Review

Uses of single-particle tracking in living cells

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ABSTRACT: Single-particle tracking (SPT) techniques have been developing rapidly in the field of cellular biology as a means of unravelling the diffusion dynamics of bio-molecules and the function of proteins in the regulation of cellular activity at single molecule sensitivity and nanometer spatial resolution. Suitable probes and technological improvements have made SPT more accessible than it used to be and paved the way for broad applications in cellular biology. This review summarizes the principles of SPT and discusses the main findings yielded by the technique and its contribution to the understanding of proteins in living cells.

Keywords: Single-particle tracking (SPT), living cell, review

1. Introduction

Over the last decade, significant advances in microscopy techniques and the introduction of novel fluorescent probes to label bio-molecules in living cells have changed the field of cell biology. Single-particle tracking (SPT), including real-time single-molecule techniques, has enabled tracking of individual molecules over time and space in living cells. SPT is a general term, and a "particle" refers in a broad sense to any probe selectively attached to a molecule of interest (*1*), such as a receptor, organelle, or virus. The variety of nomenclature in the field (*e.g.* single-virus tracing and single-molecule tracking) presents difficulty when searching the literature (*2*). SPT uses video microscopy combined with digital computer processing to monitor the motion of single molecules of interest, which are often labeled with submicrometer fluorophores. An SPT experimental system is depicted schematically in Figure 1 (*3*).

Many cellular processes involve the interaction of several individual molecules that must come together to transmit information or respond to environmental cues. Hence, importance crucial point is to understand the mechanism by which the motion of related molecules is regulated in the cell. However, molecular behavior is very inhomogeneous, and even molecules of a single species interact stochastically with distinct molecules or cellular structures in a variety of local environments. Furthermore, molecular interactions are by nature stochastic. Therefore, bulk-type observations that report on the tendency of molecular behavior averaged over all molecules under observation may not be able to distinguish various stochastic processes occurring in very inhomogeneous environments. SPT has the advantage of being able to view individual characteristics of a molecule that may be washed out in the ensemble averaging inherent in bulk studies (*4*). SPT has been used to investigate the movement of receptors or lipids in the plasma membrane (*5,6*), follow individual viruses along their infection pathway (*7,8*), or study the motion of individual complexes within living cells (*9,10*). The current review focuses on the recent progress achieved with SPT techniques, including a summary of the principle of SPT, and it discusses the main findings yielded by this technique and its contribution to the understanding of proteins in living cell.

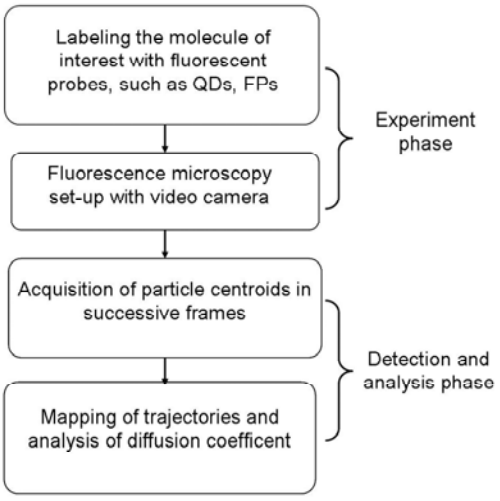

Figure 1. Schematic of an SPT experimental system.

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2. Fluorescent probes

The first step toward the realization of SPT in living cells is to label the external and/or internal molecules of interest with fluorophores, allowing imaging using fluorescence microscopy. Fluorescent probes for SPT should have a high quantum yield of fluorescence emission and high photostability. In general, two classes of probes are used for SPT: fluorescent proteins (FPs) and non-genetically encoded probes, such as organic small-molecule fluorophores and quantum dots (QDs) (*11*). To date, the most popular probe used to study the movement of proteins is QDs (*1*).

2.1. *QDs*

Inorganic nanoparticle semiconductor QDs are excellent major fluorescent probes for SPT because of their unique fluorescent properties (*12*). Their huge one- and two-photon absorption cross sections, tunable emission bands, and excellent photobleaching resistance are stimulating the development of luminescent probes for single-molecule imaging and SPT. The unique wide absorption spectra and narrow emission band that can be tuned from UV to infrared wavelengths distinguish them from conventional organic fluorophores and provide many advantages for multi-color fluorescent imaging. In addition, QDs are orders of magnitude more photostable than organic dyes and FPs, making them attractive candidates for long-term SPT experiments in living cells, where they can provide long tracking trajectories (*13*). Moreover, the developments in QD surface coating and bio-conjugation schemes have made them the most suitable probes for live cell applications. Over the years, scientists have developed a wide array of surface chemistries for QD modification (*14*). These surface coatings have not only made QDs retain the advantageous photophysical and size properties of the nanocrystal but also provide additional reactive groups for subsequent conjugation of biomolecular recognition molecules. Finally, a particular property of QDs is their alternation between "on" and "off" states, known as blinking. This adds complexity to the tracking procedure but ensures the idenfication of single QDs because signals alternate between 0 and 1 and would be fractional in the case of multiple QDs (*1*).

2.2. *Organic small-molecule fluorophores*

Organic dyes have diverse structures and photophysical properties that can be designed through organic synthesis. They are usually constructed through either chemical labeling of proteins or physical incorporation into the molecules of interest. Their small size makes them less perturbative than QD. They are, however, less bright and stable, making them suitable only for short trajectories of a few seconds.

2.3. *FPs*

FPs can be genetically engineered to be incorporated into the molecule interior of cell and they are highly specific with controlled one-to-one stoichiometry. However, they are generally bigger, dimmer, and less photostable than small-molecule fluorophores.

2.4. *Gold nanoparticles (GNPs)*

Compared to fluorescent dyes and QDs, GNPs (the size from 2 to 100 nm) have unique optical properties such as no photobleaching and absence of blinking, and the small size of the GNPs reduces the potential for probe-related artifacts. Moreover, they show no cytotoxicity under certain experimental conditions (*15*). Additionally, GNPs show strong light scattering at the plasmon resonance wavelength owing to the collective oscillation of the conduction electrons. Based on this nature of GNPs, a new technique called single nanoparticle photothermal tracking (SNaPT) has been developed to track small probes in live cells. These probes can be detected by local thermal variation after light absorption, allowing tracking experiments over a certain period of time (*16*).

2.5. *Other fluorescent probes*

In recent years, there has been enormous interest in the use of nanoparticles as tiny probes to spy on cellular machinery. Useful properties can be incorporated into the design of nanoparticles in order to study cellular functions. For example, single-walled carbon nanotubes (SWNTs) are powerful molecular fluorophores for SPT. SWNTs have interesting photophysical properties. Unlike organic dyes, the conjugated sp^2 carbon bonds in a carbon nanotube are chemically quite stable and the one-dimensional quantum confinement creates an unusual, low energy band gap in the near-infrared (NIR) range. This chemical stability translates into a high degree of photostability. Meanwhile, SWNTs have no significantly irreversible photobleaching threshold at moderate fluence and no intrinsic blinking mechanism (*17*). Fluorescent nanodiamonds (FNDs) are nontoxic and photostable nanomaterials, ideal for long-term *in vivo* imaging applications (*18*). Modifying the surfaces of FNDs with different functional groups so that they are treated as pure organic compounds has expanded these materials' applications. One type of FNDs, when exposed to green-yellow light, emits bright-red fluorescence at about 700 nm, which is well away from the spectral region (300-500 nm) where cellular endogenous fluorescence occurs. Moreover, the fluorescence shows no apparent photobleaching, thus allowing long-term probing of FND particles individually in cells (*19*).

3. Fluorescence microscopy

Labeled particles are visualized in live cells using fluorescence microscopy. Three imaging geometries are most often used, *i.e*., epifluorescence microscopy, confocal microscopy, and total internal reflection fluorescence microscopy (TIRFM). Of the three, epifluorescence microscopy is the simplest to set up. This method also has the greatest imaging depth and is often used when long-range protein trafficking or transport is being studied. However, owing to the background autofluorescence of cells, epidetection is inadequate for SPT involving only a few fluorescent molecules. To reduce the background noise, confocal detection or excitation by total internal reflection (TIF) is used. Confocal microscopy requires a rapid scanning scheme (laser scanning confocal microscopy) or multiplexed detection (spinning disc confocal microscopy) to acquire images of living cells. Using confocal imaging allows the acquisition of 3-dimensional images, but this advantage is mitigated by a relatively severe signal loss. The focus of the laser beam of a laser-scanning confocal microscope is scanned or orbited around the particle. TIRFM, which is a wide-field imaging technique that illuminates only a thin plane of the sample, can also be used for SPT. This is done to greatly reduce the background fluorescence and detect single particles in the cell membrane. However, the excitation depth of an evanescent wave generated by TIF is only 100-200 nm, limiting its use to events that occur close to the cell surface. Therefore, TIRFM imaging is typically used to monitor the mobility of membrane proteins.

In addition to these background-reduction techniques, SPT is performed with specialized video cameras. In a typical SPT system, a video camera is connected to an optical microscope. Video signals from the camera are transmitted to a recording device (*3*). As a result, the development of charge-coupled device (CCD) cameras with high quantum yields (60-80%) and rapid frame rates (1-500 ms) also represents an important technological advance that has benefited SPT technology (*7*).

4. Uses of SPT in living cells

4.1. *SPT of membrane receptor transport*

SPT techniques have offered alternative ways to explore the kinetics of receptor transport and the transport of internalized endosomes on the cell surface. It has been indispensable in understanding the details of the mechanism of cell signaling and particle transport because it eliminates ensemble averaging and provides direct information on heterogeneity and kinetics of the system.

Liang *et al.* (*20*) used SPT to efficiently explore the pathway and mechanism for the transport of α_{1A}-Adrenergic receptors (α_{1A}-ARs) in real time in single living cells. In this work, α_{1A}-ARs were specially labeled by primary antibody and Cy3-IgG, and α_{1A}-ARs internalization into cells was triggered by the agonist phenylephrine (PE). They were the first to find that the initial transport of α_{1A}-AR depended on actin filaments at a peak velocity of 0.2 μm/sec and exhibited discrete 33-nm steps. The step size, the rate constant, and the velocities were broadly in agreement with the character of single myosin molecules *in vitro*. Their results demonstrated that while transporting each endosome myosins did not work in a "tug-of-war" mode and that they did not adopt a strategy of working in coordination to boost transporting speed. These results not only offered some insight into the mode in which myosin works *in vivo* but also provided a model of transportation of internalized G-protein coupled receptors (GPCRs) in living cells. Relevant information on mechanisms of action for other GPCRs has been also obtained with SPT (*21,22*). Echarte *et al*. (*23*) investigated the biophysical behavior of retrograde transport of nerve growth factor (NGF) in living cells and in real time using QDs and quantitative SPT technology. They followed NGF trafficking inside neurites of differentiated PC12 cells by tracking trace amounts of streptavidin-coated QDs bound to biotinylated NGF (bNGF). Using this strategy, they visualized NGF binding, internalization, and trafficking and they characterized the kinetics of the NGF trafficking process. They found that a net retrograde transport of QDs-bNGF complexes with a velocity of 0.054 ± 0.020 μm/sec. Individual runs had a mean velocity of approximately 0.15 μm/sec at room temperature, and the run times were exponentially distributed. Their results demonstrated that the receptor had been internalized, like many cytoplasmic organelles. QDs-bNFG-receptor complexes exhibited bidirectional movement along microtubules after endocytosis. Based on quantitative analysis of the tracking data, they derived the rates and extent of transport in both the retrograde and anterograde directions. The new information gained through SPT is of fundamental importance in understanding normal and pathological processes in the nervous system.

4.2. *SPT of exocytosis and endocytosis*

Exocytosis and endocytosis are the basic methods of material transport between the cell and its environment. Exocytosis is a process by which the membranes of secretory vesicles fuse with the plasma membrane, releasing the contents of the vesicle into the extracellular medium. This process has been studied in particular depth with regard to the release of neurotransmitters at the synapse (*24-26*). Import into the cell is possible by the process of receptor-mediated

endocytosis, by which selected plasma membrane proteins are internalized. This not only mediates some signal transduction processes but also allows the cell to maintain or modify its electrolyte balance and control its osmotic properties. Neurons transmit information by the regulated release of neurotransmitters that bind to specific receptors at the target cell. SPT has been used to directly detect the dynamic process of synaptic vesicle trafficking in nerve terminals and to measure the release of neurotransmitters induced experimentally or evoked by normal behavior. Levitan *et al.* (*25*) used SPT to study neuropeptide vesicle exocytosis and motion at the drosophila larval neuromuscular junction (NMJ). Movement and release of single large dense-core vesicles can be resolved in nerve terminals because green fluorescent protein (GFP)-tagged neuropeptides are highly concentrated in these organelles. Utilization of SPT led to the first detection of vesicle mobilization in nerve terminals and the discoveries of activity-dependent capture of transiting vesicles and post-tetanic potentiation of neuropeptide release. Lemke and Klingauf (*26*) described a SPT technique using dual fluorescent dye labels to simultaneously visualize the movements of a single vesicle and the respective synaptic bouton during resting conditions and stimulation. They found vesicle mobility to be very low in the absence of stimulation, in accordance with previous studies (*27,28*). Interestingly, mobility was also found to be low during synaptic activity. They found that vesicles labeled preferentially *via* early, late, and spontaneous endocytotic mechanisms behaved similarly at rest and during stimulation.

Receptor-mediated endocytosis is a complex and dynamic process that is critical to the governing of cellular signaling and subsequent cell function. In order to understand receptor endocytic trafficking at the level of single or small numbers of receptors, Rajan *et al.* (*29*) developed and tested ligand nerve growth factor-bound quantum dot (NGF-QD) bioconjugates in order to image discrete receptor endocytic events inside live NGF-responsive PC12 cells. Using SPT, they investigated the molecular-scale dynamics of NGF-receptor complexes undergoing endocytic trafficking. The unique value of SPT indicated that NGF trafficking operated with a strikingly high degree of efficiency at the molecular-size scale and that diffusive and active forms of transport inside cells were rapid and efficient.

4.3. *SPT of mRNA*

SPT is a powerful technique for studying RNA dynamics in living cells. It enables the analysis of the movement of individual RNA molecules in real time and the comparison of the relationship between movement and the subnuclear structure of those molecules. Recently, SPT of mRNA has been performed in the cytoplasm (*30*) and in the nucleus (*31*). Shav-Tal *et al.* (*31*) used a cellular system for monitoring mRNA expression to characterize the movement in real time of single mRNA-protein complexes (mRNPs) in the nucleus of living mammalian cells. They found that the mobility of mRNA was not directed but governed by simple diffusion. Moreover, half the population of mRNAs diffused freely, with D = 0.04-0.05 μm^2/sec, and the other half were corralled in the nucleus (*31*). SPT of mRNA labeled with a molecular beacon was performed in the nucleus by Vargas *et al.* (*32*). They used small, hairpin-shaped oligonucleotides known as molecular beacons that possessed an internally quenched fluorophore with fluorescence that was restored upon hybridization to the complementary sequence of mRNA. Brownian motion of mRNA with D = 0.03 μm^2/sec was reported (*32*). However, these two studies were carried out with a low temporal resolution (300 ms/frame) and mRNAs were only observed over 3 s (*33*). In 2009, Ishihama and Funatsu (*33*) successfully observed the movement of individual mRNAs for more than 60 s with a temporal resolution of 30 ms using QDs. mRNA labeled with QDs at a stoichiometric ratio of 1:1 was microinjected into the nuclei of Cos7 cells. They found that almost all mRNA-QDs were in motion, and statistical analyses revealed anomalous diffusion between barriers, with a microscopic diffusion coefficient of 0.12 μm^2/sec and a macroscopic diffusion coefficient of 0.025 μm^2/sec. Diffusion of mRNA was observed in interchromatin regions but not in histone2B-GFP-labeled chromatin regions. These results provided direct evidence of channeled mRNA diffusion in interchromatin regions. QD labeling allowed SPT of mRNAs to be carried out with high temporal and spatial resolution over observations of a long duration, a feat that had not been achieved by conventional labeling with a fluorescent dye or fluorescent protein.

In order to study whether these individual mRNA species are specifically sorted into separate or common ribonucleoprotein (RNP) particles before or during transport, Katayama *et al.* (*10*) analyzed the intracellular movement of individual pairs of localized mRNA in yeast cells. mRNA pairs were tagged with tandem repeats of either bacteriophage MS2 or lambda boxB RNA sequences and fluorescently labeled by fusion protein constructs that bound to the RNA tag sequences. Using SPT with dual-color detection, they tracked the transport of two different localized mRNA species. Their observations indicated that different localized mRNAs were coassembled into common RNP particles and cotransported in a directional manner to the target site. Nonlocalized mRNAs or mutant mRNAs that lack functional localization signals form separate particles that are not transported to the yeast bud. This study revealed a high degree of co-ordination of mRNA trafficking in budding yeast.

4.4. *Single-virus tracking*

SPT studies of viruses are also known as single-virus tracking. Single-virus tracking (*7*) uses fluorescence microscopy to monitor individual virus particles or viral components in live cells. It is a powerful tool for investigating viral infection routes and characterizing the dynamic interactions between viruses and target cells. Viral infections are universally acknowledged to be complex processes that include many steps and interactions with different cellular structures. Not only are these interactions dynamic, but often the cellular structures are themselves dynamic. Furthermore, the same virus might infect cells by several different routes and most viral entry events might be futile. Therefore, single-virus tracking enables the possible elucidation of previously unknown but critical steps involved in the penetration of viruses into cells and dissemination of viruses, revealing novel therapeutic opportunities for controlling virus pandemics and pathogenesis.

A number of studies have revealed detailed information about the proteins involved in fusion for many viruses and have elucidated fundamental principles of viral fusion mechanisms, but the dynamics of the process are largely unknown. Koch *et al*. (*34*) developed a system to study the dynamics of HIV-1 entry using SPT. They generated HIV-1 particles pseudotyped with the envelope (Env) protein of ecotropic murine leukemia virus (eMLV) to study retrovirus entry at the plasma membrane using live-cell microscopy. In their experiment, they established a double labeling strategy in which a fluorescent label in the matrix (MA) domain of HIV-1 Gag was combined with another fluorescent label fused to eMLV Env (Env. YFP). Fusion events were defined as loss of Env signal after virus-cell contact. SPT of > 20,000 individual traces in two color channels recorded 28 events of color separation, indicating that the MA layer dissociated from the surface glycoproteins upon membrane fusion. Fourty-five events were detected where both colors were lost simultaneously. Furthermore, they found that virus-cell fusion appeared to be kinetically different from cell-cell fusion. This finding was in line with that from a previous study (*35*).

Dengue virus (DENV) is a mosquito-transmitted, enveloped RNA virus. DENV causes the most common arthropod-borne infection worldwide, with 50-100 million cases annually. It threatens human health, but there are presently neither vaccines nor antiviral drugs to prevent or treat dengue infection (*36*). In 2008, van der Schaar *et al.* studied the cell entry, endocytic trafficking, and fusion behavior of DENV, which was achieved using live-cell imaging, single-virus tracking, and real-time multi-color fluorescence microscopy. In the experiment, DENV was labeled with the lipophilic fluorescent probe DiD. The surface density of the DiD dye in the viral membrane allowed clear detection of single virus particles, and this labeling procedure did not affect the infectious properties of DENV. In order to test whether DENV was internalized through clathrin-mediated endocytosis, BS-C-1 cells were used; these cells stably expressed enhanced yellow fluorescent protein (eYFP) fused to the light chain of clathrin (*37*). The simultaneous tracking of DENV particles and various endocytic markers revealed that DENV enters cells exclusively *via* clathrin-mediated endocytosis. The virus particles moved along the cell surface in a diffusive manner before being captured by a pre-existing clathrin-coated pit. Following clathrin-mediated uptake, the majority of DENV particles were transported to early endosomes, which subsequently matured into late endosomes, and then fusion of the viral membrane with the endosomal membrane was detected in late endosomal compartments. This is the first report to describe the cell entry process of DENV at the single particle level and therefore provides unique mechanistic and kinetic insights into the route of internalization, endocytic trafficking behavior, and membrane fusion properties of individual DENV particles in living cells. This study has opened up new avenues in flavivirus biology and will lead to a better understanding of the critical determinants in DENV infection (*38*). In addition, this group studied the transport, acidification, and fusion of single influenza viruses in living cells (*39*). Influenza is a valuable model system for exploring the cell's constitutive endocytic pathway. The movement of individual viruses revealed a striking three-stage active transport process that preceded viral fusion with endosomes starting with an actin-dependent movement in the cell periphery, followed by a rapid, dynein-directed translocation to the perinuclear region, and finally an intermittent movement involving both plus- and minus-end-directed microtubule-based motilities 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.

4.5. *SPT of drug delivery*

Anticancer therapeutics based on active tumor targeting by conjugating tumor-specific antibodies should help to increase therapeutic efficacy and decrease systemic toxicity. Quantitative investigation of the dynamics of anticancer drug delivery *in vivo* is crucial to enabling the development of more effective drug delivery systems. Recently, SPT has been used to study drug delivery.

Tada *et al*. (*40*) tracked single-particle QDs conjugated with tumor-targeting antibody in tumors

of living mice using a dorsal skinfold chamber and a high-speed confocal microscope with a highly sensitive camera. They succeeded in capturing the delivery of single QD-antibody complexes in tumor vessels to the perinuclear region of tumor cells in live mice after QD-antibody complexes had been injected into the tail vein of mice with HER2-overexpressing breast cancer. Movement of single complexes of the QD-antibody were clearly observed at 30 frames/sec inside the tumor, and six stages were detected (Figure 2): (1) circulation within a blood vessel, (2) extravasation, (3) movement into the extracellular region, (4) binding to HER2 on the cell membrane, (5) movement from the cell membrane to the perinuclear region after endocytosis, and (6) movement to the perinuclear region. The translational speed of QD-antibody complexes in each process was highly variable, even in vessel circulation. The movement of the complexes at each stage was also found to be "stop-and-go". This indicated that the movement was promoted by a motive power and constrained by both the three-dimensional structure of the complexes and protein-protein interactions. The motive power of the movement was produced by blood circulation, diffusion force driven by thermal energy, and active transport by motor proteins. The cessation of movement is most likely induced by a structural barricade such as a matrix cage and/or specific interaction between proteins and HER2, motor proteins, and rail filaments such as actin filaments and microtubules. This study provides valuable information on antibody-conjugated therapeutic nanoparticles, which will be of use in analyzing the molecular processes of drug delivery to the tumor and in increasing therapeutic efficacy. SPT has also been use to investigate the potential of magnetic nanoparticles for drug delivery (*41*).

5. Conclusions

SPT, a very powerful technique, represents the only currently available method to clearly differentiate different types of protein movement without population averaging and will prove indispensable in understanding the details of the mechanism of cell signaling and particle transport (*42*). Among their major achievements, SPT studies have revealed the molecular details of signaling processes, receptor activation, lateral organization, dynamics of endocytosis and exocytosis, and the mechanisms of entry, trafficking, and egress of various viruses. The next 5 years should see intensive research on the *in vivo* uses of SPT. Although it has made great achievements in the field

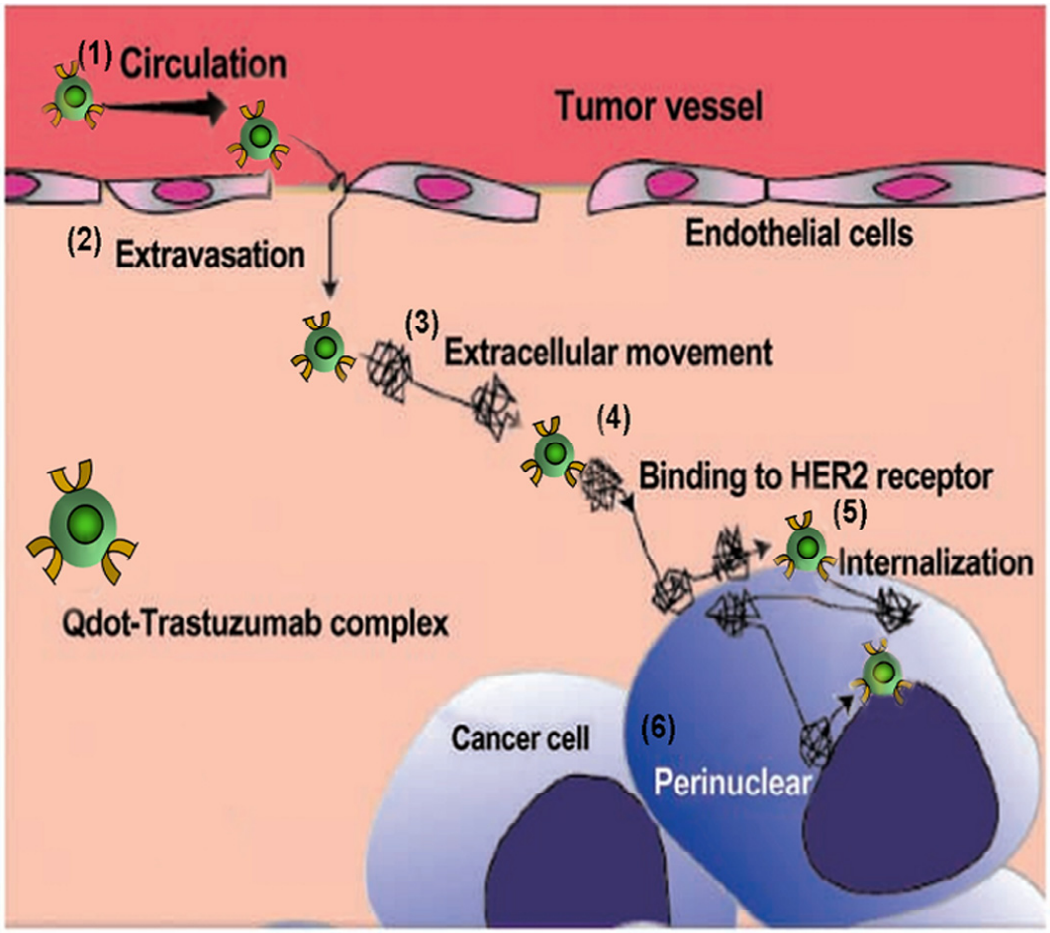

Figure 2. Schematic illustration of the delivery of single QDs-trastuzumab complex in tumor vessels to the perinuclear region of tumor cells.

of life science, many difficulties remain, such as the inconvenient labeling process, the need for a highly sophisticated imaging apparatus, and the limiting of labeling particles to living cells, and continue to represent a hindrance to the more widespread use of SPT.

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