, utilising fluoro-phores possessing a sufficient 'Stokes-shift' for there to be two differing excitation-lasers, however just a single emission/depleting laser will be required. There is now a more up-to-date design that employs equal pulsed super-continuums with excitation as well as STED beams, which then means there is no need for complex preparations of laser pulses.

The first multicolour implementation of STORM20 employed cyanine dye activator–reporter pairs for the purposes of causing 'photoswitching' behavior. The method for attaining spectrally-distinct images was by using either identical activator dyes (Cy3) together with any from the 3 differing reporter-dyes (Cy7, Cy5, and Cy5.5) or, alternatively, by employing any from the 3 spectrally-distinct activators (Cy2, Alexa Fluor 405 and Cy3) together with identical reporters (Cy5). With the first of these approaches, separate emission-spectra will take the form of multicolour read-outs, whereas in the latter case, separate activating-spectra will be utilised for the purposes of separating constant emissions temporally, which implies less-rapid implementing. With the above method, localisation precision-level for an individual fluoro-phore will be ~25 nm. 20

Multiple-colour super-resolution imaging can be achieved in further ways, which include bringing together a photo-activatible FP (rsFastLime) and antibody-labelled inorganic dyes (Cy5). Utilising just FPs in order to achieve multiple-imaging is proving harder as emission spectra from non-activated states of a PAFP frequently 'overlap' others' activated states. For overcoming this challenge, researchers at first employed non-reversible green-red PAFPs (Eos) for the purposes of changing and imaging a particular molecular species as well as, subsequently, to image green reversibly-switchable PAFPs.

In more recent times, PAGFP and PAmCherry as well as PAGFP and PATagRFP (within living cells) have been used to attain almost simultaneous two-colour imaging on fixed cells. Precision is sometimes significantly affected by photo-stability and photon count of the flourophore because not all probes provide equal super-resolution levels. Additional probe development remains necessary, mostly for green fluorophores where low photon outputs and a lack of ability to identify transfected cells before they are activated have decreased the speed of simultaneous dual colour super-resolution imaging efforts. Despite this, there are new labelling strategies that are making use of SNAP, CLIP-tag protein labelling systems and Halo. These provide the ability to covalently attach nearly any molecule-type to target proteins and they have, in fact, already been employed with STED, PALM and STORM. Super-resolution micro-scopy needs microscopists to look at a probe's photo-chemistry as well as its spectral features, which is where it differs from conventional fluorescence microscopy.

3.Measuring Dynamic Processes With Super-Resolution Technologies:

The reason for the major effect of fluorescence microscopy on the field of biology is because of its capability for visualising dynamic actions within a living system. Temporal resolution is a critical factor in order to reveal the workings of a great number of biological mechanisms. In spite of this fact, as it is usual for standard imaging optics to be used for implementing super-resolution microscopy, it is unavoidable that enhanced spatial resolution when contrasted with standard fluorescence microscopy is achieved only by losing some temporal resolution. Reading out all of the labeled proteins can be time-consuming if employing a software-based method without changing hardware. This is because it is necessary to alter the acquisition rate to make sure the fluorophores are spatially separated by the diffraction limit.

In the same way, if employing a hardware-based super-resolution technique, it is more time-consuming to move above entire fields at the more precise scan step-size that is needed the 'Nyquist-Shannon Criteria' requires. For instance, 35 micro-seconds per image with a 1.8 μm × 2.5 μm field of view at a 2D resolution of 62 nm is generally stated as being the temporal resolution level for STED microscopy. 37 Although this is true, imaging speed becomes much slower if there is a larger field of view. Researchers utilising very bright fluorescently-filled dendritic spines within living cells have observed times of 10 s/image over a 2.5 μm × 10 μm field-of-view for 50 nm resolution [6].

Despite the fact that SIM and SSIM are widefield techniques, the length of time required for collecting images can also be a problem as the excitation pattern is rotated to several different orientations for the

collection of images before the mathematical reconstructing of the super-resolution image. Usually, mechanically rotating the excitation pattern is most time-consuming stage but more current SIM usage employing ferro-electric liquid crystals on a silicon spatial light modulator for the purposes of pattern creation is now seen to be more rapid. Researchers have been able to capture image fields of 32 μm × 32 μm, as well as 8 μm × 8 μm at 3.7–11 Hz with 100 nm resolution.

A further constraint, especially in the cases of STED and SSIM, which can have a major impact on the imaging time required is photobleaching. In the case of software-based methods, it is possible for photobleaching to accelerate time requirements for collecting super-resolution image information. Gathering the highest possible number of photons within the shortest possible time-period is beneficial because localisation-precision is conditional on how many photons there are.

PAFPs provide the opportunity to follow the movement of numerous protein molecules within the same cell and to obtain high-resolution data regarding underlying cellular structures from this 'tracking' information. This is further to the nanometer-level precision for the purposes of tracking single molecules that had been possible in the past. Separately identifying highly-motile molecular species from mostly stationary ones was achieved by employing high-density particle tracking of tdEosFP-Gag and VSVG proteins in this way. [5].

Tracking single molecules and visulaising strucures have both become possible through the use of live-cell super-resolution imaging. Retrograde transport, complex morphological interactions between adhesions and elongation have been shown by'Adhesion scaffold re-modelling.' Direct characterisation of the manner in which paxillin molecules were transported into and away from adhesion scaffolds has also become possible. Such large-image-field films (of 28 μm × 28 μm) have been achieved over consecutive and repeated 25–60 second-periods, and possess a 20nm precise single-molecule localisation level (**Fig4**), although the image possesses spatial resolution of ~60 nm. It is useful to remember at this point that 'localisation precision' is the accuracy employed for identifying the a point's centroid, while 'spatial resolution' can be decided using a feature's size which is resolvable given the number of molecules which were possible to localise within a specific region.

Due to the fact that the laws governing accurate sampling in space are applicable to time as well, the acquisition-rates regarding complete images need to be sufficiently rapid (twice as rapid as the fastest event) for the purposes of capturing the target phenomena and, at the same time, keep sufficiently high level of molecular density for structural resolution purposes.

4.Dendrites' computational functions within the cerebral cortex:

When reacting to stimulation by the neurotransmitter glutamate in brain slices, 20 micrometer long segments of individual dendrites have the ability to create voltage discharges known as 'NMDA spikes' or 'plateaus'. Occurences like these will possess voltaic and glutamate 'ceilings.' It is therefore possible for individual dendrite sections to be the same computationally as decision-making 'units' found in neural network models. Due to the interaction between different sections, it is possible for a single dendrite to possess complicated and highly varied computating powers, conditional on the most up-to-date sequence of activity inside the proximate neuron network.

It is possible that NMDA spike/plateaus might give dendrites the chance to contribute to 'graded persistent firing,' which is a process that scientists believe lies berneath working memory. Further to this, they might give individual dendrites the power of orientation-selection as well as providing a 'de-coding' ability for data which has been encoded in the form of 'spike times' over a series of axons. A major research goal is finding out if dendritic NMDA spike/plateaus are important to the intact brain during normal function. This can be achieved through dendrite-observation in-vivo in order to monitor any bigger calcium-transients connected to NMDA spikes and plateaus.

Moreover, some leading scientists have undertaken investigations into interactions with NMDA spikes and plateaus in numerous dendrite areas at the moment, by employing patterned dual-photonic glutamate-uncaging within brain segments. A localalised dendritic spike/plateau potential and calcium transient can be caused by focal-iontophoresis for brief glutamate-pulses on individual-cortical pyramidal-neuron basaldendrites.

4.1 Data encoding within the cerebral cortex?

The majority of somato-sensory neurons discharge only 1/2 action potentials within a period of short-time sense stimulation like a 'whisker deflection.' Therefore, data regarding the stimulating factor (like position or orientation) could be encoded via relative-action potential-timing over wide ranges of neuron-types [7].

Calcium transients are a means of monitoring action potentials in neurons. Action potentials will most often be connected to calcium transients within the call's body as well as proximal-dendrites possessing fast-onset simultaneous with action potential. The above makes it possible to determine the an action potential up to an accuracy of just several milliseconds, in the case of an imaging techniquewith enough speed being used. It is possible to load calcium-sensing fluorescent dyes into many neurons at once. As an alternative to this, there are now strains of genetically modified mice that express artificial fluorescent proteins that can sense calcium within a neuronal sub-set. Currently, attempts are being made to bring together these methods with fast dual-photonic visualising for the cerebral cortex in-vivo. This is for the purposes of gauging relative-action potential timings over sections tens-hundreds of neurons-wide at times of stimulus to the sense.

5. Conclusion

There is not a constraining barrier anymore to the potential for achievement in light microscopy. This is due to the circumventing of the diffraction limit that was imposed by standard light microscopy. It is possible to visualise molecular structures at the right size, and resolution levels similar to electron tomography have the capability of being achieved with the benefit of very high copy number, molecularly -specific labelling. Despite this, challenges remain for the application of these new technologies. It is still necessary to attempt a balance between fast acquisition and image resolution quality. Precise molecular localization sometimes does not lead to adequate structure resolution. It is possible that living cells can move molecules faster than they can currently be imaged .

Due to the fact that the current colour pallet is constrained, there is a constant requirement for more probes – this may slow down the rate at which super-resolution is adopted in the biological sciences. What is more, placing super-resolution images in context is going to be vital. As such, light or electron microscopy have the ability to give us a useful background for interpreting the new level of ultrastructure we can see. Lastly, there is amazing potential for the biological sciences in super-resolution microscop. Super-resolution can give creative scientists the means to attain new levels of understanding regarding the molecular mechanisms involved in nanosensing.

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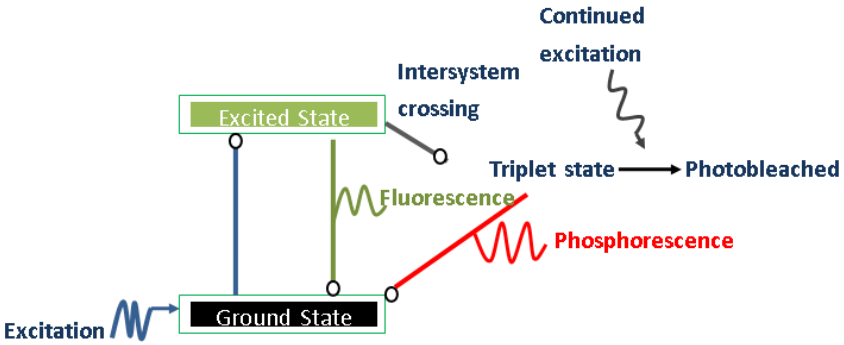

Figure(1): Simplified Jablonski diagram: Source: Harke, 2008, 1309[3]

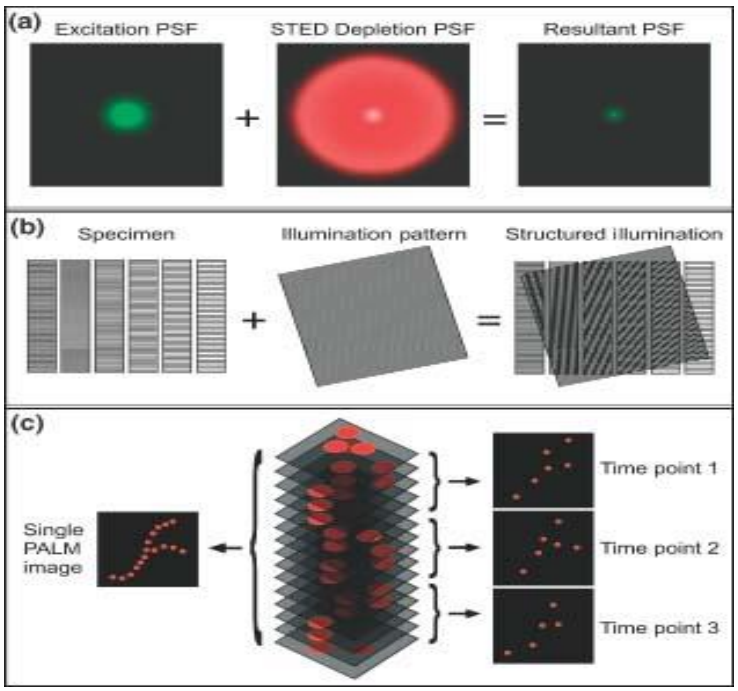

Figure (2): (a) Point spread function (PSF) and STED resolution (b) Specimen +illumination pattern = structured illumination (c) Analysis for Single PLAM image: Source: Betzig, 2006, 1645[2]

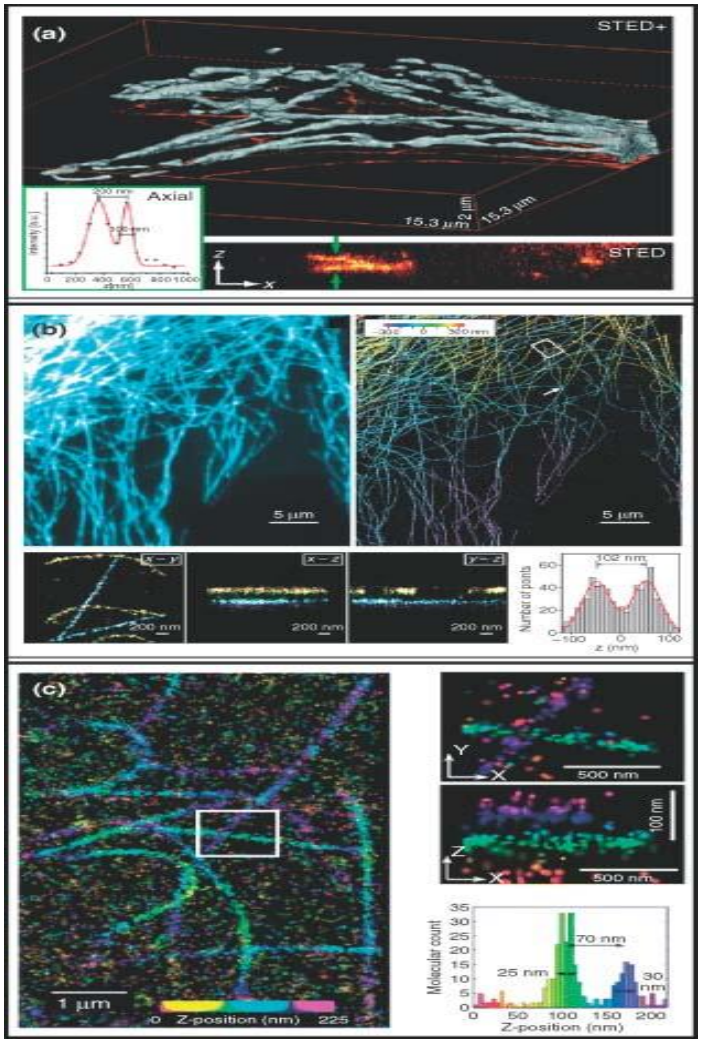

Figure (3): Use colour for detect multiple species: Source: Micheva,2007, 30[5]

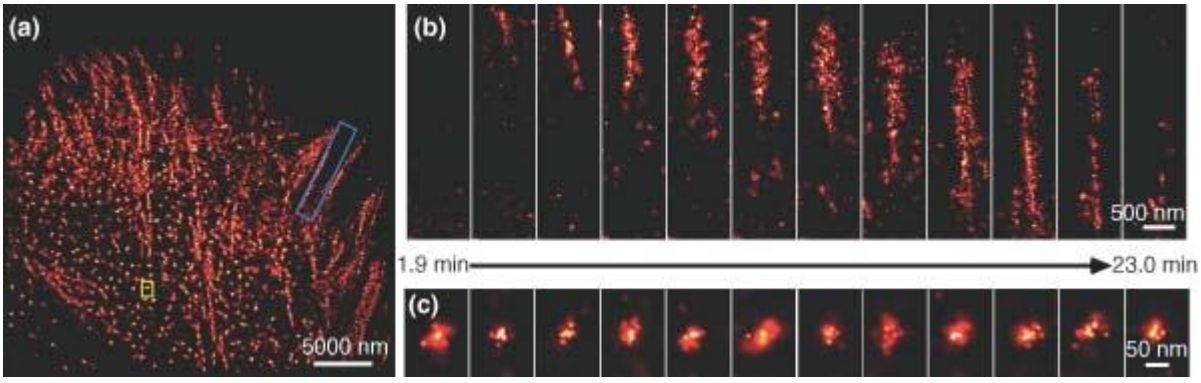

Figure (4): Measuring Dynamic Processes With Super-Resolution Technologies: Source: Shtengel, 2009, 3125[6]