Probing Molecular Cell Event Dynamics at the Single-Cell Level with Targeted Plasmonic Gold Nanoparticles; a Review

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Summary

Over the last several decades, plasmonic nanoparticles, in particular those of gold have been extensively employed in biological investigations due to their unique optical and physical properties as well as their safe use in the biological environment. In this review, the utility of gold nanoparticles to probe intracellular environments and reveal dynamic measurements in real-time at the single-cell level is described. The fundamental principles and their associated methodologies behind their unique interaction with light and the associated methodologies are discussed. Gold nanoparticle synthesis and functionalization with organelle-targeting ligands is also highlighted. The review ends with an outlook on the challenges and efforts that need to be taken in order to fully realize the potential of utilizing gold nanoparticles in the real-time molecular mapping of single cells.

Keywords

Plasmonic nanoparticles, gold nanoparticle, surface plasmon resonance, Raman scattering, Rayleigh scattering

Introduction

The exploration of time dependent cellular behavior, especially in terms of treatment response, has generally been conducted using population-based studies, such as western blots, immunoassays, reverse-transcription quantitative PCR and proliferation assays[1, 2]. While these approaches provide information about the average, the inherent heterogeneity that exists within populations as well as the heterogeneity that results from the biological environment is lost. Evidence suggests that these heterogeneous subpopulations play influential roles in treatment

resistance, especially with regards to cancer[3, 4]. Therefore, in order to fully understand how these subpopulations affect disease treatment, techniques that monitor cellular dynamics at the single cell level are becoming increasingly important. Specifically, single cell imaging techniques have been heavily utilized to investigate transcriptional/gene regulation, protein translocation, cell-cell interactions and signaling, cell fate (*i.e.* division, apoptosis) and tumor infiltration[5, 6].

Single cell analysis techniques rely heavily on fluorescence readout systems to acquire live, real-time imaging of dynamic cellular processes[5, 6]. Fluorescent imaging agents can range from cell permeable dyes to fluorescently modified proteins and firefly luciferase reporters. Although fluorescence is an invaluable tool for single-cell imaging, it still has several intrinsic limitations: i) it is susceptible to photobleaching, which limits its use in probing real-time experiments over prolonged processes such as cell division; ii) fluorescent probes commonly have overlapping emission spectra limiting the number of cellular processes that can be monitored at one time; iii) the fluorescence signal does not contain molecular vibration information, which inhibits its use in studying accurate molecular profiles inside living cells.

In an effort to complement the information obtained from live-cell fluorescence microscopy, the development of stable, bright and sensitive optical probes, mainly plasmonic nanoparticles (PNPs), have been employed to monitor long-term cellular processes[7-9]. Noble PNPs (*i.e.* gold and silver) have extraordinary optical properties stemming from their enhanced strong light scattering properties resulting from the decay of their localized surface plasmon resonance (LSPR) formed from their coherent excitation with resonant laser radiation. Moreover, as their name entails, the dimensions of the nanoparticles are on the nanometer scale allowing for their penetration across cellular membranes and interaction with various biological

molecules. Compared with fluorescence-based imaging techniques, imaging using enhanced scattering from plasmonic nanoparticles has several inherent advantages: i) the Rayleigh/Mie scattering from plasmonic nanoparticles provide very bright and stable light scattering signals, which are vital for long-term imaging of living cells, ii) the plasmonic field near the surface of the nanoparticles or between coupled nanoparticles generate “hot spots”, which can greatly enhance the Raman signals of molecules close to the plasmonic fields, allowing for sensing of the changes in the molecular microenvironment surrounding the plasmonic probes during the different cellular functions or as the cell dies, iii) the plasmonic field can also be modulated to enhance or quench the fluorescence of nearby molecules, by controlling the separation distance between them, enabling the monitoring of molecular events through the “ON” and “OFF” status of the fluorescence, and iv) the surface plasmon resonance mechanism can be used to produce multiple physical effects, *e.g.* photothermal, photoacoustic, etc., which allow for multi-modal single cell imaging.

In this review, the unique physical and optical properties of PNPs, specifically gold nanoparticles (AuNPs), as well as their functionalization to aid in organelle targeting will be discussed. Furthermore, the fundamental imaging modes using PNPs and their utilization in single-cell imaging studies will be described. Lastly, potential improvements to various imaging and analysis parameters will be discussed in an effort to move towards advanced real-time molecular mapping.

Plasmonic Nanoparticles

Fundamental Theory of Localized Surface Plasmon Resonance (LSPR)

The unique optical properties found when using AuNPs originate from the localized surface plasmon resonance (LSPR). As the complete description of the LSPR is not the focus of this review, a truncated discussion is provided. In-depth discussions of LSPR theory are available in several other works and reviews [10-12].

Fig. 1 Graphical illustration demonstrating the collective oscillation of the free conduction band electrons in plasmonic gold nanoparticles, as a result of absorption of resonant radiation giving rise to the localized surface plasmon resonance (LSPR). The collective electronic oscillation decays by either giving rise to strong electromagnetic fields (strong scattered Rayleigh or Raman light) or by exciting the lattice vibrations of the nanoparticle that is converted into lattice heating used in photo-thermal cancer therapy.

For metallic nanoparticles with a size smaller than the “skin depth”, the electric field of light can easily penetrate the nanoparticles and grasp the conduction band electrons, resulting in their coherent displacement away from the positively charged metallic ions in the lattice (Fig. 1). Then, the electric dipole of the particles applies a restoring force that pulls the polarized electrons back to the lattice. Hence, the coherent oscillation of the conduction band electrons of the nanoparticles can be approximately considered as a harmonic oscillator driven by the energy resonant light wave. Only light with a wavelength (*i.e.* energy) in resonance with the oscillation is able to excite the LSPRs. The excited plasmon oscillation can then be damped through either strong radiative scattering of the light or by non-radiative (essentially producing heat) paths. The

ratio of probabilities of these two processes depends on the size and shape of the plasmonic nanoparticle.

Using spherical nanoparticles (≤ 30 nm) as an example, according to Mie theory it is sufficient to consider only the dipolar term to solve Maxwell's equations. This approximation is also called the quasistatic or Rayleigh limit. The scattering and absorption cross-sections C_{sca} and C_{abs} of spherical nanoparticles in the Rayleigh limit are given by the following expressions [13-15]:

$$C_{sca} = \frac{8\pi}{3} k^4 a^6 \left| \frac{\varepsilon - \varepsilon_m}{\varepsilon + 2\varepsilon_m} \right|^2 \quad (4)$$

$$C_{abs} = 4\pi k a^3 \text{Im} \left| \frac{\varepsilon - \varepsilon_m}{\varepsilon + 2\varepsilon_m} \right| \quad (5)$$

$$C_{ext} = C_{abs} + C_{sca} \quad (6)$$

where a is the radius of the nanoparticles, $k=2\pi/\lambda$, ε and ε_m are the dielectric function of the nanoparticles and surrounding medium, and C_{ext} is the total extinction cross-section.

Both the radiative (*i.e.* light scattering) and non-radiative (*i.e.* heat) properties of nanoparticle relaxation can be used for bio-applications. From the above equations, we know that the efficiency of absorption or scattering depends on the particle size, *i.e.* larger nanoparticles exhibit more scattering while smaller nanoparticles exhibit higher absorption cross-sections. Therefore, larger particles (≥ 30 nm) are ideal for cellular imaging using conventional dark-field microscopy that relies on the intense light scattering from the AuNP. For very small particles with $a \ll \lambda$, the absorption, scaling with a^3 , dominates over the scattering, which scales with a^6 . Thus it is very difficult to pick out the scattering signals from single nanoparticles with sizes below 15 nm. Imaging of very small nanoparticles (< 10 nm) can thus usually only be achieved

using photothermal techniques relying on slower scaling of the absorption cross-section with size. Thus, Mie theory provides a very important principle for the use of plasmonic nanoparticles in cell imaging and sensing; the size of the nanoparticles and their absorption or scattering cross-sections should be carefully considered when designing a plasmonic-cell imaging systems.

Another unique property of the LSPR is the distribution of the plasmon field. Differing from the plasmon field at the bulk metal interface, which scales as $1/r$ (r being the distance from metal surface), the field of LSPR scales as $1/r^3$. For a spherical nanoparticle polarized in an electric field of E_0 , the distribution of the electric field inside and outside the nanoparticles can be given by [13, 16]:

$$E_{in} = \frac{3\varepsilon_m}{\varepsilon + 2\varepsilon_m} E_0 \quad (7)$$

$$E_{out} = E_0 + \frac{3n(n \cdot p) - p^2}{4\pi\varepsilon_0\varepsilon_m} \frac{1}{r^3} \quad (8)$$

$$p = 4\pi\varepsilon_0\varepsilon_m a^3 \frac{\varepsilon - \varepsilon_m}{\varepsilon + 2\varepsilon_m} E_0 \quad (9)$$

where p is the dipole moment, and n is unit vector in the direction of the point of interest, p .

From the above equations, the electric field inside the nanoparticles is homogeneous, and does not rely on the spatial position within the nanoparticle. Moreover, since the external electric field E_{out} scales as $1/r^3$, the plasmon field intensity decreases rapidly with increasing distance from the nanoparticle surface. Such properties result in a localized, high density of plasmon field confined around the nanoparticles. It should be noted that when utilizing the plasmonic field for biomolecule sensing applications (*i.e.* surface enhanced Raman spectroscopy), the highest Raman scattering intensity is observed at the plasmonic “hot spots”. These hot spots are formed between plasmonic nanoparticles that are in close enough proximity (interparticle separation $<$

$0.5 \times \text{nanoparticle diameter}$) to allow for the coupling of their LSPRs giving rise to strong Raman scattering from molecules at this hot spot location.[17]

Nanoparticle Synthesis

For single-cell imaging applications, AuNPs are extremely advantageous due to their facile tunability. AuNP preparation typically follows the colloidal synthesis method, which utilizes a metal precursor, a reducing agent and a stabilizing agent. Changing the reducing and stabilizing agents as well as the temperature and stirring rate during the synthesis can alter the size and shape of AuNPs, which can then be further modified for specific cell targeting applications. While there are many AuNP shapes, the synthesis of the most commonly used particles are described below.

Fig. 2 Common AuNPs used for investigating intracellular environments. Illustrations, electron micrographs and extinction spectra of (a) gold nanospheres, (b) gold nanorods and (c) gold nanocages. The extinction spectra of

AuNPs are influenced by size (a), aspect ratio (b) and gold deposition amount (c). Reprinted with permission from (a) [18] Copyright 1999 American Chemical Society, (b) [19] Copyright 2009 Wiley-VCH Verlag GmbH & Co. KGaA, and (c) [20, 21] Copyright 2008 American Chemical Society, 2006 American Chemical Society.

Gold nanospheres (AuNSs) are one of the most heavily used AuNPs for single-cell imaging due to their facile synthesis (Fig. 2a) [22]. AuNSs are commonly synthesized following Turkevich's citrate reduction method that results in stable, monodisperse particles with diameters between 15-50 nm [23, 24]. In this method, spheres are formed through a nucleation process which involves the reduction of Au (III) from aqueous chloroauric acid to Au(0) atoms using trisodium citrate with the aid of gentle heating and stirring. Once nucleation has occurred small AuNS (~2-4 nm) undergo aggregation to form larger spheres. Successful formation of AuNS is indicated by the color change from a colorless/yellow solution to a red wine color. Final particle diameters are determined by the stoichiometric ratios between gold and sodium citrate; large gold to sodium citrate ratio result in larger particle diameters. Recent advances have been made to increase the monodispersity of AuNS ranging from 50-300 nm in diameter through a seed-mediate growth method.[25, 26] In these strategies, AuNP seeds are first synthesized using the citrate reduction method and then added to an aqueous gold chloride solution. The gold chloride solution is then reduced using sodium citrate and either hydroquinone[25] or ascorbic acid[26]. While AuNSs with particle diameters below 10 nm are not readily used for single cell experiments, if desired they can be synthesized using the two-phase Brust method is typically used [27].

Gold nanorods (AuNRs) represent another useful nanoparticle construct for single-cell imaging as their LSPR overlaps with the biologically relevant NIR optical window (*i.e.* 700-900 nm, Fig. 2b). Jana *et al.* first reported the seed-mediated growth method in 2001 [28]. This method was then expanded upon by El-Sayed and co-workers in 2003 [29]. The seed-mediated

growth method utilizes two separate synthesis solutions to aid in nanorod growth. The first solution, a seed solution, is prepared via the reduction of aqueous chloroauric acid with NaBH_4 in the presence of CTAB (cetyltrimethylammonium bromide), and produces small gold nanospheres with diameters of 3-5 nm. A growth solution is then prepared using ascorbic acid, AgNO_3 (silver nitrate), and CTAB. The gold seeds are then injected into the growth solution resulting in the anisotropic reduction of Au^+ to AuNRs. Increasing the AgNO_3 concentration in the growth solution can elongate the longitudinal axis of AuNRs, however the transverse axis usually remains constant at 10 nm. A seedless growth method has also been reported by both the Murphy and El-Sayed groups [30, 31], and results in AuNRs with dimensions of $\sim 25 \times 5$ nm. While AuNR synthesis typically does not include stirring during the growth phase, Kozek *et. al.* showed that stirring in combination with the continuous addition of ascorbic acid does not interfere with AuNR formation and can actually improve control over the final AuNR shape and size.[32] Using this strategy, the synthesis could also be scaled up from 100 mL to 1 L. Additionally, Liz-Marzán and co-workers explored the use of 5-bromosalicylic acid (5-BrSA) as a cofactor and prereducing agent of Au(III) in the seed-mediated synthesis of AuNRs.[33] Following this method, 5-BrSA is introduced into the CTAB-growth solution to partly reduce Au(III) to Au(I). Ascorbic acid is then added to complete the gold reduction to Au(I). The addition of the seed solution causes the final reduction to Au(0) and the subsequent growth of nanorods. By increasing the 5-BrSA prereduction times the longitudinal LSPR of the AuNRs can be tuned from 850 to 650 nm.

Gold nanocages (AuNCs), which were first reported by Xia's group in 2002 [34, 35], have found an important use as cellular contrasting agents for photothermal imaging [36], photoacoustic imaging [37], multi-photon imaging [37], and optical coherence tomography (Fig.

2c) [38]. AuNCs offer excellent optical performance in the biologically transparent NIR region, similar to AuNRs, and can also be loaded with various disease fighting agents, as they are synthesized with hollow interiors. Their synthesis is regarded as relatively simple and involves the galvanic replacement of silver nanocubes and chlorauric acid [35, 39]. Briefly, 40 nm silver nanocubes are prepared by the reduction of AgNO_3 or silver trifluoroacetate (CF_3COOAg) by ethylene glycol in the presence of poly(vinyl pyrrolidone) (PVP) and trace amounts of sodium sulfide (Na_2S). The silver nanocubes are then used as the template for the formation of AuNCs using the galvanic replacement of Ag^+ atoms with Au^{3+} ions from chlorauric acid in the presence of PVP. This replacement occurs spontaneously as silver has a lower reduction potential compared to gold, causing the oxidation and displacement of silver atoms as gold ions are reduced onto the nanocube surface.

Nanoparticle Biofunctionalization to Different Cellular Components

Specific organelle targeting is an invaluable feature when targeting or studying biochemical processes at the single-cell level [40, 41]. Improper targeting can lead to an inaccurate depiction of the investigated molecular events, especially if targeted drug delivery is being explored. Fortunately, the surface of AuNPs can be easily modified to contain organelle-targeting ligands and peptides. Functionalization with biological targeting ligands usually occurs through a chemisorption covalent Au-S bond [22], as this bond is remarkably strong with a reported bond energy of $44 \text{ kcal}\cdot\text{mol}^{-1}$ [42]. In order to confirm proper targeting and intracellular localization, transmission electron microscopy (TEM) is often used. Additionally, confocal microscopy in conjunction with organelle specific fluorescent dyes can be used if the AuNPs are also functionalized with a fluorescent dye.

(a) Nucleus

The nucleus, regarded as the control center of the cell, houses important genetic material (*i.e.* DNA), and is responsible for maintaining proper cellular function. As such, targeting the nucleus has become a strategy for improving drug delivery and tracking cellular functions *via* single-cell molecular imaging [7, 9, 41, 43]. Since the nucleus is enclosed by the nuclear envelope, which is a double membrane, transport into the nucleus occurs through nuclear pore complex (NPC) structures. NPCs are equipped with a 9 nm diameter channel that limits the passive delivery of macromolecules to those that are less than 50 kDa [44]. All entities above 50 kDa require a nuclear localization signal (NLS) for successful translocation.

NLSs are placed into two groups, classical and non-classical, depending on whether or not they contain stretches of basic amino acids. Classical NLSs are equipped with the stretches of basic amino acids and can either be monopartite or bipartite. Monopartite NLSs have been best exemplified in SV40 large T antigen and have the characteristic PKKKRKV amino acid sequence [45]. NLSs with two stretches of basic amino acids separated by 10-12 spacer amino acids, or bipartite signals, have been best understood in the *Xenopus* protein nucleoplasmin and contain the sequence KR[PAATKKAGQA]KKK [46]. For successful conjugation to AuNPs, NLS peptides are modified at the C-terminus to contain a cysteine residue. This modification allows for the direct, covalent attachment of the NLS peptide to the AuNP surface through the strong Au-S bond and has been utilized extensively by our group for the successful delivery of AuNPs to the nucleus of human cells.[47, 48]

(b) Mitochondria

Mitochondria are responsible for supplying energy (*i.e.* adenosine triphosphate, ATP) to the cell through oxidative phosphorylation. These “powerhouse” organelles are also heavily involved in other important cellular functions such as apoptosis, cytosolic Ca^{2+} uptake and storage, and regulating ROS. As such, targeting the mitochondria has recently become a strategy for disease therapy and probing this environment could provide valuable information about various diseases (*i.e.* obesity, Parkinson’s, and cancer) [41].

Mitochondrial targeting is generally achieved using two methods: (1) delocalized lipophilic cations (DLCs) or (2) mitochondrial targeting sequence (MTS) peptides. The most popular DLC for conjugation with nanoparticles is triphenylphosphonium (TPP) [49]. TPP makes the nanoparticle surface cationic and induces mitochondrial accumulation in response to the high membrane potential [50]. MTS peptides exploit the sequences in endogenous proteins that are naturally translocated into mitochondria. These peptides are usually 20-40 amino acids in length and are composed of positively charged and hydrophobic amino acids.[41, 49] Additionally, they are also known to contain amphipathic α -helices. Similar to NLSs, MTS can be modified to contain a terminal cysteine to allow for Au-S covalent attachment.

(c) Peroxisome

Peroxisomes represent another energy producing organelle, as peroxisomes are a site for fatty acid degradation. Detoxification of hydrogen peroxide and glyoxylate also occurs within peroxisomes [51]. Targeting and probing this organelle could, therefore, lead to a better understanding of age-related diseases. The peroxisome is typically targeted using peroxisomal targeting signals (PTSs), which is recognized by PexP5, a peroxisomal protein receptor [52, 53]. Peroxisomal targeting signal-1 (PTS1) is the most prevalent and has a characteristic C-terminal

tripeptide SKL (serine-lysine-leucine) [54, 55]. Consensus C-terminal sequences, S/C/A-K/H/R-L/M, can also be seen in endogenous peroxisomal-tagged cargo [54, 55]. An algorithm, described in 2003, is available to predict the relative strength of a PTS1 based on the tripeptide sequence as well as the following 9 amino acids [56-58]. Peroxisomal targeting signal-2 (PTS2) is less common and contains a consensus sequence of R/K-L/I/V-X₅-Q/H-L/A, where X is any amino acid [59]. It should be noted that AuNPs, to our knowledge, have not been used to probe this organelle, but they have been shown to cross the peroxisome membrane when tagged with an SKL sequence [60].

Fig. 3 Schematic illustration of the strategy behind the surface modification, lipid binding, and AuNP immobilization on a cell membrane. (a) CTAB stabilized AuNPs are first PEGylated and then functionalized to contain a maleimide functional group. (b) The maleimide-AuNPs are then conjugated to a liposome that contains a thiolated lipid (PTE-SH, red). (c) Fusion of maleimide-AuNP decorated liposomes with the cell membrane. Reprinted with permission from [61]. Copyright 2010 American Chemical Society.

(d) Cell Membrane

The cell membrane represents an important cellular component due to its involvement in cellular protection, nutrient recognition and signaling events [62]. Disruption of the cellular

membrane can result in apoptotic or necrotic cell death. Thus, monitoring the integrity as well as cellular signaling across the membrane can provide valuable information on cell-to-cell communication, as well as responses to disease therapy and environmental changes (*i.e.* pH, temperature, *etc.*). To date, the most common use of cell membrane targeting *via* AuNPs has been to differentiate diseased cells from healthy cells [36, 63, 64]. In doing so, the general method for AuNP immobilization is through antibody-membrane receptor interactions, such as that used in 2006 by El-Sayed and co-workers when they successfully attached anti-EGFR labeled AuNRs to the surface of cancer cells [64]. Recently, in 2010, Ba *et. al.* reported an alternative immobilization strategy through functionalization with small liposomes [61]. In this approach, AuNPs are first modified with polyethylene glycol (PEG) and then maleimide. Following maleimide derivatization, the AuNPs can be conjugated to thiolated lipids within liposomes. The liposomes then allow for fusion with the cell membrane (Fig. 3). As most microscopy techniques rely on 2D imaging, Xia and co-workers have reported an I₂/KI etching method that can be employed to differentiate AuNPs that have been successfully immobilized on the cell surface from those that are internalized [65].

Utilizing LSPR for Bio-Imaging: Fundamental Principles

As AuNPs strongly absorb and scatter light at their LSPR frequency, they can be used as *in vitro* and *in vivo* imaging contrast probes. In conjunction with their excellent optical properties, their nanometer size allows their use in subcellular, quantitative imaging of biological molecules (*i.e.* DNA, proteins, viruses, *etc.*) in the cell's endogenous environment. AuNP bioimaging can be performed using the various plasmon-enhanced techniques, which are briefly discussed below.

(a) Rayleigh/Mie Scattering

Enhanced elastic light scattering (*i.e.* Rayleigh scattering) resulting from the excitation of the AuNP's LSPR has been used in various bio-imaging settings to aid in the discrimination between healthy and diseased cells, the identification of cell-surface receptors, and molecular recognition applications [64, 66-70]. As illustrated in equation 4, the scattering cross-section of a AuNP is largely influenced by the particle's size, with increasing diameters exhibiting greater scattering [71]. Thus, for imaging applications AuNPs with diameters greater than 30 nm are generally used.

Fig. 4 Rayleigh scattering of AuNPs. (a) Schematic diagram of a traditional dark field microscopy set-up for acquiring Rayleigh scattering from gold nanoparticles. (b) Rayleigh scattering images of AuNSs (top) and AuNRs (bottom) acquired using dark field microscopy. The wavelength of the strongly scattered light is dependent on the size and shape of the gold nanoparticle. Reprinted with permission from [64]. Copyright 2006 American Chemical Society.

The most common methodology used to acquire Rayleigh scattering images and spectra is dark-field microscopy [72]. Dark-field microscopy utilizes white light that is guided through a dark-field condenser that blocks the centered light rays from reaching the sample, thus only

allowing scattered light to enter the objective (Fig. 4a). Typically, a color-CCD camera is used to acquire images. It should be noted that the objective must have a lower NA than the dark-field condenser as this ensures that only the oblique light rays that interact with the sample are collected, while those that miss the sample are not collected and therefore appear dark. Since AuNPs strongly scatter light at their LSPR, they will appear as bright spots, with their color dependent on their size and shape (Fig. 4b), and provide a relatively large contrast to the complex scattering from the inherent cellular structure. In order to obtain spectral information, a spectrometer can be introduced into the dark-field microscope such that the spectrometer's entrance slit position is adjusted to the image plane of the microscope. Scattered light comprised of different wavelengths is collected in the objective and dispersed by the grating within the spectrometer to a cooled detector. Using Rayleigh light scattering for optical detection, spatial resolutions are restricted by the diffraction barrier with lateral and axial resolutions of 300 nm and 800 nm, respectively [73]. Additionally, microsecond temporal resolution with 1-2 nm spatial precision/localization has been achieved [74, 75]. While this spatial resolution does not exceed the diffraction limited resolution seen with traditional optical microscopy, the scattering cross section of AuNPs is orders of magnitude greater than that of fluorophores thus allowing for amplification of biomolecule signals. This amplification enables the live, real-time monitoring of molecular events that occur at levels below the detection limit of commonly based fluorescence techniques; flow cytometry and fluorescence microscopy require several thousand biological events to provide an ample signal to noise ratio.

(b) Plasmon-enhanced Raman (SERS/PERS)

As previously described by Equation 8, AuNPs have highly localized plasmon (*i.e.* electromagnetic) fields around their surface that scales by a factor of $1/r^3$, where r is distance from the surface of the nanoparticle. Therefore, when a molecule is located near the surface of a AuNP, the excited localized plasmon field can cause an enhancement of the molecule's Raman scattering. This form of Raman enhancement is described by an electromagnetic mechanism in which the enhancement factor, E , is determined by the local electric-field enhancement factor at the incident frequency, $E(\omega)$, and the corresponding factor at the stokes-shifted frequency, $E(\omega')$, as shown in Equation 10 [76].

$$E = |E(\omega)|^2 |E(\omega')|^2 \quad (10)$$

Under experimental conditions, the analytical Raman enhancement factor (AREF) can be defined as the integral intensities of the strongest band in the SERS spectrum to that of the conventional Raman spectrum, with also taking into account the concentrations of the analytes studied (Equation 11) [77].

$$AREF = \frac{I_{SERS}/c_{SERS}}{I_{Raman}/c_{Raman}} \quad (11)$$

In practice, isolated AuNPs can provide SERS enhancement factors of up to 10^3 - 10^4 , while aggregated AuNPs have been shown to produce up to 10^{12} enhancement [76]. Within the aggregated AuNP construct, “hot spots” are created from plasmon coupling due to the close proximity of two or more AuNPs. Plasmon coupling not only results in an enhanced electromagnetic field, but also leads to a red-shift in the resonance. As the cell is composed of many Raman active molecules that can contribute to a high background signal, targeted hot spot formation using AuNPs is extremely advantageous when investigating intracellular environments. Given the large Raman enhancement provided by AuNPs, intracellular molecules can be detected with a spatial resolution of 65 nm and a temporal resolution of 50 ms [78].

Accordingly, Kang *et. al.* were recently able to not only image but also differentiate subcellular organelles (nucleus, mitochondria and cytosol) by utilizing SERS and AuNPs functionalized with Raman reporter molecules and organelle targeting peptides [79]. It should be noted that although the molecular spatial resolution is below the diffraction-limit, imaging AuNPs themselves during SERS experiments is usually conducted using dark-field optics and thus the AuNP spatial resolution still lies at the diffraction barrier.

(c) Plasmon-modulated Fluorescence (Enhancement/Quenching)

The LSPR can also be utilized to regulate the fluorescence output of a selected fluorophore. There are three main mechanisms by which Plasmon-modulated fluorescence can occur: (1) energy transfer from the fluorophore to the AuNP resulting in fluorescence quenching, (2) an increase in the electromagnetic field at the surface of the AuNP leading to fluorescence enhancement, and (3) the alteration in the radiative decay rate of the fluorophore causing variations in the fluorescence lifetime [80, 81].

The mechanism by which the AuNP influences fluorescence is distance dependent. When a fluorophore is located in close proximity to the AuNP surface (*i.e.* 1-3 nm), the non-radiative energy transfer from the fluorophore to the AuNP dominates and quenching is observed. However, if a fluorophore is located with 4-10 nm of the AuNP surface, fluorescence enhancement is seen due to the increased electromagnetic field at the particle surface and in turn the increased photon flux. The relationship between the emitted fluorescence (F), the electromagnetic field (E), and the illumination photon flux (q_p) is described in Equations 12 and 13 [80].

$$F = \sigma q_p \phi \quad (12)$$

$$q_p \propto |E|^2 \quad (13)$$

where σ is the fluorophore's absorption cross-section, q_p is the photon flux of the excitation source, and ϕ is the fluorophore's quantum yield. The third mechanism by which the fluorophore's lifetime can be influenced via the presence of a AuNP can occur anywhere between 0-20 nm from the AuNP surface. In this mechanism, the presence of a AuNP increases the radiative rate by Γ_m , leading to an increase in the fluorophore's ϕ and a decrease in the fluorescence lifetime. The ϕ and lifetime of a fluorophore in the proximity of a AuNP are described by [80, 82]:

$$\phi_m = (\Gamma + \Gamma_m) / (\Gamma + \Gamma_m + k_{nr}) \quad (13)$$

$$\tau_m = 1 / (\Gamma + \Gamma_m + k_{nr}) \quad (14)$$

where Γ is the radiative rate, k_{nr} is the non-radiative rate, and Γ_m is a new radiative rate due to the AuNP.

As many biological systems can be modified to bind to fluorescent tags that are protein specific and even some popular chemotherapeutic drugs are inherently fluorescent (*i.e.* doxorubicin), the use of plasmon-modulated fluorescence can be a valuable tool in cell signaling and drug delivery investigations. While the diffraction barrier again limits the spatial image resolution of AuNPs themselves, the distance dependent modulation of fluorescence allows for molecular resolution below 25 nm [83]. Additionally, plasmon-modulated fluorescence can be coupled with plasmon enhanced Raman scattering as these signals are often the inverse of each other, with fluorescence signals that are turned “on” at increasing distances from the AuNP surface while Raman signals are turned “off”, and vice versa.

Fig. 5 Schematic illustration of fluorescence energy transfer between a fluorescent dye/protein and a AuNP. (a) Quenching of the fluorescent dye's emission occurs with the dye is within close proximity to the AuNP surface. (b) Restoration of the dye's fluorescent emission occurs when the linker between the dye and AuNP is cleaved.

(d) Plasmon-resonance Energy Transfer (PRET)

Plasmon-resonance Energy Transfer (PRET) involves the same principles of Förster resonance energy transfer (FRET) with the added benefit of using a plasmonic nanoparticle to extend the energy transfer distance. In FRET, when light energy is absorbed by the donor fluorophore, an electron is excited whose energy is transferred to an acceptor molecule that is at a distance less 10 nm away. The donor and acceptor must be in close proximity so that the acceptor molecule is within the electromagnetic field generated by the donor's excited electron.

Since this mechanism occurs through resonance coupling, the fluorescence emission of the acceptor increases, and the fluorescence emission of the donor decreases. The efficiency of energy transfer, Φ_T , is dependent on several factors [84]: (1) the distance between the donor and acceptor, (2) the spectral overlap of the donor's emission spectrum and the acceptor's absorption spectrum, (3) the relative orientation of the donor's emission dipole moment and the acceptor's absorption dipole moment, (4) the donor emission quantum yield in the absence of the acceptor and (5) the refractive index of the medium between the donor and acceptor. Equation 15 describes the transfer efficiency between a donor and acceptor.

$$\Phi_T = \frac{k_T}{k_D + k_T} \quad (15)$$

where k_T is the rate of energy transfer and k_D is the emission rate constant in the absence of transfer. The distance between the donor and acceptor where energy transfer is 50% is referred to as the Förster distance (R_0) and is defined by Equation 3.

$$R_0/cm = \sqrt[6]{(8.785 \times 10^{-25}) \Phi^D \kappa^2 n^{-4} J(\lambda)} \quad (16)$$

where:

Φ^D = fluorescence quantum yield of the donor in the absence of the acceptor

κ^2 = dipole orientation factor (range $0 \leq \kappa^2 \leq 4$)

n = refractive index of the medium between the acceptor and the donor

$$J(\lambda) = \int \varepsilon_A(\lambda) \cdot F_D(\lambda) \cdot \lambda^4 d\lambda \quad M^{-1}cm^3 \text{ (spectral overlap integral)}$$

where:

$\varepsilon_A(\lambda)$ = extinction coefficient of the acceptor

$F_D(\lambda)$ = normalized fluorescence spectral shape function (integral normalized to 1)

The Förster distance can be related to the transfer efficiency as shown in Equation 17.

$$E_T = \frac{1}{1+(r/R_0)^6} \quad (17)$$

where:

r = distance between donor and acceptor

Typical Förster distances range from 10-100 Å when the donor and acceptor are fluorescent dyes [84]. This small range is a major limitation to FRET. However, when the acceptor dye is replaced with a plasmonic nanoparticle, the energy transfer distance can be doubled. While the interaction between a dye and plasmonic nanoparticle still resembles a dipole-dipole interaction, it differs due to the nanoparticle's surface and the isotropic distribution of dipole vectors to accept energy from the donor [85, 86]. Moreover, as plasmonic nanoparticles are known to have orders of magnitude greater molar extinction coefficients compare to traditional dyes ($\sim 10^9 \text{ M}^{-1}\text{cm}^{-1}$ for 30 nm AuNPs vs. $\sim 10^5 \text{ M}^{-1}\text{cm}^{-1}$ for dyes), the quenching efficiency seen in PRET is greatly improved. It has also been shown experimentally that replacement of an acceptor dye with a plasmonic nanoparticle changes the distance dependence of the transfer efficiency from $1/R^6$ to $1/R^4$ [86-90]. PRET has been implemented in real-time enzyme kinetic studies [91, 92], and provides molecular resolution similar to that seen in SERS and plasmonic modulated fluorescence.

(e) Photoacoustic

Photoacoustic (PA) imaging is another attractive imaging modality due to its noninvasive nature and its relatively easy coupling with plasmonic nanoparticles for increased imaging contrast [93]. In PA imaging, acoustic waves are generated from the absorption of electromagnetic energy, usually from a short-pulsed laser (*i.e.* nanosecond laser). The generation

of acoustic waves stems results from thermal-induced expansion of the biological medium due to the absorption of light and is described by Equation 18 [94, 95].

$$\left(\nabla^2 - \frac{1}{v_s^2} \frac{\partial^2}{\partial t^2}\right)p = -\frac{\beta}{c_p} \frac{\partial H}{\partial t} \quad (18)$$

where: v_s is the longitudinal wave speed in the medium, p is pressure, β is the thermal coefficient of volume expansion, C_p is the heat capacity at constant pressure and H is the heating function describing the thermal energy per unit volume and unit time that is deposited in the medium from the pulsed excitation source.

Traditionally, PA biological imaging, specifically in regard to cancer biology, is based on the optical contrast of hemoglobin in the blood, as advanced tumors undergo angiogenesis increasing the contrast between diseased and healthy tissue. While this inherent contrast works in extreme cases, the optical differentiation between healthy and newly cancerous tissues is insufficient. In order to improve the optical contrast, targeted AuNPs have been employed due to their large optical cross-sections [93]. Unlike many of the previous AuNP imaging techniques, PA imaging relies on the enhanced light absorption, not the light scatter, of the nanoparticle. Therefore, AuNRs and AuNCs are the commonly utilized contrast agents in PA imaging as they have larger absorption to scattering ratios, and have the added ability to be tuned to the NIR region for increased tissue penetration [96-99]. The PA signal obtained is proportional to the AuNP concentration and subsequently the molecule or biological event being imaged. As PA imaging is limited by the spot size of the transducer at the focal plane, spatial resolution is typically limited to 20-30 μm ; however, this technique can provide impressive depth penetration (10-50 mm) that is ideal for in vivo imaging [100].

Applications in Single-Cell Studies

(a) *Probing cellular and subcellular environments*

AuNPs have been heavily used in bioimaging applications that aim to differentiate a diseased (*i.e.* cancerous) cell state from a healthy cell population [101, 102]. Our group, in particular, has employed enhanced Rayleigh scattering and plasmonically enhanced Raman scattering from AuNPs to not only identify human oral cancer cells, but also to selectively destroy them with AuNP assisted photothermal therapy [64, 103, 104]. Other groups have also used AuNPs for the selective labeling and visualization of cancerous cells, both *in vitro* and *in vivo*, using various antibody-AuNP combinations in conjunction with hyperspectral imaging, SERS, and fluorescence imaging [105-109]. Moreover, Jokerst *et. al*, employed silica coated AuNRs for photoacoustic imaging of mesenchymal stem cells (MSCs) in both *ex vivo* and *in vivo* mouse models [110]. Emelianov and co-workers also utilized AuNPs, in conjunction with ultrasound and photoacoustic imaging, to conduct longitudinal studies of MSCs in *in vitro* and *in vivo* models (Fig. 6) [100]. This work demonstrated that coupling AuNPs with established imaging techniques could provide noninvasive, quantitative, long-term imaging studies to monitor cell behavior and cell fate.

Fig. 6 The combination of AuNP and ultrasound/photoacoustic imaging for the *in vivo* monitoring of mesenchymal stem cells (MSCs). (a) Schematic illustration depicting the procedure for MSC monitoring using AuNPs in conjunction with ultrasound/photoacoustic imaging. This imaging protocol affords visualization of both MSC distribution and neovascularization in real-time. (b) Acquired ultrasound/photoacoustic images obtained in the lateral gastrocnemius of a Lewis rat with no AuNP loaded MSCs (left) and with AuNP labeled MSCs (right). AuNP loaded MSCs were clearly detectable using the combined imaging approach allowing for longitudinal tracking of the MSCs after administration. Reprinted with permission from [100].

AuNPs can also be utilized to probe intracellular environments through non-specific or organelle-specific targeting [48, 61, 106, 111-117]. Non-specific intracellular AuNPs have been utilized by Kneipp *et. al.* to probe and image the pH in live cells using SERS and surface-enhanced hyper-Raman scattering (SEHRS) [118]. Since the nucleus holds the cell's genetic information, several groups have aimed to target the nucleus with AuNPs through the use of TAT or NLS peptides [48, 113, 116, 119]. Kang *et. al.*, showed that nuclear targeting of AuNPs

can induce DNA damage at high concentrations, indicating that if AuNPs are to be used as imaging contrast agents only, that the concentration of AuNPs administered must be titrated to ensure no cytotoxicity is induced [47]. The mitochondrion is another vital organelle that has been targeted with AuNPs [114, 115, 117, 120]. Ju *et. al.* demonstrated the use of a cytochrome c binding aptamer for the successful accumulation of silica coated AuNRs in mitochondria [114]. The AuNRs were also shown to selectively deliver hydrophobic therapeutic agents to the mitochondria inducing mitochondrial driven apoptosis. The combination of organelle-targeted AuNPs and molecular imaging techniques in longitudinal studies has the potential to reveal valuable information on organelle response and thresholds to external stimuli, such as therapeutic agents.

(b) Monitoring cellular response and cell death induction

Due to the ability of AuNPs to penetrate the cell membrane and localize within a cell, they have been used to probe intracellular events involved in cell growth and death. As mentioned previously, AuNP imaging does not suffer from photobleaching, and this property has been exploited to continually track cell division in real-time using dark-field microscopy [121]. In 2012, our group demonstrated the use of nuclear-targeted AuNPs and plasmonically enhanced Raman scattering to continually monitor cell cycle progression in a live human malignant cell line (Fig. 7) [7]. The results obtained in this work correlated well with the PI-staining “gold-standard” of flow cytometry. This molecular imaging platform was further shown to accurately determine drug efficacy of popular chemotherapeutics through the identification and use of “SERS death bands” and to monitor apoptotic molecular events in real-time [9, 43]. The

observed bands were assigned to molecular events corresponding to protein denaturation and proteolysis as well as DNA fragmentation.

When long-term, continual imaging is not required, AuNPs have been coupled with fluorescence to monitor the induction of apoptosis via cytochrome c and caspase biomarkers[122-126]. In these studies, energy transfer between the AuNP and a target acceptor molecule is monitored. In 2009, Lee and co-workers used resonant quenching dips in the Rayleigh scattering spectra of AuNPs to follow the dynamics of cytochrome C in liver (HepG2) cells after apoptosis induction[126]. The quenching dips arise from the energy transfer from the AuNP to the molecular resonant peaks of cytochrome c. Recently, a AuNP construct was reported to not only induced apoptosis, but also monitored the progress of apoptosis in real-time. In this work, the AuNP was decorated with a dual-targeting pro-apoptotic peptide that targeted the mitochondria and a caspase-sensitive peptide that was conjugated to rhodamine B [123]. When apoptosis was triggered, activated caspase-3 cleaved the sensitive peptide, releasing rhodamine B from the AuNP and restoring its fluorescence. Rhodamine B fluorescence restoration, and in turn apoptosis progression, was monitored in single cells using confocal microscopy and quantitatively with flow cytometry.

Fig. 7 Plasmonically enhanced Raman scattering spectra throughout the entire 24 h cell cycle. (a) Schematic illustration of the cell cycle phases. (b) Rayleigh scattering images of human oral squamous (HSC-3) cells pretreated with nuclear-targeted AuNPs throughout the cell cycle and cell division. (c) Plasmonically enhanced Raman scattering spectra during the cell cycle. Dashed lines represent bands of interest and tentative cell cycle phase assignments (G1 – blue, S – green, G2 – orange, M – pink). Reprinted with permission from [7]. Copyright 2012 American Chemical Society.

(c) Biomolecule identification and quantification

AuNP molecular imaging strategies have also been utilized to investigate the presence of several biomolecules that have been linked to normal cellular function and therapeutic response. In particular, oxidative stress has become an important area of research due to its role in a

number of physiological and pathological processes. Therefore, several groups have investigated the ability of AuNP-fluorescence imaging constructs for the real-time tracking of oxidative stress levels in both *in vitro* and *in vivo* models.[127-129] Furthermore, Jiang *et. al.* utilized redox-sensitive AuNPs to quantitatively monitor the intracellular redox potential in hypoxic live cells using SERS in real-time [130]. AuNPs were modified to display organic reporter molecules that had distinct Raman spectra under reductive or oxidative conditions and were responsive in the transition between normoxia and hypoxia. Real-time monitoring of NADH-mediated pathways have also been conducted by tracking the changes in AuNP Rayleigh scattering using dark-field microscopy [131].

Fig. 8 Real-time detection of mRNA binding in living cells using Nano-Flares. (a) Schematic illustration of the Nano-Flare construct. AuNPs are decorated with a recognition sequence that is then hybridized with a Cy5 labeled reporter sequence. Under this state, the Cy5 fluorescence is quenched. After cellular internalization, the reporter sequence will be released from the AuNP if the cell contains the targeted mRNA sequence, restoring the Cy5 fluorescence. (b) Confocal fluorescence images and flow cytometry histograms of human breast cancer (SKBR3) cells that express surviving and in turn contain the targeted mRNA sequence. Cells treated with Nano-Flares composed of the complementary strand exhibit ~2.5 fold fluorescence signal (left) compared to cells treated with

In 2007, Mirkin and co-workers exploited the quenching ability of 13 nm AuNPs to track mRNA levels in live cells (Fig. 8)[132]. The probe entitled “nano-flare”, was composed of a AuNP decorated with oligonucleotides which were hybridized with Cy5-reporter sequence. When complimentary mRNA was not present the Cy5 emission was quenched, however if the correct target mRNA sequence was in proximity to the nano-flare the reporter sequence was released from the AuNP and its fluorescence was imaged using a standard confocal microscope. Qiao *et. al.* also used a AuNP-molecular beacon approach to detect two forms of tumor mRNA in breast cancer cells by using bi-color fluorescence imaging [133]. Furthermore, AuNPs have been used to monitor enzymatic activity and cellular metabolism using fluorescence quenching and SERS [91, 131, 134, 135].

(d) Drug action and release

Drug delivery systems (DDS) are attractive strategies for ensuring selective and specific release, while maintaining or even improving the efficacy of the drug. Many DDS have been explored, with AuNPs being a popular platform due to their biocompatibility and the ease of visualizing successful delivery through optical means. Doxorubicin (DOX), a popular chemotherapeutic agent, is a model drug for such applications as it is inherently fluorescent and when loaded onto a AuNP will adopt a quenched state. Upon successful release, DOX's fluorescence will be restored allowing for real-time drug delivery dynamic measurements to be conducted. Several groups have reported successful variations of this DDS format [136-138]. Our group has utilized this DDS in conjunction with the strongly enhanced Raman scattering from the DOX molecule to monitor the dynamic release of DOX in live human oral squamous

cell carcinoma cells [8]. In this work, the relationship between DOX's Raman and fluorescence signal are inversely related, resulting in the disappearance of the DOX Raman spectra with the simultaneous restoration of its fluorescence emission, signifying the successful delivery of the drug (Fig. 9). Ock *et. al.* also utilized SERS to monitor the glutathione (GSH) controlled thiopurine anticancer drug release from AuNPs, both in vitro and in vivo [139]. In this system, 6-mercaptopurine or 6-thioguanine were adsorbed onto the surface of AuNPs, and the distinct Raman bands of the two anticancer agents were monitored before and after external GSH administration. After delivery of GSH, the Raman bands of both drugs were reduced, indicating their successful desorption from the AuNP surface.

Fig. 9 Real-time monitoring of intracellular drug release using AuNPs and the inverse relationship between Raman scattering and fluorescence near the nanoparticle surface. (a) Schematic illustration of the pH-sensitive drug release of doxorubicin (DOX) from AuNPs and the resulting state of DOX's Raman scattering and fluorescence signals. Real-time Raman scattering spectra (b, d) and fluorescence images (c, e) throughout DOX release from the AuNPs. The characteristic DOX Raman band (460 cm^{-1}) decreases over time while the fluorescence signal increases. This inverse pattern confirmed drug release. Reprinted with permission from [8]. Copyright 2013 American Chemical Society.

Perspective: Moving Towards Real-Time Molecular Mapping

In this review, we have given an overview of current advances in using plasmonic gold nanoparticles to probe cellular dynamics within single cells. Here we would like to give several

outlooks about the challenges and efforts that should be taken to move this technique towards the real-time molecular mapping of single cells.

Spatial resolution: By using high-aperture dark field optics, single gold nanoparticles with size of 30 nm could be imaged in living cells. To achieve single-molecule probing in single cells, higher imaging resolution might be required. The spatial resolution depends not only on the imaging optics, but also the size of the plasmonic probes. For example, promise has been shown on super-resolution imaging of single molecules in living cells, by using a 5 nm gold nanoprobe and detecting the light absorption instead of scattering via photothermal imaging optics [140]. Future efforts should involve the synthesis of smaller plasmonic gold nanoprobe, as well as developing new imaging principles to overcome the diffraction barrier.

Temporal resolution: Theoretically, the temporal resolution of optical imaging mainly depends on the signal intensity and the detector sensitivity. The plasmonic scattering from gold nanoprobe has shown advantages of excellent photostability and intense single-particle brightness compared with fluorescence dyes. If combined with a cooled electron multiplying charged coupled device (EMCCD) with high sensitivity, the temporal resolution of plasmonic-based imaging has the potential to achieve microsecond or even nanosecond resolution. This resolution would be beneficial for monitoring many fast molecular events occurring within living cells. For plasmonically-enhanced spectroscopy (i.e. SERS/PERS) the temporal resolution also depends on the ability of the plasmonic nanoprobe to enhance the signal, as well as the speed of the spectral data read-out by the spectrometer. New probes with ultrahigh Raman enhancement factors, and also a high-speed Raman mapping techniques, such as stream-lined Raman, are

expected to achieve video-rate or even faster spectral mapping of the molecular events and dynamics in single cells.

Conjugation/Delivery strategy: Multi-type bioconjugation and targeting strategies have been established to deliver the plasmonic nanoprobe to different compartments of single cells, such as the cell membrane, mitochondrion, peroxisome, cell nucleus and so on. Compared to the well-established immunofluorescence techniques, which have covered almost all the intracellular targets, novel conjugation and delivery strategies are still desired to expand the scope of plasmonic nanoprobe to target specific intracellular organelles or even specific molecules in single cells.

Multifunctional probes: Differing from the fluorescence-based optical imaging technique, the plasmonic-nanoprobe based imaging not only provides the spatial-resolution, but also allows for the detection of the nanoprobe microenvironments in single cells. Novel multifunctional probes are expected to be designed to enable simultaneous sensing of intracellular temperature, pH, pressure, enzyme activity and other molecular states related to cellular functions or biological progresses.

Data analysis: With the development of advanced imaging/spectroscopic principles, methods and instruments, multi-dimensional data (spatial, temporal, spectral, etc.) can potentially be obtained simultaneously from the same cell sample. Such data usually contains complex information about molecular structure, spatial distribution and dynamic processes. Analysis of

such large data sets requires the use of advanced statistical data models and methods, including principal component analysis (PCA) and validation of the support vector machine (SVM) model.

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Graphical Abstract