

Brief Review

## Optical detection of single molecules in living cells

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**Abstract:** Single molecule detection is a technology of studying biomolecules with high spatial and temporal resolution. By exploiting recent technical advances, we are able to observe, detect, even manipulate individual molecules and study their conformational changes and dynamic behaviors. New information can be obtained from the single molecule research, which is otherwise hidden or averaged out. In recent years, the development of single molecule detection techniques has opened up a new era of life science. In this review, we introduce the advances of the techniques for detecting single molecules in cell biology and review the development of single molecule detection in living cells.

**Key words:** single molecules; optical detection; living cell

## 光学显微镜技术在活细胞单分子检测中的应用

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**摘 要:** 单分子检测是一门以高度的时间以及空间分辨率研究生物单分子的技术。近来, 科学技术的探索发展使我们可以观察、检测甚至操纵单个分子并且研究它们的构象变化和动力学行为。这一发展使得以前被传统系统研究体系平均化所隐藏的新信息被揭示出来。单分子检测技术的发展已经揭开了生命科学研究的新篇章。在本文中, 我们将介绍有关活细胞中单分子检测技术的发展以及活细胞内单分子检测的现状。

**关键词:** 单分子; 光学检测; 活细胞

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In the past, a variety of biochemical methods have been widely used in biological research. Detections *in vitro* were averages of many events occurring at the same time. The information obtained revealed the integrated events of a large population of molecules, but never answered the individual behavior of single molecules. Biomolecules as small as nanometer exist in the complex and well-organized micro-environment of cells. They work very efficiently and accurately in the cells. However, their individual behaviors in the test tube and in the cell are not identical. It is very important to study the molecular behavior under physi-

ological conditions. Recent progress in single-molecule detection techniques has allowed us to visualize the individual biological molecules inside the living cells. Single molecule visualization in living cells has been proven useful for quantitative analysis of the dynamics. Single molecule detection is a technology to study biomolecules with a high spatial and temporal resolution, such as the studies on DNA transcription<sup>[1]</sup>, enzyme reactions<sup>[2]</sup>, molecular motors<sup>[3]</sup>, protein dynamics<sup>[4]</sup>, and cell signaling<sup>[5]</sup>. In this review, we will discuss the development of single molecule detection in the living cells.

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### Single molecule detection optical techniques in living cells

Recently, single molecule detection techniques have made rapid progress. These techniques have allowed us to record the behavior of individual molecules in real time. For a long time, biologists have dreamed of seeing the individual molecular event within the living cell. Now it became true. In the next we will review the experimental techniques that have the potential to detect single molecules in living cells. Applied optical spectroscopy, the techniques providing optical and spectroscopic information, enhancing the resolution along the optical axis has been obtained by reducing the excitation and detection volume within the sample using scanning techniques like confocal microscopy<sup>[6]</sup>. They are a set of techniques that provide optical and spectroscopic detail at very high resolution. In recent years, the visualization and localization of single molecules by optical techniques has had remarkable progress<sup>[7-9]</sup>. These techniques provide spectroscopic data in the single molecule level. The major prerequisite for any optical detection of single fluorophores is the reduction of background signals, which mainly arise from auto fluorescence. New fluorescent probes<sup>[10]</sup>, confocal microscopy<sup>[11]</sup>, total internal reflection fluorescence microscopy<sup>[12]</sup>, and spectral imaging<sup>[13]</sup> are the powerful new technique for live cell imaging. They will provide an insight into the future possibilities of the imaging technology.

#### Fluorescent probes

Fluorescent probes for single-molecule detection in living cells must have a high quantum yield of fluorescence emission, and emit a large number of photons before photo-bleaching. In living cells, fluorescent probes with longer light wavelengths for both excitation and emission are also advantageous to separate the fluorescence signal from cellular auto fluorescence.

#### Fluorescent protein

Green fluorescent protein (GFP) and its derivatives have proven useful in bioimaging studies of living cells. GFP has been identified in the jellyfish *Aequorea victoria*; it is a 27 kDa protein that forms a barrel-like structure and in its center a fluorophore is formed by three amino acids<sup>[14]</sup>. Generally speaking, GFP is a monomeric protein and therefore it is very suitable for investigation of protein-protein interactions and subcellular distribution<sup>[15]</sup>. It has an emission around 488 nm. So its image can be interfered by auto fluorescence of living cells when detecting single molecules. And also its quantum yield of fluorescence is a weakness for single molecule detection. DsRed has been cloned from

a coral of the genus *Discosoma*<sup>[16]</sup>. It has very attractive properties since it has an emission maximum around 600 nm by which it can be imaged without interference of auto fluorescence and it has a rather high quantum yield of fluorescence. However, the currently available DsRed proteins still have drawbacks since they mature slowly and form oligomers (tetramers)<sup>[17]</sup>. Although GFP is a rather small protein, it still represents a large group in fluorescent chimeras and in some cases it affects the activity of its host protein.

#### Quantum dots (QDs)

The use of organic fluorophores for live-cell applications is subject to certain limitations. Luminescent quantum dots — semiconductor nanocrystals — are a promising alternative to organic dyes for fluorescence-based applications. QDs, such as CdSe-ZnS core-shell nanoparticles, are inorganic fluorophores that potentially circumvent those limitations and are thus a promising alternative to organic dyes. When organic fluorophores are restricted by their narrow excitation spectra, QDs can be excited by any wavelength from UV to red. QDs have narrow emission spectra. Moreover, in contrast to organic fluorophores, QDs are highly resistant to chemical and metabolic degradation and have a higher photo-bleaching threshold.

#### Other fluorescent probes

Furthermore, the dye such as Cy3, Cy5, tetramethylrhodamine and Texas Red can be labeled to the detected target to be the fluorescent probes for single-molecule imaging in living cells. And a promising tag was designed by the group of Tsien<sup>[18]</sup>. They designed a receptor domain of only 6 amino acids. It contains 4 cysteine residues. When introduced in a helix the spatial separation used allows the 4 thiol groups to form a receptor side on one side of the helix. With trivalent arsenic compounds it can be excited fluorescence. It was reported that the labeling of recombinant protein molecules with trivalent arsenic compounds has been used successfully in animal systems.

#### Fluorescence resonance energy transfer

Fluorescence resonance energy transfer (FRET) is a physical process by which energy is transferred from an excited molecular fluorophore (the donor) to another fluorophore (the acceptor). The current advances in fluorescence microscopy and new fluorescent probes make FRET to be a powerful technique for studying molecular interactions inside living cells. If FRET occurs, the donor channel signal will be quenched and the acceptor channel signal will be sensitized or increased. FRET imaging methods have been used in detecting the functional organization

and tracing the movement of proteins inside living cells<sup>[19]</sup>. A single-molecule FRET method has been developed to observe the activation of the small G protein Ras at the level of individual molecules<sup>[20]</sup>. FRET between different chromophores (hetero-FRET) has opened the way to studying protein interactions<sup>[21,22]</sup> and biochemical reactions<sup>[23-25]</sup> in living cells. FRET can also occur between like chromophores (homo-FRET). This process, which can only be monitored by fluorescence anisotropy. It was used recently to study protein structure<sup>[26]</sup>, oligomerization<sup>[27,28]</sup> and the organization of membrane proteins at the cell surface<sup>[29]</sup>.

#### *Single molecule detection optical techniques*

As a single molecule detection technique should be considered four things: high-efficiency collection optics, speed of acquisition, careful elimination of background fluorescence by various means, and the viability of the specimen.

Wide-field detection collect light emitted from the entire depth of the specimen, and it made a highly flexible system for live cell imaging with fast acquisition and flexible excitation at low cost. Usually charged coupled device (CCD) allows fast acquisition of the whole field simultaneously. So wide-field microscopy is the simplest and most widely used technique. For example, the measurement of  $\text{Ca}^{2+}$  signals in the cytosol and organelles<sup>[30]</sup> and imaging of dynamic protein tyrosine kinase activities in living cells<sup>[31]</sup>. Scanning confocal systems are now a commonly used tool for live cell imaging. But its scanning speeds limit acquisition rates.

The total internal reflection fluorescence microscopy (TIR-FM) is a technique used to observe the interface between two media with different refractive indexes such as glass and water<sup>[32]</sup>. Using the evanescent field for excitation, the excitation depth of a fluorescence microscope can be limited to a very narrow range. This is one of the most effective ways to reduce background of fluorescence microscopy to achieve single-molecule imaging. TIR-FM is one of the techniques widely used for single molecule detection *in vivo*<sup>[33-35]</sup>. Yasushi Sako *et al.*<sup>[5]</sup>, have studied the process of early signal transduction by using the total internal reflection fluorescence microscopy.

And the fluorescence correlation spectroscopy (FCS) is a particular example of fluctuation correlation techniques. The volume element is defined by the focus of the laser beam, approximately  $1 \times 10^{-15}$  liters. Smaller molecules will move more quickly through the confocal volume than larger molecules. The fluorescence is measured from the volume. The number density of the fluorophores in solution can be

calculated from the magnitude of the autocorrelation. One important application of FCS in the biochemical sciences is the determination of thermodynamic and kinetic parameters. It has been shown that FCS measurements in living cells are feasible<sup>[36,37]</sup>. The most recent FCS work has centered on the determination of chemical rate constants and even probing inside the cell nucleus.

In the end, the scanning near-field optical microscopy (SNOM in Europe and NSOM in North America) is high-resolution optical microscopy realized by scanning a small spot of "light" over the specimen and detecting the reflected. Single molecules have often been observed using SNOM. It included the imaging of biological samples<sup>[38]</sup> and the detection of single dye molecules<sup>[39,40]</sup>.

Progress in technology has enabled us to visualize and manipulate single molecules in living cells. These techniques have allowed us to record the behavior of individual molecules in real time.

#### **Real-time single-molecule imaging of the infection pathway of virus**

The viral infection process is a very interesting interaction in nature. Georg Seisenberger *et al.*<sup>[41]</sup> described a method, based on single-molecule imaging system, that allows the real-time visualization of the infection pathway of single adeno-associated viruses (AAV) in living cells (Fig.1).

For the study, AAV was covalently labeled with Cy5 dye. They analyzed 1 009 trajectories of single AAV-Cy5 particles in 74 cells at different stages of the infection. Trajectories of single AAV-Cy5 particles indicated infectious entry pathways of AAVs into a living HeLa cell. To demonstrate how cells constituents were determined, trajectories are projected onto a phase contrast image of the investigated cell cross-section, taken with a commercial CCD (Coolpix, Nikon). The traces showing single diffusing virus particles were recorded at different times. They describe various stages of AAV infection, e.g. diffusion in solution, touching at the cell membrane, penetration of the cell membrane, diffusion in the cytoplasm, penetration of the nuclear envelope, and diffusion in the nucleoplasm. Diffusion trajectories with high spatial and time resolution show various modes of motion. The real-time visualization of the single AAVs infection shows a much faster infection than was generally observed so far. Their single virus tracing measurements have allowed, for the first time, a detailed observation and quantitative description of the infectious entry pathway of single virus particles into living cells.

Melike Lakadamyali *et al.*<sup>[42]</sup> have studied the transport,

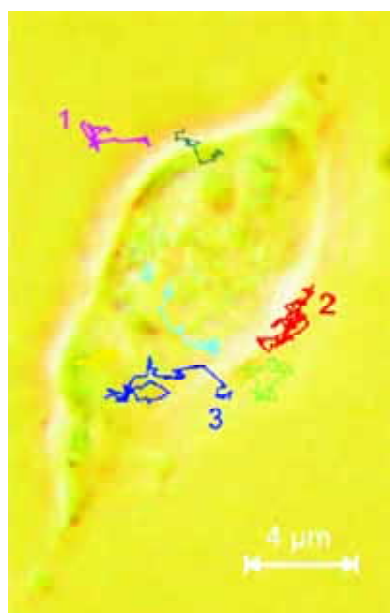


Fig.1. Real-time visualization of the infection pathway of single adeno-associated viruses (AAV) in living cells<sup>[41]</sup>.

acidification, and fusion of single influenza viruses in living cells by using real-time fluorescence microscopy. Influenza virus X-31 was labeled with three dyes, DiD, Cy3 and CypHer5. And they have dissected individual stages of the viral entry pathway. Their single-virus trajectories clearly dissect the viral endocytic pathway and unambiguously demonstrate that the viral transport is composed of three distinct stages before virus-endosome fusion, with stage being an actindependent active transport in the cell periphery, stage being a rapid and dynein-directed movement on microtubules toward the perinuclear region, and stage being an intermittent active transport involving both plus- and minus-end-directed motor proteins on microtubules in the perinuclear region. Surprisingly, the majority of viruses experience their initial acidification in the perinuclear region immediately following the dynein-directed rapid translocation step. This finding suggests a previously undescribed scenario of the endocytic pathway toward late endosomes: endosome maturation, including initial acidification, largely occurs in the perinuclear region (Fig.2).

### Single molecule detection of signaling molecules in living cells

Single-molecule imaging is an ideal technology to study molecular mechanisms of biological reactions *in vitro*<sup>[43,44]</sup>. Recently, this technology is extended to real-time observation of signal transduction. The early events in signal transduction from the epidermal growth factor (EGF) receptor

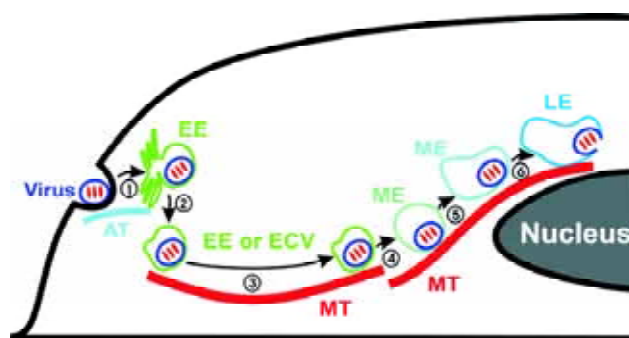


Fig.2. A model of the endocytic pathway toward late endosomes<sup>[42]</sup>.

(EGFR) are dimerization and autophosphorylation of the receptor, induced by binding of EGF. Yasushi Sako *et al.*<sup>[45]</sup> observed these events in living cells by visualizing single molecules of fluorescent-dye-labeled EGF in the plasma membrane of A431 carcinoma cell. As a probe for detection of single molecules in living cells, they used a fluorescent dye, Cy3, conjugated to mouse EGF. As mouse EGF has only one reactive amino residue (at the amino terminus), it can be labeled with amino-reactive Cy3 dye with a dye: protein ratio of exactly 1:1. Under an objective-type total internal reflection fluorescence microscope, Cy3-labelled EGF (Cy3-EGF) was added to the culture medium of A431 cell, and individual Cy3-EGF molecules could be visualized on the apical surface. Single molecule tracking reveals that the predominant mechanism of dimerization involves the formation of a cell-surface complex of one EGF molecule and an EGFR dimer, followed by the direct arrest of a second EGF molecule, indicating that the EGFR dimers were probably preformed before the binding of the second EGF molecule. Single-molecule fluorescence-resonance energy transfer shows that EGF-EGFR complexes indeed form dimers at the molecular level. Use of a monoclonal antibody specific to the phosphorylated (activated) EGFR reveals that the EGFR becomes phosphorylated after dimerization.

Ryota Iino *et al.*<sup>[46]</sup> successfully imaged single green fluorescent protein (GFP) molecules in living cells. GFP linked to the cytoplasmic carboxyl terminus of E-cadherin (E-cad-GFP) was expressed in mouse fibroblast L cell, and observed using an objective-type total internal reflection fluorescence microscope. Based on the fluorescence intensity of individual fluorescent spots, the majority of E-cad-GFP molecules on the free cell surface were found to be oligomers of various sizes, many of them greater than dimers, suggesting that oligomerization of E-cadherin takes

place before its assembly at cell-cell adhesion sites. The translational diffusion coefficient of E-cad-GFP is reduced by a factor of 10 to 40 upon oligomerization. Because such large decreases in translational mobility cannot be explained solely by increases in radius upon oligomerization, an oligomerization-induced trapping model is proposed in which, when oligomers are formed, they are trapped in place due to greatly enhanced tethering and corralling effects of the membrane skeleton on oligomers (compared with monomers). The presence of many oligomers greater than dimers on the free surface suggests that these greater oligomers are the basic building blocks for the two-dimensional cell adhesion structures (adherens junctions). Based on the single GFP imaging technique for live cells, oligomerization levels and the movement of E-cad-GFP were directly observed for the first time.

Monitoring the behavior and reaction of signal-transduction molecules using single-molecule techniques will be indispensable in fully understanding the mechanism of intracellular signalling.

### Single molecule detection of ion channels in living cells

One main goal in cell biology is the *in vivo* determination of the local distribution of cellular components and its dynamical changes. This first visualization of individual membrane proteins in live cells by fluorescence labeled ligands with 40 nm 3D positional resolution opens new perspectives for the study of cellular organization and processes at the molecular level. Gerhard J *et al.*<sup>[47]</sup> labeled hongotoxin (HgTX1-Cy5) to the potassium channel KV1.3 in T-lymphocyte cell membranes and employed single dye tracing (SDT) for imaging single ion channels optically. Well approximated by a Gaussian distribution, resolution of channel positions to within  $\pm 40$  nm was obtained in all three dimensions. The positional resolution along the optical axis (z-direction) was obtained from the accuracy of estimating the position of minimum defocusing for a single molecule. For this, the width of the fluorescence peaks in consecutive images, taken at different degrees of defocusing, were shown to accurately match the theoretical prediction, yielding  $\sim 40$  nm accuracy of finding the z-position of the labeled channels. This potential opens a whole scenario of studies promising new insights, valuable for basic pharmacology and for the drug finding process.

L-type  $\text{Ca}^{2+}$  channels are an important means by which a cell regulates the  $\text{Ca}^{2+}$  influx into the cytosol on electrical

stimulation. To describe their structure and dynamics in the plasma membrane can help us in-depth understanding of their function. Construction of a fluorescent variant by fusion of the yellow-fluorescent protein to the ion channel and expression in a human cell line allowed us to address its dynamic embedding in the membrane at the level of individual channels *in vivo*. Gregory S *et al.*<sup>[48]</sup> reported on the observation of individual fluorescence-labeled human cardiac L-type  $\text{Ca}^{2+}$  channels using wide-field fluorescence microscopy in living cells. The fluorescence and electrophysiological data indicate that L-type  $\text{Ca}^{2+}$  channels tend to form larger aggregates, which are moveable in the plasma membrane.

### Single molecule detection by single-quantum dot tracking

QDs, which are intermediary in size, are more photostable than conventional fluorophores, and have been seen as promising fluorescent probes. Maxime Dahan *et al.*<sup>[49]</sup> used QDs to track individual glycine receptors (GlyRs) and analyze their lateral dynamics in the neuronal membrane of living cells. The entry of GlyRs into the synapse by diffusion was observed by QD-tagged receptors and further confirmed by electron microscopy imaging. The properties of QDs make it possible to record the mobility of individual molecules at the neuronal surface, even in confined cellular compartments.

QDs have also been demonstrated their use for long-term multicolor imaging of live cells. Jyoti K *et al.*<sup>[50]</sup> have developed procedures on uptake of QDs and selective labeling of cell surface proteins with QDs conjugated to antibodies. Live cells labeled using these approaches were used for long-term multicolor imaging. For labeling with QD-antibody bioconjugates, cells were first washed with PBS, then incubated at 4 °C for 45~60 min with the specific antibody-QD conjugates in 1% BSA. Unbound QDs were removed by washing with PBS. HeLa cells were stably labeled for over a week with no detectable effects on cell morphology or physiology. Even after continuous growth for 12 d, the cells were still labeled.

QDs offer a favorable compromise between small fluorophores and large beads for single-molecule experiments in living cells and will be invaluable tools for ultra sensitive studies of the dynamics of cellular processes.

### Future perspective

Single molecule detection techniques have made rapid advances in the research of life science. These techniques

allow us to record the behavior of individual molecules in the living cell in real time. Though it has opened up a new era of life science, there are many difficulties to be gone over, such as reduction of background signals of single fluorophores which mainly arise from auto fluorescence, improving spatial and temporal resolution and location in three-dimensional in the living cell. After all we can say that the development of single molecule detection techniques has opened a new horizon for the research of single molecule under physiological conditions. And we will learn more about the truth of nature by it.

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