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# **Advanced imaging approaches to reveal molecular mechanisms governing neuroendocrine secretion**

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## **Abstract**

Identification of the molecular mechanisms governing neuroendocrine secretion and resulting intercellular communication is one of the great challenges of cell biology to better understand organism physiology and neurosecretion disruption-related pathologies such as hypertension, neurodegenerative or metabolic diseases. To visualize molecule distribution and dynamics at the nanoscale, many imaging approaches have been developed and are still emerging. In this review, we provide an overview of the pioneering studies using transmission electron microscopy, atomic force microscopy, total internal reflection microscopy and super-resolution microscopy in neuroendocrine cells to visualize molecular mechanisms driving neurosecretion processes, including exocytosis and associated fusion pores, endocytosis and associated recycling vesicles, and protein-protein or protein-lipid interactions. Furthermore, the potential and the challenges of these different advanced imaging approaches for application in the study of neuroendocrine cell biology are discussed, aiming to guide researchers to select the best approach for their specific purpose around the crucial but not yet fully understood neurosecretion process.

## Introduction

Neuroendocrine secretion involves a succession of intracellular compartments, *i.e.* endoplasmic reticulum, Golgi apparatus and secretory granules, structures and molecules [1], whose distribution and dynamics analysis requires efficient resolute imaging techniques. In neuroendocrine cells, secretory granules ensure the release of neurohormones/neuropeptides and neurotransmitters upon stimulation. These vesicular organelles, with a diameter ranging between 60 and 300 nm in their mature state depending on neuroendocrine cell type [2], are generated by budding from the *trans*-Golgi membrane and convey their content to the cell periphery through a microtubule/kinesin-dependent transport at a mean speed of 100 nm/s [3]. Near the plasma membrane, actin filaments trap secretory granules through a myosin-dependent manner to keep a pool of vesicles ready for neuroendocrine cell response to extracellular solicitations [4]. Neuroendocrine cell stimulation provokes an increase in cytosolic calcium concentration and then the fast fusion of secretory granules with the plasma membrane, approximately 130 ms, to release neurohormones more or less rapidly and totally (in a mean time of 1-2 s for a total release) into the blood stream [5]. Regulated exocytosis is a fast, multi-step process controlled by calcium which, for example, induces actin filament depolymerization and the activation of fusion proteins such as soluble N-ethylmaleimide-sensitive factor-attachment protein receptors (SNAREs) [6]. The major part of the molecular actors involved in exocytosis are sorted from the lumen and the membrane of the *trans*-Golgi compartment during secretory granule formation. At the level of this compartment in neuroendocrine cells, a family of proteins called granins, and among them more particularly chromogranin A (CgA), play a pivotal role by interacting with both (*i*) soluble neuropeptides, leading to their aggregation and sorting, and (*ii*) Golgi membrane lipids and proteins, leading to their selection, to finally induce the Golgi membrane budding. All of these events occur at the origin of functional secretory granule formation [7,8].

The distinct steps of the secretory granule journey from biogenesis at the Golgi level to fusion at the plasma membrane need to be tracked with high spatial and temporal resolution to clarify the subtle molecular mechanisms underlying the vesicular/conventional neuroendocrine secretory pathway established in response to physiological needs [7]. The last two decades have seen a tremendous development in high resolution microscopy techniques to visualize and track the molecular mechanisms governing the finely tuned steps of neuroendocrine secretion, e.g.,

fusion pore expansion. In this review, we will compile existing microscopy techniques, including total internal reflection fluorescence microscopy (TIRFM) and others which belong to the last developed super-resolution microscopy (SRM) family, such as structured illumination microscopy (SIM) and stimulated emission depletion (STED), and which complement confirmed techniques such as atomic force microscopy (AFM) and transmission electron microscopy (TEM). AFM is a powerful technique which specifically studies surfaces at the atomic or sub-nanometer scale [9]. TEM is used to spatially map plasma membrane domains through the preparation of plasma membrane sheets [10]. High resolution imaging may also be achieved using SRM. This fluorescence technique was a major breakthrough for biological samples examination since, more recently, it has been used to overcome the optical diffraction barrier [11]. The impact of SRM was acknowledged when the scientists who developed the technique were awarded the Nobel Prize for Chemistry in 2014. The use of both AFM and SRM techniques is increasing in numerous fields including cell biology, plasmonic, catalysis and biomaterials. The goal of all these techniques is to overcome the physical resolution barrier in order to determine precise molecule localization and, possibly, interactions at the level of cell membranes.

This review describes the technical advantages of using AFM, TEM, SRM and TIRFM to study neuroendocrine secretion by highlighting the works that used these imaging approaches in neuroendocrine cells. Its main objective is to enable neuroendocrinologists to choose the most suitable microscopic method to analyze molecular mechanisms and interactions during the secretory process.

## **Atomic Force Microscopy**

AFM is a member of a large family of surface-scanning probe microscopies introduced in 1980s by Binning and Rohrer with their invention of the scanning tunneling microscope [12]. AFM has been applied in a wide range of disciplines, including solid-state physics [13], molecular engineering [14,15], polymer chemistry and physics, surface chemistry, molecular biology and cell biology [16,17], and medicine [18,19]. In biological domains, AFM is a powerful tool for visualizing and manipulating biological structures with nanometer resolution [9,20]. It can be used with light microscopes, especially in the biosciences [21]. An overview of the AFM method is shown in Box 1. For example, AFM has been used on bacterial surfaces, revealing nanoscale net-

like structures [22]. Since the direct “seeing” of single proteins and of eukaryotic wall-free cell surface was a challenge for the first AFM, the ability to provide 3D information of the scanned surface led to its use on isolated protein complexes and assemblies, such as microtubules, to study their structure and mechanical properties [23]. AFM has been used on fixed whole cells (Box 1), for example to distinguish cancer cells and normal cells based on their hardness, to evaluate interactions between a specific cell and its neighboring cells in a competitive culture system [24], or to indent cancer cells in order to study how cells regulate the stiffness or shape of their plasma membrane [25]. AFM can also be used to detect exocytotic fusion pores when neuroendocrine cells are stimulated [26] since, at the resting state, the cell surface is quite flat, with little fluctuations and, after secretion stimulation, the cell surface exhibit numerous depressions (Box 1). These plasma membrane depressions were identified using a primary antibody raised against a secretory granule transmembrane protein, recognized by a secondary antibody coupled to a 30-nm gold particle. Exocytic sites can be detected after cell stimulation, since the gold nanoparticles, observed at the level of plasma membrane depressions, reveal the number, membrane distribution and morphological characteristics of fusion pores obtained using distinct types of secretagogues [26]. In another study, Tsai *et al.* used AFM scanning to analyze endocytosis and vesicle recycling by measuring the size of the invagination pits [20]. It must be noted that the scanning head may not reach the bottom of the invagination because of the limitation of the AFM tip length, leading to underestimation of the depth [25,27]. AFM can also be used on plasma membrane artificial models. For example, a recent study demonstrated the membrane molecular remodeling impact of a granin protein, CgA [28]. Using supported membrane bilayers, this study revealed that addition of the granular protein CgA to giant liposomes enriched with Golgi phosphatidic acid (PA) increased the number, height, and surface of thicker domains in a concentration and time-dependent manner [28]. This modification of membrane topography could result from either CgA aggregation, membrane deformation, microdomain formation, or any combination of these, indicating, in any case, that CgA induces the remodeling of the supported membrane bilayers through its interaction with Golgi PA.

Kelvin probe force microscopy mode (KPFM) is a derivative, non-contact mode of the AFM allowing investigation of the electrical properties of materials, including biomolecules, using a conductive cantilever [29]. When the tip of the AFM is close to the plasma membrane, the surface potential caused by the charged molecules can be revealed at high resolution. In Tsai’s study, the

AFM images revealed that the plasma membrane surface of neuroendocrine PC12 cells is not flat and exhibits a potential that is not homogenously distributed [29].

While AFM is more often applied to study of the plasma membrane surface of fixed cells, its potentiality on living cells has been demonstrated [30,31]. AFM using a PeakForce Tapping mode has been used to analyze structure, dynamics and nanomechanical properties of the plasma membrane from living cells, which enables the visualization of microvilli of epithelial cells [32]. AFM using a nanomechanical measurement protocol enables researchers to follow real-time changes of the plasma membrane during receptor-based endocytosis in neuroendocrine cells [33]. More recently, high speed AFM (HS-AFM) has been developed to provide nanometer structural information and dynamics with subsecond resolution to analyze fast cellular events [34]. This AFM technique relies on the development of ultrashort cantilevers with microsecond time resolution, and fast electronics and piezoelectric scanners which allow 1000-fold faster imaging rates to be reached. As an example, HS-AFM has been used to image living mammalian cell endocytosis without damage [35].

All these studies demonstrate AFM's potential to dissect cell processes and molecular dynamics associated with exocytosis and endocytosis in neuroendocrine cells. AFM is a versatile tool which is in a state of constant evolution and improvement. In combination with other microscopy techniques (e.g., TIRFM), as detailed in the dedicated section below, it may provide further insights into the study of neuroendocrine secretion.

### **Transmission Electron Microscopy**

Although AFM is a powerful resolute technique, only plasma membrane topography is achieved and is reconstituted after filtering during data analysis that might alter the original topographical features. TEM allows the earliest views of the plasma membrane through the use of electron-dense cellular stains and fixatives. In neuroendocrine cells, TEM has, for decades, focused on analysis of secretory vesicle morphology [36]. The fusion model of the full collapse of the secretory granule membrane with the plasma membrane during exocytosis was proposed on the basis of TEM data obtained 40 years ago [37]. This model was discarded very recently with the advent of super-resolution techniques allowing the study of living cells. Indeed, even if high-performance TEM still offers a resolution ~10 times higher (Table 1), current super-resolution

microscopy techniques enabled to show shrinking fusion spots rather than growing fusion spots that subsequently disappear as would be expected from full collapse [38,39].

TEM can also be used to study the molecular structure of membrane sheets prepared from resting or stimulated cells (Box 2) [10,40]. This protocol enables determination of the number of secretory granules docked at or fused with the plasma membrane after cell stimulation. The use of immunogold labelling helps identify docked secretory granules and exocytic sites on the inner face of the plasma membrane [41].

Using correlated light and electron microscopy (CLEM) by combining optical (fluorescence) microscopy and EM can improve the precise localization of molecules in subcellular compartments [42,43]. In an integrated CLEM system, the sample is imaged using an electron beam and a light path simultaneously. The electron microscope provides high-resolution information down to the nano-scale, while the fluorescence microscope highlights the regions of interest. This technique can be used to help find the 3D position of the endocytic protein epsin on clathrin-coated structures at the plasma membrane [44], or to uncover a relationship between the distance of secretory granules to the plasma membrane and the association between SNAREs proteins of respective membranes [45].

## Super-Resolution Microscopies

The advent of SRM allows observation of the plasma membrane deformations during exocytosis and endocytosis processes, observations vital to the study of molecular mechanisms involved in neuroendocrine secretion (Box 3). The resolution obtained by wide-field microscopy is limited by the Abbe's diffraction limit, defined as the minimum distance between two closely localized structures that can be distinguished from each other [46]. The achieved resolution for understanding cellular and subcellular structures is restrained to ~250 nm in the lateral plane and ~500–700 nm in the axial dimension [47,48]. This limit was overcome by fluorescence nanoscopy, also referred to as SRM. In this section, we review the most relevant contributions to the development of SRM for the study of molecular events in neuroendocrine cells. The three main types of SRM used to study neuroendocrine secretion are stimulated emission depletion (STED), single molecule localization microscopy (SMLM) and structured illumination microscopy (SIM). These techniques are briefly described in Box 3.

### *Stimulated Emission Depletion (STED)*

The main advantage of STED is it allows image acquisition at a resolution below the diffraction limit, STED microscopy achieving a resolution of ~40 nm laterally and ~150 nm axially (Box 3). STED microscopy has been used in several recent studies to examine the dynamics and implications of fusion pores in living neuroendocrine chromaffin cells. Shin *et al.* were able to observe the opening, expansion, constriction and closure dynamics of 0-490 nm pores, and then determine the fusion and fission efficiency during exocytosis and endocytosis events, efficiency being defined by the rate of releasing contents and retrieving vesicles [49]. In another study, Abbineni *et al.* analyzed the behavior and implications of fused granule membranes using a GFP-tagged pleckstrin homology domain of PLC δ1 (PH-GFP). The authors observed the diffusion of the PH-GFP/PtdIns(4,5)P<sub>2</sub> complex from the cytosolic leaflet of the plasma membrane to the fused secretory granule membrane and were able to determine that diffusion would occur within tens of milliseconds rather than milliseconds [50]. Other studies using STED demonstrated that the dynamic assembly of several proteins (F-actin, N-WASP and formin) mediates omega-profile merging of the fusion pore by providing sufficient plasma membrane tension to shrink the omega-profile [39,51].

### *Structured Illumination Microscopy (SIM)*

The SIM technique is based on the illumination of the sample with a series of excitation light patterns (Box 3), which cause a lateral resolution approximately twofold higher than that of confocal microscopy [52]. Compared to other SRM methods, SIM has the particularity to image thicker sections for 3D- and live-cell imaging (Table 1). Moreover, subsecond temporal resolution can be obtained without high illumination power, making SIM appropriate for use with living cells [53]. In chromaffin cells, SIM has been used to visualize and analyze the spatial correlation between secretory granules and F-actin [54,55]. SIM has also been used to show that the cellular distribution of secretory granules was not affected by t-SNARE and Munc18-2 overexpression [56]. SIM imaging has allowed the tracking of secretory granule biogenesis, revealing that secretory granules separate from an intermediate Golgi compartment, mature in its proximity for about 1 hour, and then travel to the plasma membrane [57]. At a molecular level, 3D-SIM has revealed that the exocytic machinery composed of vSNAREs and synaptotagmins, is most likely

acquired during secretory granule maturation, and that recycling of secretory granule membrane proteins is achieved in less than 2 hours [58]. In Tawfik et al.'s study, 3D-SIM, through its high lateral resolution and its ability to distinguish single objects in distinct channels, has also revealed that CgA-containing secretory granules are distinct from synaptotagmin spots.

#### *Single Molecule Localization Microscopies (SMLM)*

Photo-activated localization microscopy (PALM) and stochastic optical reconstruction microscopy (STORM) are widefield fluorescence microscopy methods (as opposed to point scanning techniques such as laser scanning confocal microscopy) that enable single molecule imaging with a resolution beyond the diffraction limit (Box. 3). The methods were proposed in 2006 in the wake of a general emergence of optical super-resolution microscopy methods, and were featured as methods of the year for 2008 by the Nature Methods Journal. The high resolution obtained with STORM has revealed three-dimensional ultrastructure of the actin cytoskeleton [59] and nano-sized syntaxin clusters [60] in fixed cells. PALM has been used to study fixed cells but also live cells, its combination with single-particle tracking (sptPALM) allowing a high temporal resolution (Table 1) [61]. Then, this SMLM technique enabled to analyze the distribution and mobility of individual synaptic proteins in membrane nanoclusters, such as syntaxin1A [62–64], voltage gated  $\text{Ca}^{2+}$  channels (VGCCs) [65,66], postsynaptic AMPA receptors [67,68], and actin [69].

#### **Total Internal Reflection Fluorescence Microscopy (TIRFM)**

One of the first fluorescence methods developed to study the real-time dynamics near/of the plasma membrane, was TIRF microscopy, also known as evanescent field microscopy [70–72] (Box 4). Fluorescence excitation is limited to an optical slice near a glass/water interface. Only a small thickness of the ventral part of the fluorescent dye-loaded cell body is illuminated. To create the evanescent excitation, a high numerical aperture ( $> 1.4$ ) objective is used to allow the total reflection of the laser at the interface and to collect the signal [73]. In this configuration, the greater the laser reflection angle, the thinner the evanescent field. The field of excitation light can be 50 nm depth, which illuminates the membrane vicinity but leaves the rest of the cell unstimulated/dark, producing a high-contrast, low background image of the glass-attached plasma membrane [74]. TIRFM is widely used to localize focal adhesion zones and to study local

dynamics of structures at the level of the plasma membrane, such as synaptic vesicles, at millisecond time resolution in living cells [75,76]. Evanescent excitation significantly limits the photodamage of the specimen which explains the potential of TIRF microscopy to track dynamics of single secretory granules fusing with the plasma membrane, endocytic vesicles retrieving cargo, and single-receptor dynamics at the nanometer scale in living cells [70,77]. To improve the image resolution, it must be noted that TIRFM has recently been applied to isolated plasma membranes coming from live neuroendocrine cells encoding a fluorescent granular protein to study endocytosis and exocytosis events [78,79].

Many improved versions of TIRF adapted to modern TIRF setups have been developed, such as variable-angle TIRFM (vaTIRFM) with a nanometric axial resolution, typically 10–20 nm. This setup has been used to measure the axial motion of secretory granules in the basal side of living cells [80]. Another modification of TIRF has been obtained by varying the polarization of the excitation beam to generate a polarized excitation field [50,81]. Recently, this method has been used to monitor the real time shape of exocytic secretory granules during fusion [50] and the curvature of clathrin-coated pits during endocytosis [82] in chromaffin cells. These studies have provided direct insights into how membranes bend during fusion and fission events, showing the dynamic heterogeneity of these plasma membrane/organelle systems in living cells. Abbineni *et al.* have shown that the fusion pore curvature of CgA-containing secretory granules is long lived, indicating that fusion pore expansion can be controlled by a lumen protein during regulated exocytosis in chromaffin cells [83]. TIRFM has been coupled to patch-clamp membrane capacitance measurements and carbon fiber amperometry to demonstrate that, in chromaffin cells, exocytosis could be  $\text{Ca}^{2+}$ -independent and tightly coupled to membrane depolarization to support secretion during action potentials at low basal rates [84]. A new approach coupling TIRFM with a microelectrode array serves as a powerful platform to record and visualize simultaneously the action potentials and vesicular exocytosis in neuroendocrine PC12 cells [85]. The combination of optical and electrical techniques enables mapping of neuron connectivity revealing the relevance of electrical and chemical activities in the neuronal model. Other modifications of TIRFM that have gained prominence over the last decade are the result of its merger with super-resolution techniques. Harris *et al.* combined TIRFM and AFM force clamp to identify the nanomechanical properties of the secretory granule-plasma membrane tether by force clamp measurements [86]. They prepared plasma membrane sheets from PC12 cells to locate and track the movement of

secretory granules attached to the AFM cantilever tip. Their findings showed that the frequency and length of tether unfolding events was consistent with those of exocyst complex helices, indicating the tether extensions play a significant role in the secretory granule-plasma membrane link. A distribution of tether extension steps was observed around ~5 nm. Evanescent field illumination has also been combined with SIM and SMLM in order to reduce out-of-focus signal [87]. One of these methods, called TIRF-SIM, is able to break the diffraction limit by using patterned illumination that leads to the reconstruction of an image with a resolution twice as good as that of a conventional TIRF microscope without compromising imaging speed [52,88]. As a result, objects that have structures below the diffraction limit (e.g. actin filaments, microtubules) can be tracked and studied over time in multiple colors [54,89]. Combination of SMLM with TIRF described the nano-scale locations and dynamic behaviors of single ion channels in relation to single vesicles in living cells [89].

### **How to choose the most suitable imaging approach to study exocytosis and endocytosis in neuroendocrine cells?**

All the nanoscopy techniques mentioned above have different advantages and limitations (Table 1). It is therefore crucial to choose the ideal technique to answer the desired scientific question. The best spatial resolution is acquired with AFM (typically 1 nm, down to 0.1 nm). The major difference between AFM and competing technologies (e.g., light microscopy, TEM) is that AFM does not use lenses or beam irradiation, both of which induce cell damage. Moreover, it is not limited in spatial resolution due to diffraction and aberration, and neither preparing a space for guiding the beam (by creating a vacuum) and staining the sample are necessary. Therefore, this technique has been used to provide a precise measurement of the 3D-morphology of fusion pores after neuroendocrine cell stimulation. Even if AFM is more often used to study fixed cells, its evolution goes towards an application on living cells [30,31].

TEM offers better resolution for visualizing secretory granule morphology than what can be achieved by current super-resolution microscopy techniques. However, electrons passing through or scattering off the sample interact only weakly with carbon-based matter which explains the unavoidable sample treatment with stains and fixatives [36]. For the aforementioned reasons, fluorescence microscopy has two advantages: (1) the requirements for sample preparation and

observation are modest and make fewer changes to the original form, and (2) dynamics events may be observed and studied in living cells. The main limitation of fluorescence techniques is that fluorescent protein overexpression may change organelle behavior, as has been reported for secretory granule fusion during the exocytosis process [83]. Although it is not necessarily true for all advanced, super-resolution, light microscopy realizations, in principle, light microscopy allows observation of structures inside a living cell in real time. Among the types of scanning microscopy techniques including scanning probe microscopy (*i.e.* AFM, scanning tunneling microscopy, STED and electrochemical AFM), STED provides intermediate spatial and temporal resolution and fast data acquisition. STED improvement enables better image quality by removing autofluorescence while preserving the desired signal, and the separation of fluorescent species even when their emissions fully overlap. Moreover, since STED uses visible, infrared or even terahertz light to illuminate the sample, image resolution is not constrained by the diffraction limit. The major limitation of this technique is the restricted choice of dyes resistant to the photobleaching induced by STED illumination mode. This characteristic also induces cell photodamage, which led to the use of STED more often on fixed cells. Recently, this super-resolution technique used lifetime imaging which enables to explore the molecular mechanisms involved in multiple dynamic events simultaneously in living cells (*e.g.*, exocytosis and endocytosis in neuroendocrine cells) [90]. A brief overview and practical comparison of commercial implementations of STED, SIM and SMLM has been published [91]. While the fastest data acquisition is achieved using STED, SIM has fewer requirements regarding sample preparation and gives the best signal-to-noise ratio. Of all commercial implementations, SMLM is likely to provide the best image resolution (typically 20 nm, down to 5 nm in special cases), with a high temporal resolution in the case of sptPALM (Table 1). However, it requires acquisition of thousands raw frames to produce an image and large data processing, which do hamper live cell imaging [92]. SMLM and STED also require the use of special probes (photoswitchable dyes) and experimental conditions. Therefore, SMLM is preferred for static measurements where maximum resolution is needed and when information at the single molecule level is desired. Since a high density of labeling is necessary to achieve optimal resolution and the dye structure can have a significant effect on molecular properties, these aspects can result in perturbation of the structure of interest. Special care must be taken in the experimental design and sample preparation to prevent this from occurring. The validation of SMLM clustering methods remains a challenge. Potential

future directions using multi-modality imaging (e.g., SMLM coupled to electron microscopy) might help validate quantitative SMLM image analysis methods [93]. TIRFM and SIM enable easy and reasonably fast imaging with standard fluorophores. SIM and TIRFM also require less illumination power than SMLM and STED, and, therefore, are suggested for photosensitive samples. The z resolution of TIRF (typically 50 nm) is better than SIM (typically 300 nm) and worse than that of SMLM and STED, but the confinement of excitation light to a thin, near-interface layer in TIRFM configuration results in background reduction and contrast enhancement without being invasive and causing damage.

Because of these characteristics, SIM, TIRFM and STED methods of cell imaging are commonly used to study various aspects of the neurosecretion process in living neuroendocrine cells, (e.g., secretory granule biogenesis, exocytosis and retrieving, including fusion pore and recycling vesicle dynamics, structure and single molecule dynamics) at the nanometer scale. Optimized versions of pioneer resolute techniques have been developed and are currently used to finely visualize and track single molecules, such as CLEM and HS-AFM. It is interesting to note that, to study the molecular interaction between secretory granule and plasma membranes during exocytosis in living cells, several fluorescence microscopy techniques (TIRFM, SIM, SMLM) can be implemented with some specialized fluorescence characterization techniques, e.g. Förster resonance energy transfer (FRET), Fluorescence Lifetime Imaging (FLIM), and with Fluorescence Correlation Spectroscopy (FCS) module. Among these techniques, the FLIM-FRET combination can discriminate nanoclusters of synaptic proteins exhibiting variable degrees of interactions with labeled binding partners [94,95], and FCS allows the precise quantification of interaction-induced modifications of diffusion characteristics of membrane molecules at the nanoscale, occurring within short period of times [96].

## Future

High resolution microscopy, such as SMLM, STED, TIRFM and SIM, each have distinctive and valuable techniques for the study of neuroendocrine cell structure and dynamics, ideally complementing TEM and AFM. High-resolution microscopy alone is not sufficient to adequately study neuroendocrine secretion therefore studies still must rely on TEM. CLEM, which combines super-resolution microscopy and EM, has been developed to solve this problem. The purpose of these nanoscale techniques is to visualize the precise localization of single proteins

within the machinery driving exocytosis and endocytosis processes thereby highlighting the molecular mechanism at the origin of secretory granule fusion and retrieving. Current studies are attempting to obtain 3D resolution in multi-color towards the nanometer scale and high-speed imaging to determine the molecular mechanisms governing neuroendocrine secretion at the cellular level. Recently, another point-scanning fluorescent method with a high 3D imaging performance, called minimal emission fluxes (MINFLUX) nanoscopy, has been developed to break the diffraction limit while imaging dynamic single events in living cells [97]. This technique requires fewer detected photons than standard camera-based localization and allows the use of a larger range of fluorophores. Consequently, MINFLUX enables nanometer scale tracking of molecules with temporal resolution less than one millisecond [98]. While these technological developments have made significant contributions, it is essential to develop reference standards for quality control, counting of protein copy numbers in complexes, and to develop software to extract specific and quantitative information from SRM data for biological interpretation. Furthermore, fluorescent techniques are often applied in studying over-expressed fusion proteins that can help obtain the labelling required for high resolution, but can result in aberrant protein expressions with functional consequences [99]. To overcome the biological disruption, recent studies reported advances in the field using the CRISPR/Cas9 approach towards studying endogenously tagged proteins for single molecule imaging techniques [100,101]. The challenges of all these advents will benefit to the neuroendocrinologist community in the sense that they aim to provide the more precise visualization, tracking and quantification of molecule association *in situ* in living neuroendocrine cells, with the least damages and/or modifications, facilitating our understanding of molecular interactions that drive neurosecretion in any physiological context.

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## **Conflict of interest**

The authors declare that no competing interests exist.

## **Author contributions**

L. Riachy wrote the manuscript with significant inputs from T. Ferrand (TIRFM section), S. Chasserot-Golaz (TEM section), L. Galas (super-resolution section) and S. Alexandre (AFM section); M. Montero-Hadjadje coordinated the writing of the paper.

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### Box titles and inserted comments (there are no figure legends)

#### Box. 1. AFM and fusion pores

(a) The sharp tip fixed to the free end of the small spring-like cantilever is used to feel the sample surface. Optionally, a piezoelectric element (typically made of a ceramic material) oscillates the cantilever. The detector records the deflection and motion of the cantilever. The sample is mounted on the sample stage. A piezoelectric xyz scanner permits to displace the sample stage and the sample in x, y, and z directions with respect to the tip apex. Eventually, the z scanner may be used to oscillate the sample below tip. Although this panel shows the drive attached to the sample, the drive can also be attached to the tip, or independent drives can be attached to both, since it is the relative displacement of the sample and tip that needs to be controlled. Controllers and plotter are not shown here. The AFM signals, such as sample height, cantilever deflection or phasing of the signal, are recorded on a computer during the x-y scan. Eventually, during imaging, these informations are computed to obtain additional information on the physico-chemical properties of the sample. These informations are plotted in a pseudocolor image (b), in which each pixel represents an x-y position on the sample, and the color represents the recorded/computed signal. AFM has three major abilities: imaging (topography, nanomechanics,...), force measurement, and

manipulation. (c) Typical 3D representation of the cell surface topography. A portion of the cell surface contained in the delimited zone in (b) was scanned using AFM and revealed the presence of depressions with a dark brown color on the plasma membrane of a chromaffin cell under acetylcholine stimulation. Some of these membrane deformations correspond to regulated exocytosis-related fusion pores with a width ranging between 90 and 130 nm.

#### **Box. 2. TEM and secretory granule fusion**

TEM operates on the same basic principles as the light microscope but uses electrons instead of photons to pass through the specimen. Because the wavelength of electrons is much smaller than that of light, the optimal resolution attainable for TEM images is many orders of magnitude better than that from a light microscope. Thus, TEM reveals the finest details of cell internal structure - in some cases as small as individual atoms. TEM is frequently used to study organelle morphology. (a) Preparation of plasma membrane sheets for TEM imaging. (b) Images of plasma membrane sheets obtained using TEM. Few secretory granules docked at the plasma membrane are observed on plasma membrane sheets of resting cell, while exocytic sites are observed on plasma membrane sheets of stimulated cells after secretory granule fusion (white arrow heads). Exocytic sites are identified using an immunogold protocol. Scale bar 100 nm.

#### **Box. 3. An overview of Super Resolution Microscopy techniques**

*Stimulated Emission Depletion (STED)* microscopy technique relies on the use of a specific illumination method and photo-physics to generate directly highly resolved images. Super-resolution images are achieved by reducing the size of the effective point spread function (PSF). This reduction is obtained by illuminating the sample with two aligned beams: a confocal excitation beam to excite fluorescence and a donut shaped beam. The resulting spot in the middle of the donut, from which the normal emission occurs, can be as small as 40 to 80 nm large. Basically, only the fluorophores close to the center of the donut will emit, resulting in a reduced PSF and therefore improved resolution. The generation of an image is similar to classical confocal microscopy but due to the very small voxel size, the scanning speed is relatively slow. Typically, STED can reach a lateral resolution of 150–300 nm with a temporal resolution of seconds, comparable to that of a confocal microscope. *Single Molecule Localization Microscopy (SMLM)* encompasses several related techniques (such as STORM, PALM) that obtain many thousands of

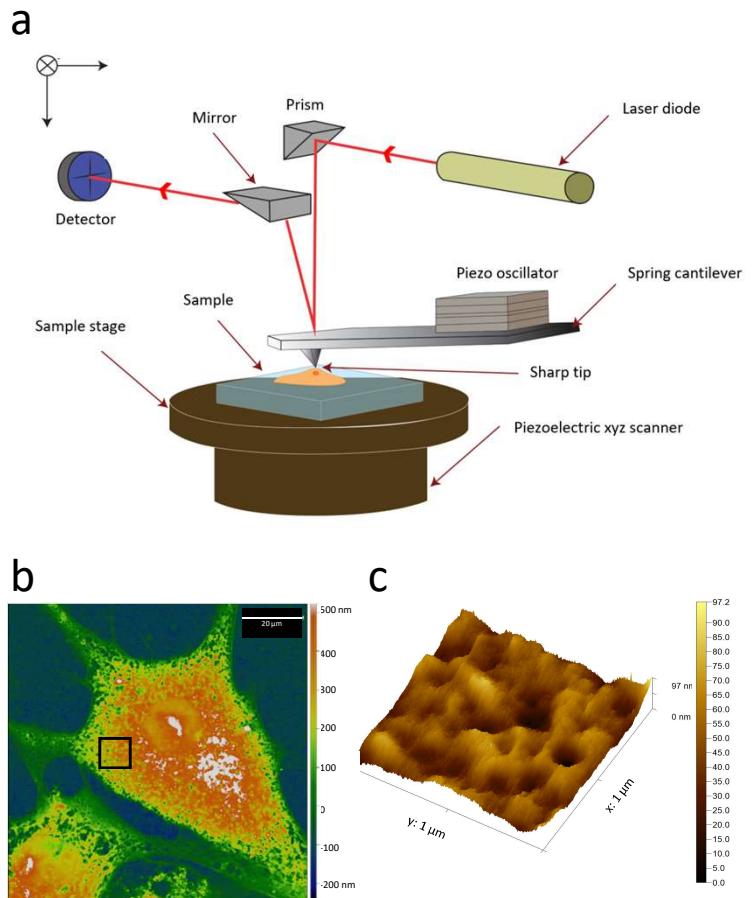
images of a given sample in which only a very small number of fluorophores are fluorescing at a given time. Precise localization of the geometric centers of individual fluorophores within the thousands of collected images can then be used to reconstruct a super-resolutive image. *Structured Illumination Microscopy (SIM)* technique relies on the illumination of a sample using high spatial frequency patterns. The excitation light has parallel stripe lines. Then, the combination of the patterned illumination and the sample generates large, detectable interference patterns. The stripe pattern of light, which is applied to the cell, is shifted and rotated, so that the entire cell is illuminated within several images. These super-resolution images contain sub-diffraction limited structural information, and can be mathematically deconvolved from the interference signal.

#### **Box 4. TIRFM and neuropeptide release kinetics**

(a) TIRFM is an optical technique that provides the excitation of fluorophores in a thin axial region. The method is based on the principle that the optical sectioning of TIRF relies on the excitation confinement of the evanescent wave generated at the glass/cell interface. Since the intensity of the evanescent wave exponentially decays with distance from the surface of the coverslip, only fluorescent molecules within a few hundred nanometers of the surface are efficiently excited. In living neuroendocrine cells expressing CgA-EGFP, TIRFM was used to track CgA-containing secretory vesicles near to the plasma membrane upon time after secretion stimulation. (b)The images resulting from a single secretory granule tracking display a fluorescence intensity variation that is plotted and revealed a fast and high increase of fluorescence (arrow) due to the CgA-EGFP release consecutive to secretory granule fusion during exocytosis.

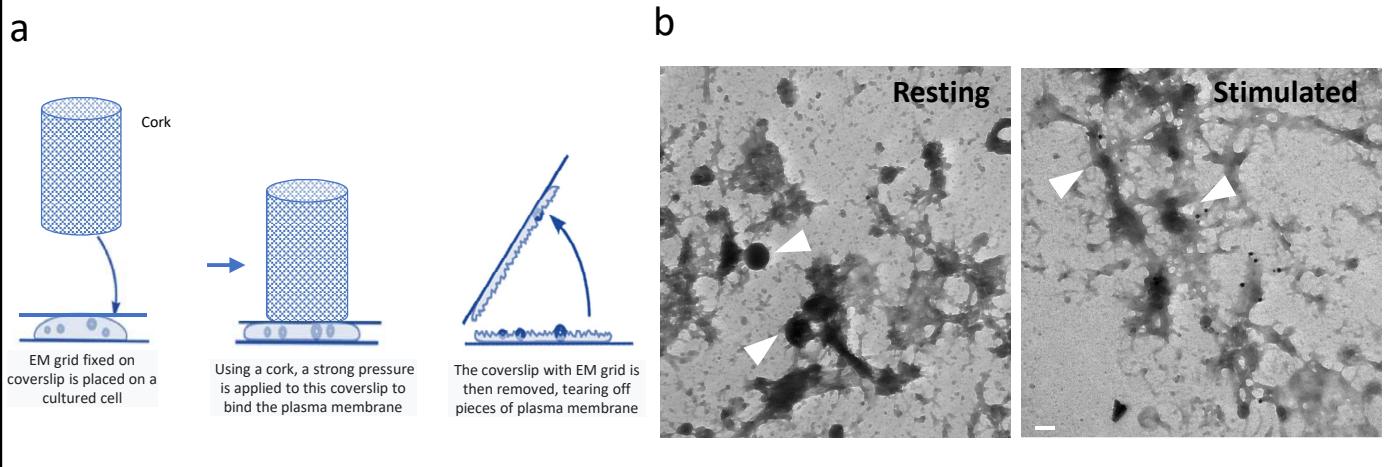
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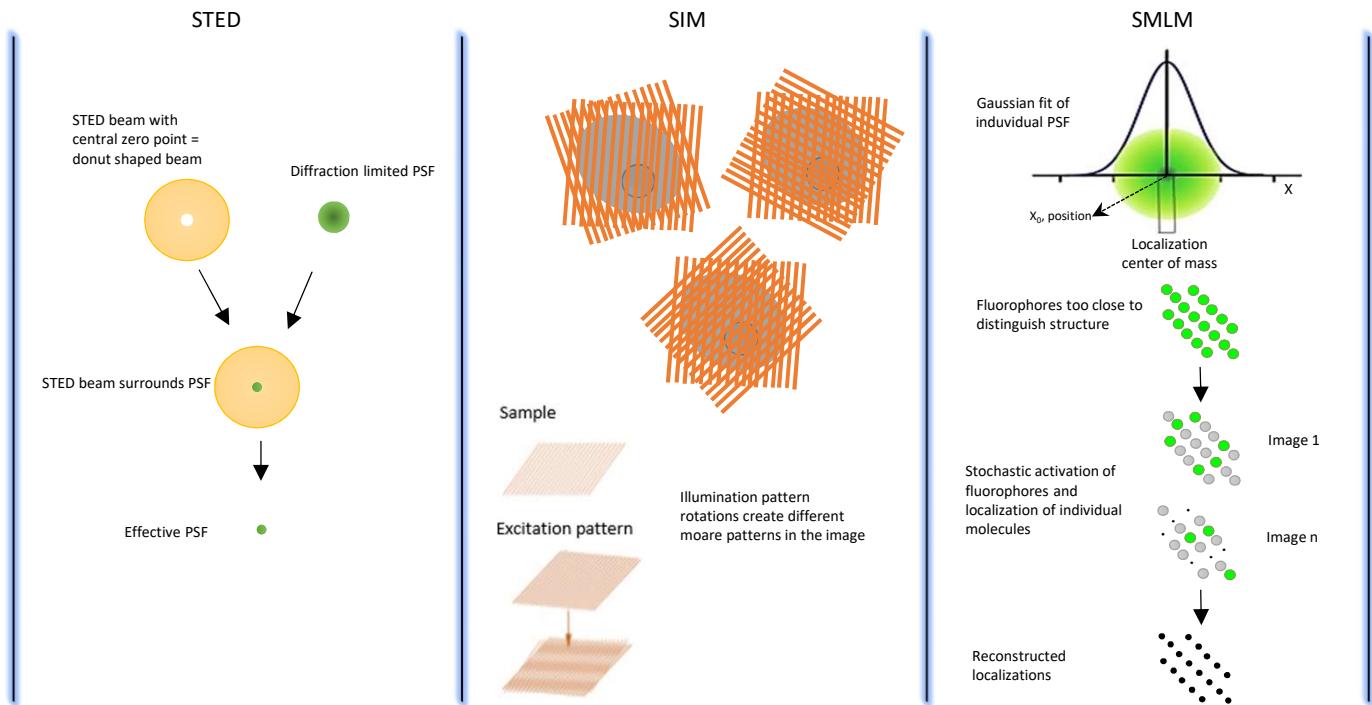
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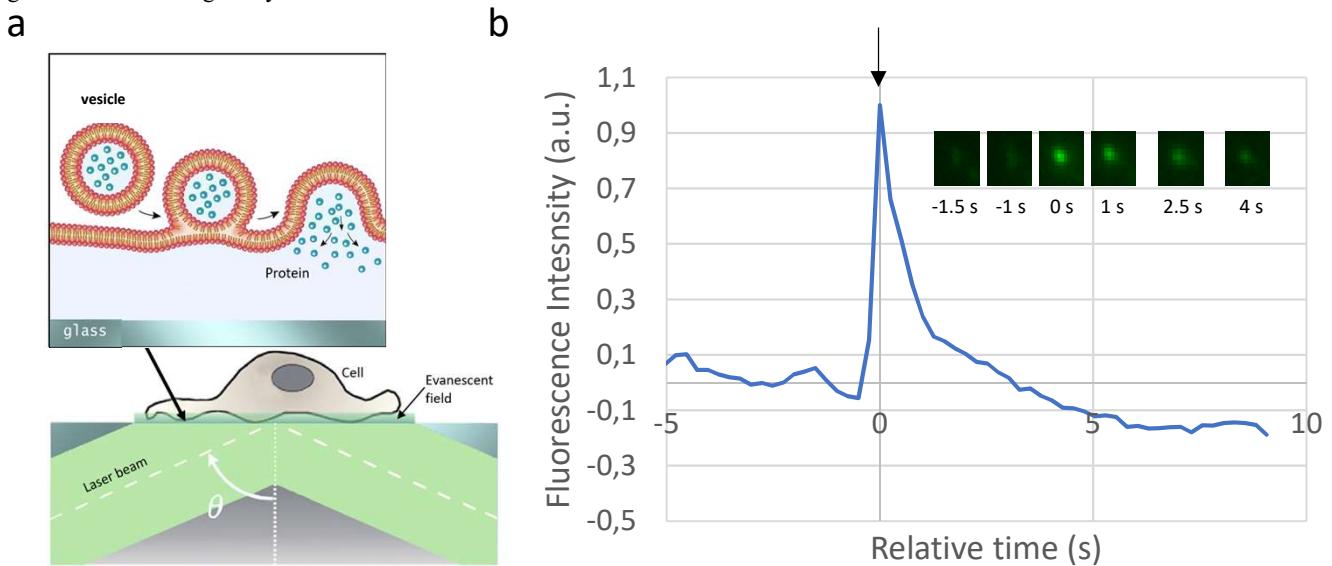


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**Table 1. Summary of the advantages/limitations and applications of imaging approaches used to study neuroendocrine secretion**

	<b>AFM</b>	<b>TEM</b>	<b>STED</b>	<b>SIM</b>	<b>SMLM</b>	<b>TIRFM</b>
<b>XY resolution</b>	0.1 - 1 nm 10 nm (HS-AFM)	0.1-5 nm	40-80 nm	120 nm	20-50 nm	250 nm
<b>Z resolution</b>	0.1 nm	1 nm	150-300 nm	300 nm	10-25 nm	50 nm
<b>Temporal resolution</b>	4 ms - 8 ms 5 µs – 25 µs (HS-AFM)	N/A	5 ms - 2s	10 ms – 500 ms	1 min – 1 h 400 µs – 40 ms (with sptPALM)	10 ms – 1 s
<b>Source</b>	Laser	Electron Beam	Laser	Laser	Laser	Laser
<b>Application</b>	Fixed and living cells	Fixed cells	Fixed and living cells	Fixed and living cells	Fixed cells (STORM) Living cells (PALM)	Fixed and living cells
<b>Duration of acquisition</b>	Minutes Seconds (HS-AFM)	N/A	Ten of milliseconds to minutes	Milliseconds to minutes	Seconds to minutes	Ten of milliseconds to minutes
<b>Data processing requirement</b>	Optional	No	No	Yes	Yes	No
<b>Fluorophore choice</b>	N/A	N/A	Restricted (with appropriate STED characteristics)	Broad	Restricted (preferentially photoswitchable / photoactivatable)	Restricted (to fluorophores resistant to photobleaching)
<b>Advantages</b>	Imaging under native conditions  High signal-to-noise ratio  Can be combined with fluorescence microscopy (TIRFM)	Access to atomic structure of proteins  Molecular resolution of intracellular structures  Can be combined with fluorescent microscopy techniques (CLEM)	Access to cellular structures in 3D  Direct super-resolved images  High spatiotemporal resolution  New implementations reducing irradiation to improve live cell imaging  Can be combined with FCS	Standard sample preparation  High acquisition speed  Improved signal-to-noise ratio  Easy to use  Thick sample imaging for 3-D and live cell study  Can be combined with FCS	Very high 2D-, high 3D- resolution  Single molecule detection  Can be combined with other fluorescence microscopy techniques (TIRFM)  Advanced mode with FRET	Depth of the optical section <0.1 mm, illumination restricted to a thin section  Can be easily coupled to existing optical microscopes, SIM and AFM  Advanced mode with FRET  Limited sample photodamaging
<b>Limitations</b>	Restricted to surfaces  Slow imaging (if AFM is not high speed optimized)  Artefacts may occur due to tip contamination  Imaging in liquid limits the observation area  Interaction force may damage the sample	No live processes  Dehydrated samples  Sample artefacts due to sample preparation, beam damage  Prone to cell photodamaging	Imaging restricted to STED consistent fluorophores  Limited multicolor choice	Limited improvement of the resolution	Not for thick samples (<10µm)  Slow imaging (if without SPT)  Need of special buffers/probes  Prone to reconstruction artefacts  Difficulty of sample preparation	Limited improvement of the resolution
<b>Applications in neuroendocrinology</b>	Detection and analysis of exocytotic fusion pores [26], endocytosis and vesicle recycling [20]  Study of endocytic activity (HS-AFM) [35]  Study of molecular interaction on supported membranes [28]  Tracking of the real-time changes of the plasma membrane during receptor-based endocytosis [33]	Observation of secretory vesicle morphology [36,37] and shrinking fusion spots [39]  Identification of docked secretory granules and exocytic sites on membrane sheets [40,41]  Elucidation of the 3-D position of exocytic proteins [45]	Analysis of fusion pore dynamics [49]  Observation of expanding fusion pores [50]  Study the role of the dynamic assembly of filamentous actin [39,51]	Visualization and analysis of the spatial correlation between secretory granules [54,55]  Analysis of the distribution of secretory granules [56]  Tracking of secretory granule biogenesis [57]	Analysis of the distribution and mobility of individual synaptic proteins [62], voltage gated Ca <sup>2+</sup> channels [63,65,66], postsynaptic AMPA receptors [67,68]  Characterization of the size and distribution of actin and syntaxin clusters [60,64,69]	Study of the distribution and dynamics of synaptic vesicles during exocytosis [75,76]  Tracking of endocytic vesicles and single-receptor dynamics at the nanometer scale [70,78]  Study of endocytosis and exocytosis on isolated plasma membranes [77,79]
				Analysis of the molecular composition of the exocytotic machinery [58]	3D ultrastructure of the actin cytoskeleton [59]	Monitoring real time shape of secretory granules during fusion [50] and curvature of clathrin-coated pits during endocytosis [82]
						Simultaneous recording and visualization of action potentials and exocytosis [85]