In vitro imaging of embryonic stem cells using multiphoton luminescence of gold nanoparticles

D Nagesha[1]
GS Laevsky[2]
P Lampton[3]
R Banyal[1]
C Warner[3]
C DiMarzio[2]
S Sridhar[1]

[1]Electronic Materials Research Institute and Department of Physics, [2]Electrical and Computer Engineering Department, [3]Department of Biology, Northeastern University, Boston, MA, USA

Abstract: Recent advances in nonlinear optical techniques and materials such as quantum wells, nanowires and noble-metal nanoparticles have led to advances in cellular imaging wherein various nanoparticles have been shown to improve both in vitro and in vivo visualization. In this paper, we demonstrate in vitro imaging using multi-photon photoluminescence of gold nanoparticles from two different cell types – Dictyostelium discoideum and mouse embryonic stem cells. By observing nanoparticles we show that embryonic stem cells maintained their ability to proliferate for several passages while grown in the presence of gold nanoparticles. The advantages of multi-photon luminescence using gold nanoparticles have important implications for use in stem cell proliferation experiments and in vitro experiments to monitor differentiation.

Keywords: gold nanoparticles, multiphton absorption induced luminescence, mouse embryonic stem cells, visualization

Introduction

Fluorescence microscopy using multiphoton excitation is a powerful technique that takes advantage of non-linear optical effects for high resolution imaging of biological samples. The underlying principle is to excite the materials to a high energy state by a simultaneous absorption of two or more photons of red or near infrared (NIR) light. The emitted photon has a much shorter wavelength which can be easily extracted from background illumination using a spectral filter. Several organic and inorganic flurophores have been well characterized and researched in past studies for fluorescent labeling. However, due to toxicity and bleaching, their role is practically limited in a biological environment. In some instances, the inability to clear the particles may result in toxicity or the particles themselves may be toxic. Finally, in some cases particles are not phototoxic but the method used in imaging might lead to toxicity.

Quantum Dots® (QDs) (Chan et al 2002; Gao et al 2002) are a popular fluorescent platform that can be used with single or multi-photon imaging techniques. Though QDs are extremely bright and do not bleach, they sometimes blink during excitation and are manufactured with the toxic materials cadmium, selenium, or tellurium. For these reasons there is a continuing search for new nanoparticle imaging platforms that do not suffer from these deficiencies.

In recent years, the use of noble-metal nanoparticles has emerged as a potential alternative to the fluorophore-based staining and labeling of biological samples. The observation of photoluminescence (PL) from gold and copper metals was first reported by Mooridian by subjecting the metal foils to electromagnetic excitations at different optical wavelengths (Mooradian 1969). The resulting PL signal had extremely low quantum yield ($\approx 10^{-10}$) and the phenomenon was attributed to the radiative recombination of photo-excited electrons (in the s-p conduction band) with holes in the *d*-band. In subsequent experiments, the quantum yield of the PL signal was drastically increased by several orders of magnitude ($\approx 10^{6}$) by electric field enhancement in the proximity of

Correspondence: Srinivas Sridhar
Department of Physics, Northeastern University, 360 Huntington Avenue, Boston, MA 02115, USA
Tel +1 617 373 4774
Fax +1 617 373 8589
Email s.sridhar@neu.edu

roughed metal surface (Boyd et al 1986). The effect is even more pronounced in material structures at submicron scale. The metal nanostructures that are significantly smaller than the wavelength of light exhibit strong resonant interactions with incident radiations.

This technique is referred to as Multiphoton-Absorption-Induced Luminescence (MAIL), where the absorption of multiple photons from a near-infrared femtosecond pulsed laser can lead to robust luminescence of metal nanoparticles (Farrer et al 2005). The local field enhancement under the plasmon resonance conditions is a unique feature of nano-sized materials which lead to modification of various linear and nonlinear optical processes at nano-scale. Therefore, optical properties of noble-metal nanoparticles provide enhanced functionality of their usage in a wide range of biological and physical applications. One such application has been in utilizing gold nanoparticles and nanorods for biological cellular imaging. A gold nanorod suspension was injected into the tail vein of a mouse and in-vivo multi-photon luminescence was observed in the ear lobe of nude mice (Wang et al 2005).

Stem cell-based therapies are expected to have a huge impact in the treatment and cure of various diseases and disorders in the future. These cells, given the right conditions, have the ability to differentiate into the constituent cells of various organs (Bjornson et al 1999). When stem cells are used in therapies, it is imperative to have a robust imaging technique to be able to track these cells through migration and differentiation steps. Magnetic nanoparticles have been used to track stem cells through magnetic resonance imaging (MRI) (Wang et al 2005). However, MRI is not a convenient method for routine in-vitro analysis and therefore there is a need for an alternate method for stem cell imaging. In this paper we have demonstrated the use of the MAIL technique to image Au NPs and track their internalization in Dictyostelium discoideum (Dictyostelium) and mouse embryonic stem cells.

Experimental procedures

Cell culture with Dictyostelium discoideum

Our method to visualize and determine phagocytosis of Au-NPs is similar to previously described experiments using bacteria expressing DsRed by Maselli et al (2002). Briefly, vegetative Dictyostelium were cultured in HL-5 axenic medium (Sussman 1987). Cells were harvested, washed, and resuspended in 1.5 mL 20 mM phosphate buffered saline (PBS) at a concentration of 4×10^6 /mL. Stock 10nm Au-NP solution (Ted Pella, Redding, CA, USA) was added to cell suspension at a concentration of 2.5×10^{19}/mL Au NPs for every 4×10^6 Dictyostelium. Au NP solution was used as received without any surface treatment. The cell-particle suspension was placed in a 50 mL beaker and rotated at 130 rpm at room temperature. To distinguish internalized bacteria from those bound to the surface, the assay was developed so that sodium azide was used to release surface-bound particles (Maselli 2002). After 60 minutes, 0.5 mL aliquots were removed and placed into ice cold PBS supplemented with sodium azide (0.1%), washed 3 times, and then resuspended in HL-5. Cells were then placed in a Lab-Tek®II Chamber Coverglass System (Nunc, Naperville, IL, USA) and allowed to attach and spread for approximately 30 minutes.

Cell culture with mouse embryonic stem cells

Mouse embryonic stem (mES) cells, R1, were obtained from Mt. Sinai Hospital (Toronto, ON, Canada). To maintain an undifferentiated state, mES cells were cultured on a feeder layer of mitomycin c-treated primary mouse embryonic fibroblasts (PMEF) in medium containing 80% DMEM, 15% ES cell qualified fetal calf serum (Atlanta Biologicals, Lawrenceville, GA, USA), l-glutamine, 1 mM sodium pyruvate, 1% non-essential amino acids, penicillin/streptomycin, ß-mercaptoethanol and 1,000 units/mL of leukemia inhibitory factor (LIF) (Chemicon, Temecula, CA, USA).

The ability of gold nanoparticles to label undifferentiated mES cells was tested by addition of gold nanoparticles to the culture medium throughout three passages of the embryonic stem cells. Presence of Au-NPs in cell culture medium does not seem to have any affect on cell proliferation and their viability. A similar labeling method has been previously reported for embryonic stem cells with iron oxide nanoparticles (Sykova and Jendelova 2005). The cells were passaged every two days via trypsinization and fed daily with fresh medium containing gold nanoparticles (approximately 10^{18} particles/mL). On the third passage, the fibroblasts and mES cells were plated on a glass-bottom imaging dish (MatTek Corp., Ashland, MA, USA). Prior to imaging, unincorporated gold nanoparticles were removed and unbound from the cells by several washes in fresh medium and a single wash in 1× PBS containing 1% bovine serum albumin (BSA) and 0.1% sodium azide.

Sodium azide treatment was used to maintain similar experimental conditions with the Dictyostelium experiments. The mES cells were imaged via differential interference contrast (DIC) and two-photon luminescence two days after the third passage.

Photon luminescence microscope setup

For MAIL excitation and detection, we used a fusion microscope invented and constructed at Northeastern University, the W.M. Keck 3D Fusion Microscope (Townsend et al 2005, Warger et al 2007). A tunable Titanium-Sapphire laser (Tsunami, Spectra-Physics, Carlsbad, CA, USA) was used in ultra-fast (femtosecond) pulsed mode at 80 MHz for illumination. The range of excitation wavelengths is 700–1100 nm. The laser is coupled into a Nikon TE 2000U microscope (Nikon, Japan) via a Lucid Electronics (Rochester, NY, USA) breadboard that contains a rotating polygon mirror to produce 480 lines per frame and a galvanometric mirror for a xy raster rate of 10 fr/sec (640 × 480 pixels, 325 ns pixel dwell). We used a Nikon 1.45 NA 60× objective for illumination and collection. The returning fluorescent light was not descanned. Instead, a detector was placed in the right camera port as close to the objective lens as possible, to minimize throughput losses and increase detection sensitivity. A "hot mirror" (reflects light × 700 nm and transmits light $\leq$700 nm (03MHG009 (Melles Griot) reflects the scanned illumination light toward the objective lens and transmits the returning fluorescent light toward the detector. To avoid scanning of the returning fluorescent beam across the PMT's active area, the PMT cathode was placed conjugate to the pupil of the objective lens (where the light is stationary). If the PMT were placed conjugate to the image plane, the fluorescent light would scan across the PMT's cathode and any non-uniformity in the active area would appear in the image. On the path to the detector, luminescence passes through a G-2E (565LP–620/60) cube filter set (Nikon) and is detected with a Hammamatsu PMT (HC1024–02, Hamamatsu, Bridgewater, NJ, USA) with a photosensitivity of 89.5 volts/nW.

Results and discussion

The mechanism of two photon luminescence is similar to single photon luminescence in that it is due to the electron-hole pair recombination. In the case of gold nanoparticles, the electron-hole pair is formed when the electrons are excited from the *d*-level to *s-p* level. In general, the two photon luminescence signal is weaker than single photon luminescence but can be enhanced by several orders of magnitude through increased field enhancement effect. Multiphoton absorption cross section is higher when these noble metal structures have a degree of asymmetry associated with them in their structure. It has been reported that luminescence efficiency of these particles is size dependent and is higher from larger particles which have a higher degree of asymmetry associated with them. However, luminescence efficiency can be improved from smaller nanoparticles by exploiting the field enhancement effect. This phenomenon is stronger at the edges and intersection of two or more particles. Therefore, by having a higher concentration of small-sized particles, luminescence intensity or efficiency can be significantly improved. Multiphoton luminescence has also been observed from gold nanoparticles of different shapes such as nanorods and nanostars. Recently, multiphoton luminescence from gold nanosystems has been exploited as a biological imaging modality (Wang et al 2005).

In Figure 1a, a 10 µl drop of a 10 nm gold nanoparticles suspension was placed on a coverslip, and drying was facilitated using a "smear" technique. The incident excitation wavelength was 790 nm which gave the best signal to noise ratio. The image is a representative slice (10 fr/ave.) of a 10, 1 µm step z-stack. The bright spots correspond to luminescence from a cluster of gold NPs. The optical resolution of the light microscope limits the detection of luminescence from individual gold NPs and is therefore detected only from clusters of NPs. Figure 1b is a corresponding transmission electron micrograph (TEM) of gold NPs on a copper TEM grid. The individual gold NPs are 10 nm in size and surrounded by stabilizer molecules. However, during the sample preparation step while drying, gold NPs tend to form aggregates.

The internalization of nanoparticles, through endocytosis, is an important process that has great potential in several areas including drug and gene delivery, particle tracking, and metabolic characterization. The method allows transport of small particles and other biomolecules across the cell membrane into the cells and can be made cell-specific by exploiting the receptors on the cell surface to induce receptor-mediated endocytosis. A gold nanoparticle-based system using a heterobifunctional PEG spacer was used for intracellular tracking and delivery in model cancer cell lines (Shenoy et al 2006). Using thiol chemistry, the surface of these gold nanoparticles was readily modified to attach Coumarin and their internalization was monitored through fluorescence microscopy. Recently, gold nanoparticles have been synthesized to achieve organ-specific localization (Kannan et al 2006). In the work reported here, gold NPs were internalized and subsequent two photon luminescence was observed from two different cell types – Dictyostelium and mES cells.

Dictyostelium was selected since this is a model organism used for the visualization of binding, internalization, and metabolic breakdown of various substrates including tungsten beads, fluorescent latex beads, and externally and internally

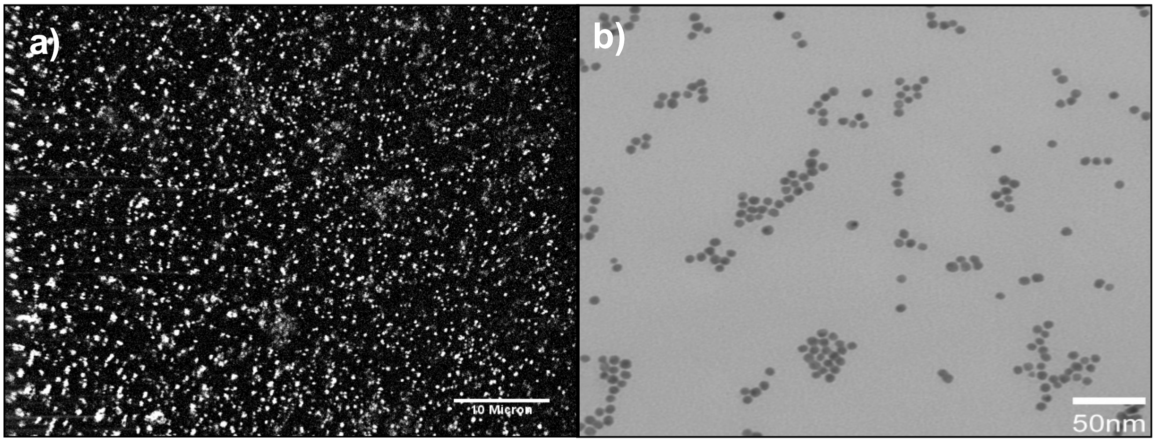

Figure 1 Photoluminescence and structural characterization of gold nanoparticles. (**a**) Multi photon absorption induced luminescence from gold nanoparticles dried on a cover slip and (**b**) Transmission electron microscope (TEM) image of 10 nm gold NPs.

labeled bacteria (Vogel et al 1980; Cornillon et al 2000; Maselli et al 2002; Montet et al 2006). The kinetics of these procedures is well characterized. Vegetative Dictyostelium cells in suspension were exposed to Au-NPs for 60 minutes on a rotary shaker to facilitate internalization. Dictyostelium cells rapidly bind and internalize Au-NPs. When excited with 80-fs pulses at a repetition rate of 80 MHz with a center wavelength of 790 nm, Au-NPs provides a bright internal bioluminescent signal that is not degradable in the phagolysosomal pathway, does not bleach, and does not blink.

Figure 2 shows the results of internalization after repeated washings, to visualize internalized particles in Dictyostelium. In the left brightfield image, aggregates of particles are clearly seen within the cell. The right two-photon panel is a maximum intensity z-projection of the acquired two-photon stack (using Image J) (Rasband 1997–2000). Previous studies have determined that the azide method of washing clearly releases bound particles, and allows only internalized particles to be visualized (Maselli 2002). In addition, further analysis revealed almost 80% of the cells internalized some amount of particle (data not shown). It is clear from this image that the presence of gold NPs does not have any adverse reaction to normal function of these Dictyostelium.

The process of phagocytosis is defined as the internalization of particles greater than 0.5 µm in diameter. Receptor-mediated endocytosis has been shown to be an effective method to label several different types of cells, including stem cells, tumor cells, and acinar cells of the pancreas (Gupta et al 2003; Kim et al 2006). Fluidic uptake of nutrients from the surrounding medium occurs via the process of pinocytosis. The Au NPs we used in this study are 10 nm in diameter. Therefore, by definition, the process of

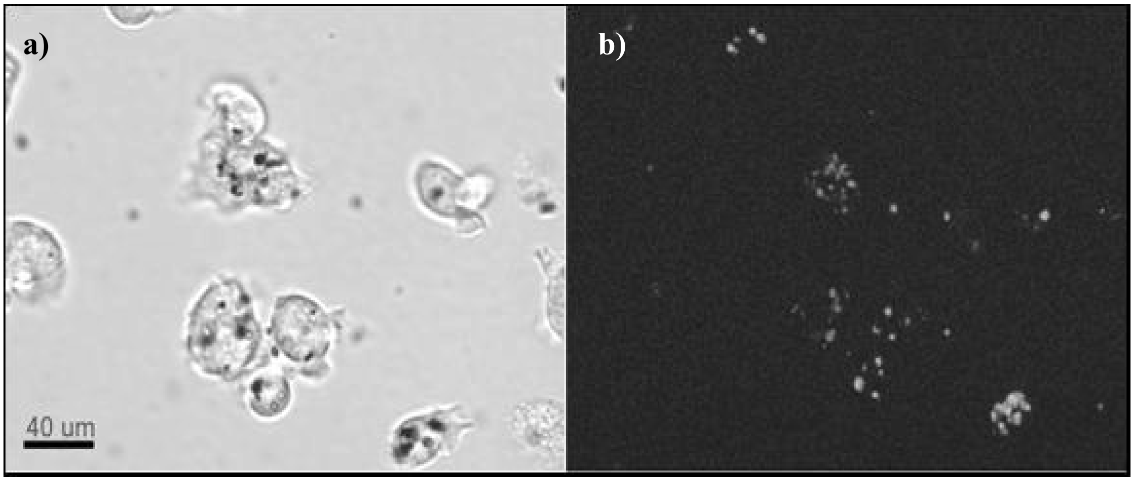

Figure 2 Internalization of Au-NPs in Dictyostelium. Brightfield image (left) and corresponding z-projection of the acquired 2-photon stack (right).

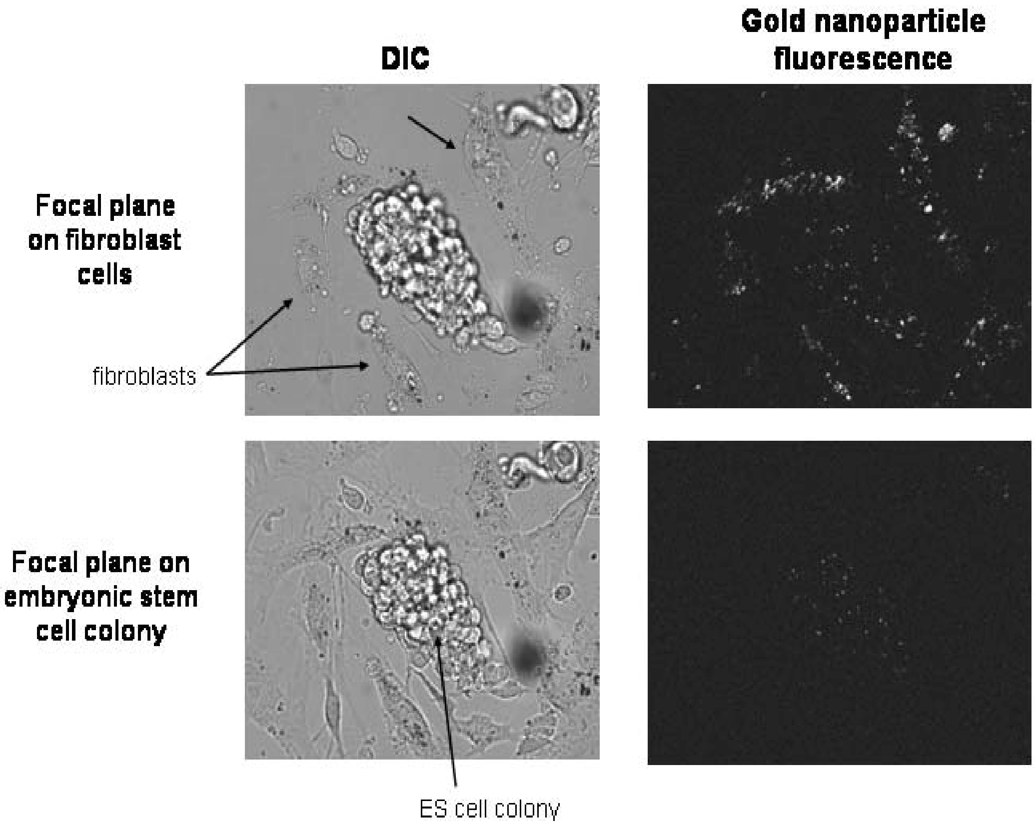

Figure 3 Gold nanoparticles detected via two photon luminescence in mouse embryonic stem cells and mouse embryonic fibroblast cells. In the top panels, the focal plane is on the fibroblast feeder layer, which readily endocytose the particles. A fainter, yet detectable signal is visible when the focal plane is within the embryonic stem cell colony (lower panels).

internalization must be considered either receptor-mediated endocytosis or pinocytosis.

Mouse Embryonic Stem Cells (mESCs) were incubated with gold NPs and were visualized, in vitro, via two-photon luminescence using an excitation wavelength of 790 nm. Luminescence was observed in both the mouse embryonic fibroblasts and undifferentiated mouse embryonic stem cells. To our knowledge, this is the first report of two-photon detection of gold nanoparticle uptake by mammalian stem cells. The cells were passaged every two days and medium replaced along with fresh gold NPs. It is necessary to have a high concentration of gold NPs in the medium to facilitate endocytosis. A similar method was reported in experiments for MRI tracking of stem cells using iron oxide NPs.

The embryonic stem cells grow as a multi-layered colony on top of a layer of nondividing embryonic fibroblast cells (flatter, larger cells) that act as a feeder layer for the stem cells. Since mESCs and fibroblast are both in the same medium gold NPs were internalized in both these cells. The images in Figure 3 were taken as a z-stack on multiple focal planes but only two focal planes from the stack are shown. Top images of Figure 3 focus on the fibroblast feeder layer, while the bottom images of Figure 3 focus on the stem cell colony and therefore, luminescence from the out of focus cells is not visible.

The embryonic stem cells were cultured in the presence of gold nanoparticles for several passages and continued to divide and grow, indicating cell viability. The fibroblasts were labeled to a much greater extent than the mES cells, presumably due to greater endocytosis of the unlabeled gold nanoparticles. The gold nanoparticles are clearly visible within the fibroblast cells and are therefore not merely bound to their surface (Figure 3). The luminescence signal by the gold nanoparticles within the fibroblast cells was bright in comparison to the signal detected within the embryonic stem cells.

Gold nanoparticles could also be detected via two-photon luminescence throughout the multi-layered embryonic stem cell colony, indicating cellular uptake of the particles by the embryonic stem cells. Within the z-stack images obtained (Figure 3), the images in a higher focal plane corresponded to the multi-layer mouse embryonic stem cells, which grow as multi-layer colonies on top of the fibroblast feeder layer. Although we have not proven endocytosis as the method of internalization of the gold nanoparticles, it is likely that the gold nanoparticles were endocytosed by the mouse embryonic stem cells.

There are several advantages to using multi photon luminescence from gold NPs as an imaging tool. Embryonic stem cells labeled with gold NPs may allow in vitro tracking of these cells as they differentiate into more specialized cell types. For example, mESCs could be labeled with gold NPs and cultured in the presence of unlabeled cells (either different populations of embryonic stem cells or a different cell type). Using the capabilities of the Keck 3D Fusion microscope, growth rate and morphology of live, dividing cells could be analyzed and different cell types distinguished via two-photon detection of these gold NPs. Recent observations have shown that undifferentiated embryonic stem cells may have phagocytic characteristics and gene expression similar to macrophages (Charriere et al 2006). Two-photon tracking of gold NPs uptake within embryonic stem cells may be useful in further analysis of this observation.

Summary and conclusions

The direct observation of luminescence from gold nanoparticles eliminates the use of additional labeling through organic fluorophores. Other nanoparticles-based imaging systems such as inorganic semiconductor nanoparticles or quantum dots are available but they have their own limitations. Gold nanoparticles offer advantages over these systems in that they are non-cytotoxic (Connor et al 2005), do not photobleach, and do not blink.

Using MAIL on gold nanoparticles (Au-NPs) internalized during biological processes has many advantages. By definition, the non-linear excitation method of multiphoton particle excitation allows for precise optical sectioning characteristics not available using conventional confocal fluorescence microscopy (CFM) techniques. In addition, the non-toxic nature of Au NPs is an added benefit compared to the heavy metals used in the manufacture of quantum dot based fluorescent NPs. The MAIL imaging also provides three-dimensional optical sectioning without absorption (which would lead to photobleaching and phototoxicity) above and below the plane of focus.

This assay using MAIL from gold nanoparticles will be useful in investigations of the molecular machinery of endocytosis, post-internalization vesicle trafficking, lineage tracking, and cellular motility assays. High percentage uptake, coupled with the increased resolution of two-photon imaging, could be a benefit to studies involving the lineage tracking of stem cells. Currently, these tracking methods include the use of QDs, transgenics (Tanaka et al 2005), transiently transfected cells (Kouskoff et al 2005), and preliminary gene therapeutic strategies (Kume et al 2000). While these methods all have their advantages, the Au-NPs are non-toxic, do not need any additional conjugates, they are easily introduced into a model system, and they are not susceptible to metabolic breakdown. The last of these advantages, resistance to breakdown, needs to be further analyzed as this could be a detriment to the system. The axial resolution that can be accomplished using the two-photon imaging method will also allow us to more closely follow the progress of the particles/vesicles through the cell during protein trafficking and localization studies.

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