

Semiconductor Nanocrystals for Biological Imaging

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Summary of Recent Advances

Conventional organic fluorophores suffer from poor photo stability, narrow absorption spectra and broad emission feature. Semiconductor nanocrystals, on the other hand, are highly photo-stable with broad absorption spectra and narrow size-tunable emission spectra. Recent advances in the synthesis of these materials have resulted in bright, sensitive, extremely photo-stable and biocompatible semiconductor fluorophores. Commercial availability facilitates their application in a variety of unprecedented biological experiments, including multiplexed cellular imaging, long-term *in vitro* and *in vivo* labeling, deep tissue structure mapping and single particle investigation of dynamic cellular processes. Semiconductor nanocrystals are one of the first examples of nanotechnology enabling a new class of biomedical applications.

Introduction

Semiconductor nanocrystals, also called quantum dots (QDs), are a new class of fluorescent biological labels. Originating from quantum confinement of electrons and holes within the nanocrystal core material, the fluorescence from QDs is unique compared to that from traditional organic fluorophores. For example, QDs exhibit high photo stability, broad absorption and narrow and symmetric emission spectra, slow excited state decay rate and large absorption cross section [1]. Their emission color can be continuously tuned from ultraviolet to visible and infrared wavelengths by changing the size and chemical composition of the semiconductor core nanocrystal. Growing a semiconductor shell with a larger band gap improves the quantum confinement resulting in very bright and highly stable, chemically as well as optically, semiconductor

fluorophores [2,3]. QDs offer an exciting potential to overcome many of the limitations encountered by traditional organic dyes and genetically engineered fluorescent proteins. Since their introduction into biological imaging in 1998 [4,5], an enormous body of research has emerged focusing on the synthesis, photophysical property characterization and bioconjugation [6-9] of QDs. Advanced molecular and cellular imaging with QDs has also been realized [10,11].

Biocompatible QDs find utility not only as a basic bio-labeling tool, but also as a key building block for complex multi-functional bio-probes. Their large surface area may be tailored to bind both target selective molecules and therapeutic molecules, enabling spontaneous delivery of treatments to a probed disease area. Complex nanostructures formed by linking QDs and gold nanoparticles through DNA hybridization or streptavidin-biotin interaction have also been realized [12-14] and applied in sensing biomolecular concentration [14]. Although QDs have been utilized in a broad range of imaging applications to date, their versatility for advanced biomedical applications remains to be fully explored. In this article, we will focus on applications in biological imaging, with a brief introduction about their unique optical properties, followed by a discussion of recent progresses in both *in vitro* and *in vivo* imaging as well as their application in neurobiology. Toxicity issues are also addressed.

Optical Properties

The size dependent optical properties of QDs result from their quantum-confined electronic states [15]. Just as in the “particle in a box” model, excitons in smaller nanocrystals experience stronger quantum confinement, resulting in larger photoluminescence energy. Figure 1 shows the typical absorption and emission spectra of

water-soluble QDs. Their emission wavelength can be continuously tuned from 400 nm to 2000 nm by changing both the nanocrystal size and composition [16].

In contrast to conventional fluorophores, QDs have broad absorption and narrow and symmetric emission spectra. These features allow concurrent imaging of multiple entities in a single biological experiment, a quite difficult task with standard fluorophores since their relatively narrow excitation and broad emission spectra often result in spectra overlap [2]. Another advantage of QDs is that they are highly resistant to metabolic degradation and are hundreds of times more photo stable than conventional fluorophores. In addition, QDs often have a large Stokes shift, that is, a large separation between the excitation wavelength and the emission maxima, this has the effect of reducing autofluorescence, resulting in a several fold increase in sensitivity versus organic fluorophores [17].

For QDs, quantum yields can be as high as 0.89 at room temperature [18]; molar extinction coefficients, about 10^5 - 10^6 $M^{-1}cm^{-1}$, are 10 to 100 times larger than most organic dyes [19]; and they have orders of magnitude larger two-photon absorption cross section [20]. Optical properties of QDs are usually unaffected by conjugation to biomolecules. Thus they are both highly stable and bright probes, especially suitable for photon-limited *in vivo* studies and continuous tracking experiments over extended time periods. A more extensive discussion of photophysical properties of QDs is presented by Grecco *et al.* [21].

***In Vitro* Imaging**

QDs have been very successful in immunofluorescent labeling. With continuous efforts in developing high quality biocompatible QDs, nanoparticles conjugated to antibodies, peptides and DNA have been prepared and targeted to cells and tissues specifically, allowing multiplexed labeling and long term studies that can not be achieved by using standard dyes [22-29]. Although QDs and organic dyes can have comparable quantum yields, the larger absorption cross-section of the nanocrystal results in a much stronger photoluminescence signal. The sustained strong signal from a single nanoparticle was used to track dynamic cellular processes over time scales unavailable using organic fluorophores [10]. Recently, Dahan *et al.* developed a method to study single nanocrystal fluorescence patterns using defocused microscopy [30]. By relating these patterns to the structures of the nanocrystal emission dipoles they were able to determine the three-dimensional orientation of the nanoparticles, and successfully applied this technique to track the orientation of single membrane receptor in live cells (Figure 2). With continuous efforts in elucidating the photophysics of single QDs, there will be increasing interest in their application as fluorescent emitters for studying dynamic biophysical processes. For example, Yildiz and Selvin demonstrated that total internal reflection microscopy used in conjunction with organic fluorophores can produce fluorescence imaging with one nanometer accuracy (FIONA) [31]. In this technique, a large number of photons are collected over time from a single dye molecule, allowing researchers to locate the center of the fluorescent pattern with high precision. This technique has been applied to unravel the walking mechanism of the molecular motors myosin V, myosin VI and kinesin. Although the presented experimental results were

from an organic dye, the authors believed that using QDs would provide at least a 10-fold improvement in time resolution and are extending the applications of FIONA in motor movements with QDs.

QDs can be uptaken by live cells with no need of any functionalization, possibly due to the characteristic size range and good biocompatibility. Pellegrino *et al.* studied the phagokinetic tracks left on a homogenous layer of silanized CdSe/ZnS, and demonstrated that QDs can be used as a two-dimensional *in vitro* invasion assay for discriminating between non-invasive and invasive cancer cell lines [32]. This technique provides a new tool for qualifying tumor cell invasiveness. Internalized QDs are also powerful probes for long-term studies of cell-cell interactions. They have been used to examine the interactions of human mammary epithelial tumor cells with normal cells growing in a 3-D culture system. The tumor cell behavior around polarized normal cell clusters was clearly demonstrated when labeling tumor cells and normal cells with nanocrystals of different emission colors. The high photostability of the QDs is critical in the tracking and imaging of these cocultures for extended time periods (up to 14 days) and cannot be replaced by organic fluorophores (Figure 3, RM Boudreau, unpublished results).

Applying semiconductor nanoparticles for *in vitro* labeling allows fluorescent and electron microscopy [10,18,33] imaging of the same probe, so that information on both temporal dynamics and high-resolution cellular localization can be obtained [10]. The fluorescence and electron density properties of QDs were also utilized by Nisman *et al.* to label a nuclear protein on cell sections and to correlate the fluorescence and TEM data. They also employed QDs in conjunction with immunogold to colocalize proteins at the ultrastructure level. Moreover, by obtaining cadmium elemental maps of CdSe/ZnS

distributed on a nuclear structure, the authors demonstrated the potential of using quantum dots as tags for electron spectroscopic imaging to colocalize multiple proteins [33].

***In vivo* Imaging**

The extreme brightness of QDs and their resistance to photobleaching enable continuous exposure under laser illumination for an extended period of time, making them especially useful for *in vivo* imaging. Progress in nanocrystal synthesis, coating and surface modification has significantly enhanced their applications in tracking and imaging. Efforts in optimizing the surface coating for *in vivo* imaging have shown that specific polyethylene glycol (PEG) coatings result in longer circulation time [18,34], enhanced stability [18], and minimal nonspecific deposition [18,35], which are essential elements for *in vivo* imaging.

While the first *in vivo* targeting experiment imaged the histological sections of mouse organs after intravenous injection of peptide conjugated QDs, recent applications primarily focus on live animal imaging combined with multi-photon microscopy or with the use of near infrared nanocrystals.

The large two-photon absorption cross section of QDs allows for more efficient probing of thick specimens by multiphoton excitation microscopy [36]. With the use of this technique, fluorescence signals were able to be detected hundreds of microns deep through the skin of live mice [20] and thick tissue specimens [37]. Stroh and colleagues recently explored the use of QDs in anatomical imaging with multiphoton microscopy. Unlike traditional fluorescence labeled dextran vessel markers, the nanocrystals distinctly

differentiate tumor vessels from perivascular cells and matrix. This group also assessed the ability of nanocrystals to monitor tumor and cell trafficking [38]. These findings show the potential uses of QDs in designing drug delivery particles and tumor pathophysiological studies.

Tracking and imaging nanocrystals in live animals has been achieved by Nie's group. QDs were conjugated to the antibody specific for the prostate cancer cell marker PSMA. After injection into mice that had been transplanted with human prostate cancer cells, the quantum dot-tagged PSMA antibodies recognized and bound at the tumor site and were clearly imaged *in vivo*. Due to the large absorption coefficient and long lifetime, *in vivo* images of nanoparticles were much brighter and more sensitive than images with green fluorescent protein [34].

One challenge in live animal imaging is the significant autofluorescent background. Several strategies can be applied to solve this problem. One approach is to use spectral imaging or emission scanning microscopy to separate the nanocrystal fluorescence signal from background noise [34,37]. Since nanocrystals have narrow emission bands, this method also allows for multicolor tracking of up to five different nanoparticles *in vivo* [37]. An even more effective solution is to move from visible light to near infrared (NIR) since most tissue chromophores absorb weakly at such long wavelengths. Another advantage of NIR imaging is deeper penetration. Kim and co-workers first demonstrated the use of NIR QDs to map sentinel lymph nodes (SLN) during surgical procedures [11]. The nanoparticles, after intradermal injection into the animal, entered the lymphatic system, and were followed using an intraoperative imaging system. The surgeon followed the flow of nanocrystals in real time with NIR image

guidance, and quickly identified the position of the SLN in a precise and rapid surgical procedure. NIR nanocrystal imaging of blood vessels and beating heart through 1-2 mm of skin and tissues were also reported [39].

Semiconductor Nanocrystals for Neurobiology

One common approach to studying neurotransmitters involves the use of radiolabeled substrates or antagonists that can be monitored with high sensitivity. However, the cost and complexity involved in using radiolabeled material is high. Additionally, real time monitoring of the transporter activity is not possible. In contrast, a fluorescence based approaches allow for the localization and direct monitoring of real-time activities.

Owing to their high degree of photostability and brightness, QDs are more suitable probes than organic dyes for studies of neuronal protein or receptor dynamics over an extended period of time. Semiconductor nanoparticles have been used to track individual glycine receptors, a major inhibitory neurotransmitter receptor, on the surface of cultured spinal neurons [10] (Figure 5). Compared to Cy3 dye, fluorescent nanoparticles had significant higher signal-to-noise ratio and allowed for tracking of single glycine receptors for at least 20 min, which is 200 times longer than Cy3 dye. Also, due to their small dimensions, nanoparticles are able to access dense synaptic regions and provide dynamical analysis that cannot be achieved with the use of 500 nm latex beads, one of the probes typically used for studying single molecule properties in live cells.

Besides single molecule studies of neurotransmitters, QDs have been used to study neurotransmitter localization and signal pathways. Nanocrystals conjugated with peptides, antibodies, or other small molecules have been shown to recognize their target cell surface receptors [25,40-42]. It has also been shown that nanocrystal probes, after binding to their targets, can modulate receptor functions by either inhibiting ligand transportation [41] or activating downstream signaling [25,42].

Rosenthal and co-workers used nanocrystals conjugated with the neurotransmitter serotonin to target serotonin transporters on transfected cells [41]. Serotonin labeled nanocrystals specifically interacted with the serotonin receptor, and also inhibited the transportation of free serotonin in a way similar to antagonists.

Recently, Mason and coworkers studied norepinephrine (NE) and dopamine (DA) transporter (NET and DAT) locations and activities with semiconductor nanocrystal linked antibodies and peptides [40]. With streptavidin-biotin interaction, nanocrystals can specifically bind to NET in transfected cells as well as surface protein Limbic Associated Membrane Protein (LAMP) in hippocampal cultures. Since the activity of Ang II receptor is closely correlated with NET, the authors studied the localization of this receptor using nanocrystal-neuropeptide Ang II conjugates. This approach would allow for future study of Ang II receptor redistribution and dynamics in relation to NET activity in real-time.

To investigate whether QDs can serve as fluorescent nano-devices to evoke specific cell physiological responses, Vu et al. linked beta the subunit of neuron growth factor (β NGF) to the nanocrystal surface and used this complex to target tyrosine kinase A (TrkA) receptors of PC12 cells [42]. They reported that nanocrystal- β NGF activated

TrkA receptor initiated downstream signaling that resulted in conversion of PC12 cells to a neural phenotype.

These experiments show that QD-ligand conjugates are promising imaging probes for studying receptor-mediated activities and will have a wide range of applications in pharmaceuticals and therapeutics.

Toxicity

Cadmium and selenium are known to be toxic [24]. Therefore, concerns have arisen about semiconductor nanocrystal toxicity and their environmental impact. Most of the above cell and animal experiments showed that when properly capped by both ZnS and hydrophilic shells, no obvious CdSe nanocrystal toxicity was observed under normal experimental conditions. Several groups have varied parameters such as synthesis, surface coating and incubation concentration to further investigate the potential toxicities of nanocrystals [24,43-45]. Cytotoxicity was observed when Cd^{2+} or Se^{2+} ions were released. This occurred when the nanoparticle surface coating was not stable, exposing the CdSe to oxidation by air or UV damage [24,45]. Surface molecules also play a role in QD cytotoxicity [44,45]. While cells can tolerate PEG-silica coated QDs at concentrations up to 30 μM , mercaptopropionic acid coated QDs have deleterious effect at $\sim 6 \mu\text{M}$ [45].

Besides cytotoxicity, the degradation and metabolism of nanocrystals in the body remains to be investigated and there are reports that injected nanocrystals can accumulate in kidney, liver and spleen [34,35]. Whether nanocrystals can ultimately be cleared from

the body is not known. More research in this area must be completed before they can be used as probes for diagnostic applications.

Conclusions

In the past several years, there has been increasing amount of interest in using QDs in an expanding variety of biological applications. Although they will not replace traditional fluorophores in biological imaging, QDs have been gradually accepted as a better alternative probes with enhanced signal-to-noise, extremely high stability, and improved specificity suitable for studying important biological problems.

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Figure Legend

Figure 1. (a) Excitation (dashed) and fluorescence (solid) spectra of fluorescein (A) and a typical water-soluble QD sample (B) in PBS. The nanocrystals have a much narrower emission (32 nm compared with 45 nm at half maximum and 67 nm compared with 100 nm at 10% maximum), no red tail, and a broad, continuous excitation spectrum. **(b)** A, Size- and material-dependent emission spectra of several surfactant-coated QDs in a variety of sizes. The blue series represents different sizes of CdSe QDs with diameters of 2.1, 2.4, 3.1, 3.6, and 4.6 nm (from right to left). The green series is of InP QDs with diameters of 3.0, 3.5, and 4.6 nm. The red series is of InAs QDs with diameters of 2.8, 3.6, 4.6, and 6.0 nm. B, A true-color image of a series of silica-coated core (CdSe)-shell (ZnS or CdS) nanocrystal probes in aqueous buffer all illuminated simultaneously with a handheld ultraviolet lamp. Reproduced with permission from [4].

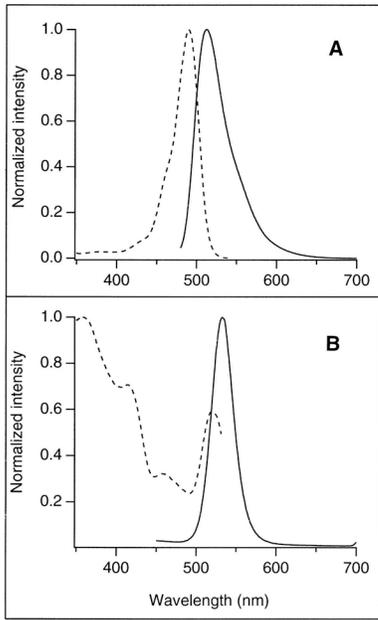
Figure 2. Defocused microscopy images of QD coupled glycine receptor in the membrane of a Hele cell. The contour intensities (dotted lines) can be fitted (solid lines) to determine the orientation (Θ , Φ) of each QD. Reproduced with permission from [30].

Figure 3. QDs were used to study mixed cell interactions in a 3-D Matrigel culture system. **(A)** Human mammalian epithelial MCF 10A cells (labeled with green emitting silica coated QDs) form acini structures after growing in growth-factor reduced matrigel for 10 days. **(B)** After the acini were formed, human breast tumor MDA-MB-231 cells (labeled with red-emitting silica coated QDs) were added to the culture. After

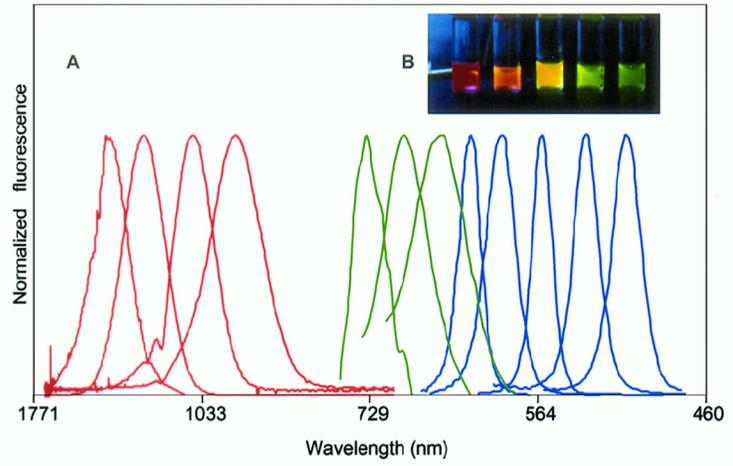
14–16 h of incubation, the tumor cells had attached to the acini. **(C)** The contact was fatal to the tumor cells, which were found dead surrounding the MCF 10A acinus. Most of the tumor cells had lysed, leaving transparent ghosts loosely attached to the acinus, but a few newly attached cells still retained red-emitting QDs. **(D)** The MCF-10A acini and all invading tumor cells; it is a superimposition of all sections, displaying the sharp edge of each cell followed by a projection of color-coded depth information so that red is the uninvolved lower portion of the MCF-10A acini and the tumor cells are shades of orange through green. Bar = 10 μm . From manuscript in preparation.

Figure 4. Near infrared (NIR) QD sentinel lymph node mapping in the mouse. The mouse was injected intradermally with 10pmol of NIR QDs in the left paw. Left, pre-injection NIR autofluorescence image; middle, 5 min post injection white light color video images; right, 5 min post-injection NIR fluorescence image. An arrow indicates the putative axillary sentinel lymph node. Reproduced with permission from [11].

Figure 5. QDs as marker for glycine receptor (GlyR) localization in neurons. QD labeled GlyR (red) was detected over the somatodendritic compartment identified by microtubule-associated protein-2 (green). Arrows mark clusters of QD labeled GlyRs located on dendrites. Reproduced with permission from [10].



(a)



(b)

Figure 1

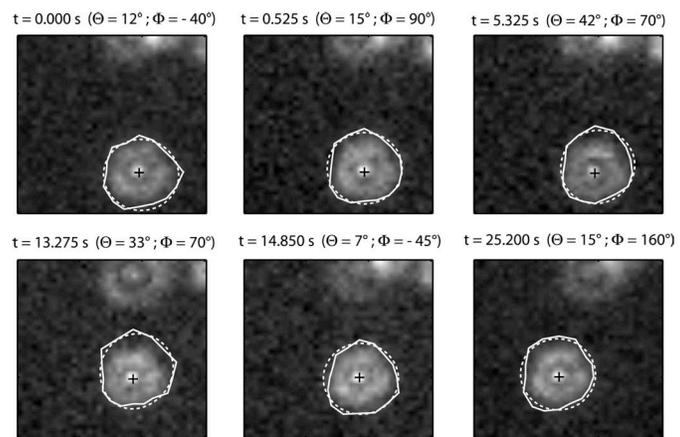


Figure 2

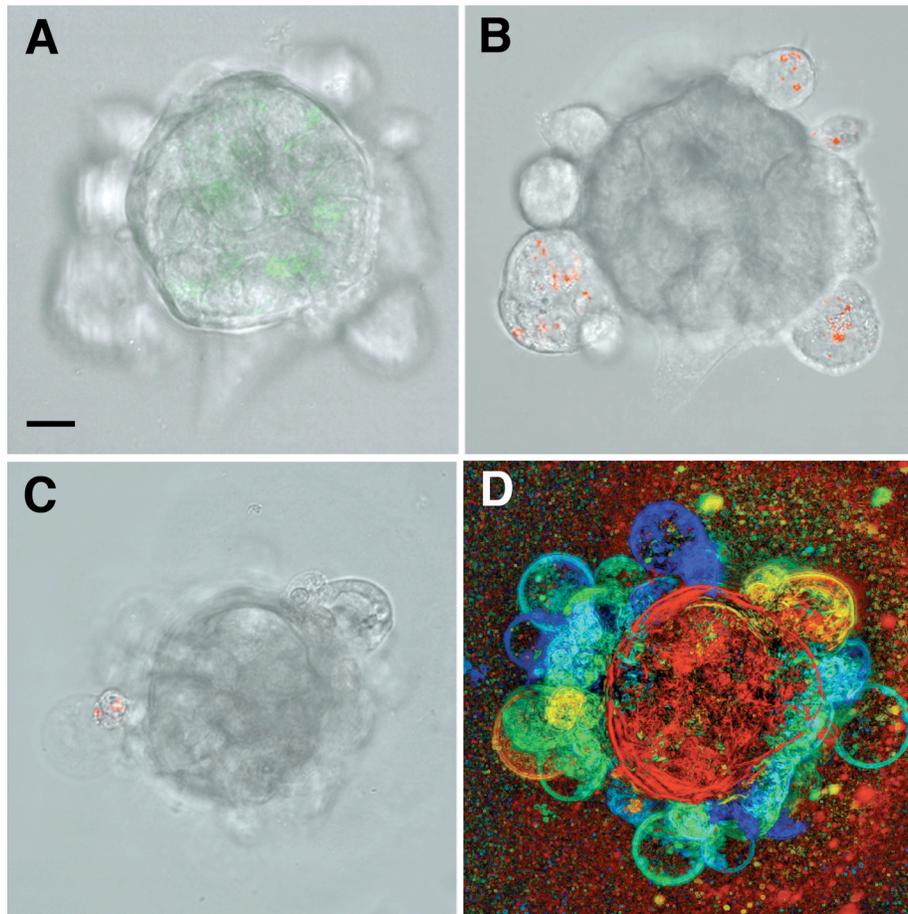


Figure 3

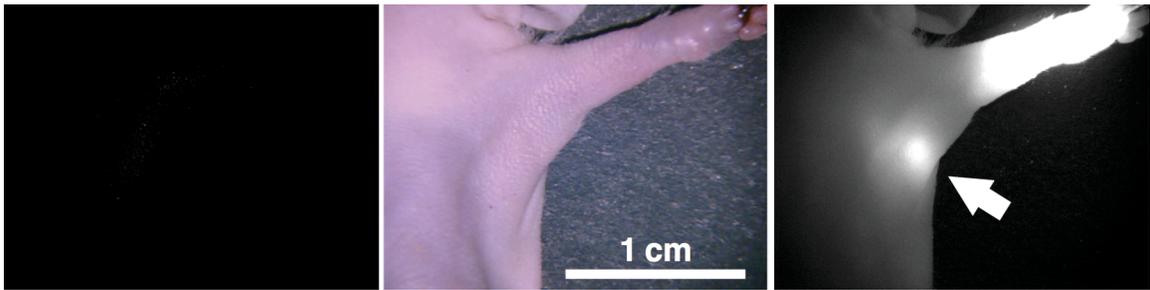


Figure 4

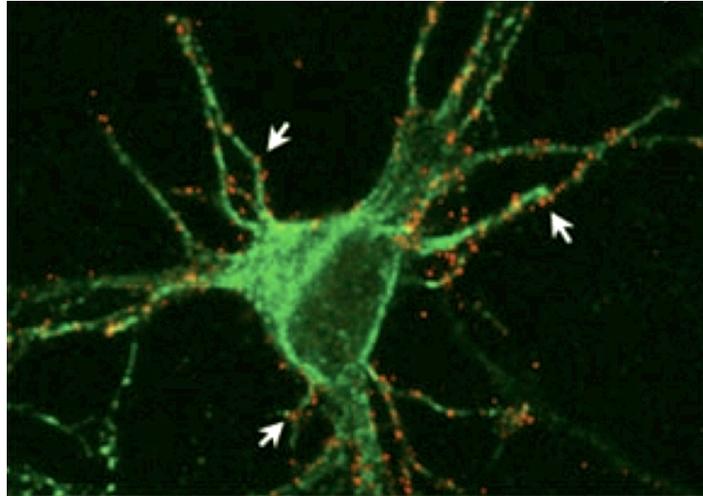


Figure 5