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Surface Enhanced Raman Scattering (SERS) Cytometry

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I. Introduction

A key element of modern cytometry is the ability to measure many parameters simultaneously, with the demand for an increasing number of parameters driving cytometry technology development over the last decade. As new fluorescence probes have been developed and adapted to biological reagents such as antibodies, cytometry instrumentation has expanded the numbers of light sources and detectors to the point where the entire visible spectrum, from the UV to the far red, is now fully employed to measure different fluorescence labels. Further increases in the number of parameters that can be measured simultaneously necessitate one or more of the following paths: A) the adaptation of non-optical methods of detection, and thus the development and validation of entirely new instrumentation and reagent sets; B) expansion of optical methods beyond the visible, requiring new probes, detectors, light sources, and optical components; or C) more efficient use of the currently accessible spectrum by using alternative optical techniques. Each of these options is the subject of active research and development, and each has specific advantages and challenges. In this Chapter, we discuss the challenges of expanding the numbers of fluorescent labels that can be measured in cytometry, and introduce surface enhanced Raman scattering (SERS) tags with extremely narrow spectral peaks as an approach to make more efficient use of the optical spectrum and increase the number of parameters in cytometry.

II. Multiparameter Fluorescence Measurements

The development of multiparameter flow cytometry has been enabled by increasing the number of lasers, the number of detectors interrogating the sample stream at each laser intersection point, and the availability of fluorophores with appropriate excitation and emission spectra. The number of discrete parameters (or colors) that can be measured with a flow cytometer is determined by the useful spectral range of the instrument, and the absorption (excitation) and emission spectra of available fluorophores. The useful spectral range for a flow cytometers is about 350 nm to 900 nm, and is limited by the transmission of optical components at shorter wavelengths and by the poor sensitivity sensitivity of commonly available detectors at longer wavelengths. Many fluorophores are available with excitation and emission within this range of wavelengths, but the width of a typical emission spectrum is 50–100 nm wide. Some degree of spectral overlap between fluorophores can be accommodated though compensation, but this adds noise to the data and complicates experimental design and analysis.

A common configuration for multiparameter flow cytometry is illustrated in Figure 1. A blue laser can excite the small green-emitting organic fluorophores such as fluorescein, and the large fluorescent protein phycoerythrin (PE) and its tandem conjugates, and by using appropriate dichroic mirrors, band pass, and long pass filters it is possible to distinguish these fluorophores. The addition of UV, violet, or red laser allows the excitation of additional fluorophores that can be measured with additional mirrors, filters, and detectors.

However it is easy to see that because of the width of the fluorescence emission spectra it is difficult to detect more than four or five fluorophores per excitation laser.

One approach to increase the number of parameters is to use fluorophores with more suitable excitation and emission characteristics. A notable recent example is the adaptation of semiconductor quantum dots (QDots), which are efficiently excited in the violet range of the spectrum, and which can be tuned to emit from the blue to the far red. This allows as many as 7 different labels to be excited with a single laser. Another approach is to increase the efficient excitation of fluorophores by using additional lasers more closely matched to the absorption spectra of fluorophores of interest. For example, green (532 nm) excitation more efficiently excites PE and its tandem conjugates compared to a 488 nm laser. Thus, the configuration of commercial flow cytometers optimized for highly multiparameter (or polychromatic) applications feature 4 or more lasers and multiple banks of detectors to allow detection of as many as 17 different fluorochromes. However, these increases in instrument complexity have reached a point of diminishing returns in terms of increased number of parameters, as the fundamental constraints of useful spectral range and fluorescence emission spectral width have not changed.

An alternative approach to increase the number of labels is to make more efficient use of the available spectrum. Raman light scatter, in comparison to fluorescence, has very narrow spectral features (Figure 1D) that originate from the interaction of the exciting light with molecular vibrations of chemical bonds in a material. Raman scattering has been used for label-free analysis of cells and tissues as well as for the development of bright labels for antibodies or other targeting molecules.

III. Raman Scattering in Cytometry

Raman scattering originates from the interaction of light with molecular vibrations in chemical bonds. The inelastic scattering results in scatter at longer wavelengths proportional to the energy that is lost as a result of the interaction with the scattering material. The correspondence of the Raman spectral fingerprint of a sample with its molecular composition has made Raman spectroscopy a popular tool in chemical analysis, and there has been interest in applying Raman analysis to biological systems as well, although the spectral signatures are typically not specific enough to distinguish individual proteins, for example. From the standpoint of labels for cytometry, Raman is interesting because it offers the possibility of encoding many distinct labels in a relatively narrow region of spectral space.

Like fluorescence, Raman scattering occurs at longer wavelengths from excitation (anti-Stokes Raman scattering at shorter wavelengths also occurs, but we will not focus on this here due to the very low intensity of the anti-Stokes scattering signal). Unlike fluorescence, Raman scattering can be observed with any excitation wavelength and is always shifted the same degree (in terms of frequency, expressed as wave numbers in units of inverse cm) relative to the excitation wavelength. Raman scattering is not subject to saturation or photobleaching, making it possible to increase signal by increasing laser power and measurement times.

A. Intrinsic Raman Scattering

There is significant interest in using Raman scattering to detect and quantify different biochemical species from biological samples. For example, protein, DNA, carbohydrate and lipid have distinct Raman spectra (Mourant et al., 2006), and this has been used to estimate the relative abundance of each of these classes of biological molecules in tumorigenic and nontumorigenic cultured cells (Mourant et al., 2005)(Short et al., 2005). While such

measurements have the advantage of being label-free and thus relatively non-invasive, they do not have a great deal of molecular specificity compared, for example, to a measurement using a labeled antibody to detect a specific protein. A second disadvantage is that Raman scattering in endogenous compounds produces relatively weak signals that require long measurement times (seconds). Still, there is significant interest in using these approaches to develop diagnostic tools for cancer and other diseases (Kendall et al., 2009)(Nijssen et al., 2009)(Owen et al., 2006).

Stimulated Raman scattering techniques, including coherent anti-Stoke Raman scattering (CARS) (Rodriguez et al., 2006)(Krafft et al., 2009), are multiphoton methods that can be tuned to specific Raman scattering frequencies and can produce significantly stronger signals, allowing label-free live imaging of particular biochemical constituents in cells. A notable example is the real-time imaging of lipid droplets within living cells (Nan et al., 2003)(Evans et al., 2005). However, like intrinsic Raman scattering, CARS lacks the molecular specificity of methods that use antibodies or other targeting molecules.

B. Surface Enhanced Raman Scattering (SERS)

More than 30 years ago it was recognized that the intensity of Raman scattering was greatly increased near certain metal surfaces (Jeanmaire and Van Duyne, 1977). In recent years, improvements in the ability to reproducibly make uniform nanostructures have enabled significant advances in the theoretical understanding and experimental control of SERS (Stiles et al., 2008). SERS combines the molecular information provided by Raman scattering with a very bright signals, enabling, in some cases, single molecule analysis (Kneipp et al., 1997)(Nie and Emory, 1997). This combination makes SERS an extremely promising analytical technique, and numerous groups are working on the development of substrates for the direct detection of biological analytes. For example, several groups have reported the detection of SERS from bacterial (Patel et al., 2008)(Jarvis et al., 2006)(Jarvis and Goodacre, 2008) or viral (Shanmukh et al., 2008)(Hoang et al., 2010)(Shanmukh et al., 2006)(Driskell et al., 2010) pathogens, or their metabolites (Zhang et al., 2005)(Daniels et al., 2006)(Evanoff et al., 2006). In some cases, multivariate analysis approaches such as principal components analysis (PCA) were used to resolve the slight spectral differences among species. While these direct detection approaches have the advantage of enabling label free analysis, they also used relatively pure samples of cultured organisms. Extending this work to complex environmental samples, with low levels of targets and high levels of irrelevant species or background, is likely to be very challenging (Golightly et al., 2009) (Efrima and Zeiri, 2009).

Similarly, direct detection of biomolecules such as proteins and nucleic acids using SERS has been demonstrated. For example, it has been shown that the phosphorylation of a peptide substrate by a kinase can be detected using SERS (Yue et al., 2009)(Moger et al., 2007)(Sundararajan et al., 2006). Such an approach is feasible for an in vitro assay of enzyme activity, but is unlikely to be applicable to cytometry applications using single cells, whether live or fixed. The challenge of interpreting intrinsic SERS spectra directly from a complex sample such as a cell are illustrated by reports of SERS spectra obtained from single cells incubated with gold or silver nanoparticles. Spectra obtained with such approaches allow only the most general components of cells to be resolved, and offer little promise of useful cytometric measurements.

In order to provide information with relevant molecular specificity, as with fluorescence, SERS benefits from the use of a targeting molecule such as an antibody or oligonucleotide. In contrast to the previously described direct detection methods that measure SERS from endogenous compounds, in indirect methods the SERS signal serves as an exogenous label for the targeting model, allowing exploitation of the photostability and narrow spectral

features of SERS for multiplexed analysis. The use of SERS as a label can be implemented in many popular detection formats currently used for fluorescence, including solid phase assays such as immunoassays, planar microarrays, and lateral flow assays.

Early examples focused on nucleic acid detection, and employed oligonucleotides bearing Raman-active compounds, whose scattering is enhanced upon hybridizing near an appropriate metal surface (Vo-Dinh et al., 1994)(Isola et al., 1998), or when metal is deposited over the hybridized probe (Cao et al., 2002). A variation on these two approaches exploits the formation of SERS “hotspots” upon aggregation of silver or gold colloids during binding of Raman-tagged oligonucleotides (Fabris et al., 2007). Similar approaches have been used to detect the binding of Raman-tagged antibodies in immunoassays (Grubisha et al., 2003) and to detect DNA-protein interactions. These detection schemes all require either the assay to be performed on a SERS-active surface, or the aggregation or deposition of SERS-active metal onto the sample.

A further evolution of the indirect or extrinsic SERS detection approach involves the development of discrete SERS tags, in which the plasmonic nanoparticle, Raman-active compound, and targeting molecule are incorporated into a single entity that can be used to label a sample. This format of labeling is likely to be the most useful for the analysis of single cells, whether by flow or image cytometry, and is the focus of this chapter.

IV. Reagents and Instrumentation

A. Anatomy of a SERS Tag

At the most general level, a SERS tag (Figure 2) is composed of A) a plasmonically-active nanoparticle with a resonance at a desired excitation wavelength, B) a Raman active compound, which is ideally also resonant at the excitation wavelength and gives the SERS tags its distinctive spectral fingerprint, and C) a coating to stabilize the SERS tag, provide a surface for conjugation to a targeting entity such as an antibody and passivate the surface to reduce non-specific binding. In this section we will discuss each of these components, as well as specific examples of SERS tags.

1. Plasmonic Nanoparticles—The starting material for a SERS tag is a plasmonic nanoparticle. Noble metal nanoparticles support localized surface plasmon resonances (LSPRs), a collective oscillation of electrons that strongly couple to light at specific wavelengths, and produce extremely high electromagnetic fields near the nanoparticle surface.

In bulk measurements, the LSPR results in distinctive changes in the extinction of a nanoparticle suspension. The extinction is the result of increases in both nanoparticle absorbance and scatter, and the wavelengths at which plasmonic nanoparticles interact with light can be tuned by varying the composition, shape, and size of the particle (Figure 3). Gold and silver nanoparticles are the most commonly used plasmonic nanoparticles as they absorb and scatter light in the 350–900 nm spectral range. For example, solid silver spheres exhibit a resonance in the blue, shifting to longer wavelengths as the diameter increases. Solid gold spheres have resonances at green wavelengths, also red-shifting as the diameter increases. Thin shells of silver or gold over a non-conducting material such as silica also support a LSPR that can lead to strong SERS enhancements (Oldenburg et al., 1999). In this case, the resonance wavelength depends on the ratio of the shell thickness to the core diameter. Asymmetric structures such as nanorods and spheroids support strong resonances on their long axes, the wavelength of which varies with the particle's aspect ratio (Orendorff et al., 2006)(Murphy et al., 2008)(Hao and Schatz, 2004). Beyond spheres, shells and rods, a variety of other nanoparticle structures have been shown to produce SERS including

nanocubes (McLellan et al., 2006) nanostars (Khoury and Vo-Dinh, 2008), nanoflowers (Xie et al., 2008), and nanorice (Wiley et al., 2007).

The electric field intensity resulting from the LSPR varies across the surface of the nanoparticle, and depends on the shape of the nanoparticle, its orientation relative to the exciting light, and the polarization and wavelength of that light. The LSPR is predicted to be highest at particle edges, points, or surface irregularities, which has led to the concept of SERS “hot spots” as being responsible for generating the highest SERS enhancement and signals. Additionally, the LSPRs of adjacent nanoparticles can couple, creating hotspots at the junctions between aggregated particles (Camden et al., 2008). The engineering of metal nanoparticle systems to provide the highest and most uniform LSPRs and SERS enhancement is a key goal of SERS tag design.

2. Raman Tag—The Raman tag is a compound that exhibits a distinctive Raman spectrum, or fingerprint, when adsorbed to the surface of a plasmonic particle that can be distinguished from other Raman tags. The electromagnetic field is strongest nearest the nanoparticle surface, so physical adsorption of the Raman compound directly to the metal surfaces is required for the strongest SERS enhancement. Often, the Raman tag is a thiol-containing compound, which takes advantage of the affinity of that moiety for gold and silver surfaces, although in principle any compound that adsorbs to the metal surface is suitable. Small organic thiols such as mercaptobenzoic acid (MBA) have been the subject of many fundamental SERS studies. As mentioned above, if the Raman tag has a molecular absorbance that is resonant with the exciting light, an additional signal enhancement is obtained. Highly absorbing fluorophores such as rhodamine 6G (R6G) have been used to demonstrate single molecule SERS detection. Such surface enhanced resonant Raman scattering (SERRS) is a special case of SERS that produces the strongest Raman scattering enhancement and thus the brightest SERS tags. Isothiocyanate versions of many common fluorophores combine the advantages of a resonant tag with a metal binding sulfur group. While fluorescence is often a significant source of background in Raman measurements, in the case of fluorophores used as resonant Raman tags, the fluorescence is fortuitously quenched by proximity to the metal surface.

3. SERS Tag Coating and Functionalization—SERS tags are generally coated or encapsulated to stabilize and protect the tag from environmental components and to provide functional groups for conjugation to targeting molecules such as antibodies. Silica is a popular coating that provides chemical and mechanical stability, as well as a surface that is readily functionalized for conjugation to targeting molecules. SERS tags have also been coated with polymers such as polyethylene glycol (PEG) to stabilize the tags, provide functional groups for conjugation, and passivate the surface to reduce non-specific binding.

B. Examples of SERS Tags

1. SERS Tags Based on Metal Spheres—Some of the first SERS tags were based on gold or silver spheres. In early examples based on gold spheres, organic thiols (Mulvaney et al., 2003) or sulfur-containing resonant dyes (Doering and Nie, 2003) served as the Raman tags, and a silica coating encapsulated the tags and served as the surface for functionalization. Subsequent variations employed resonant dyes (Gong et al., 2006) and polymer (Merican et al., 2007) or protein (Lee et al., 2007) coatings. SERS tags based on metal spheres can be made to be fairly uniform, but do not provide the brightest SERS signals compared to SERS tags made with other nanoparticles due to the relatively low electric field intensities found near the nanosphere surface.

2. SERS Tags Based on Aggregated Spheres—Empirical observations led to the recognition that aggregates of nanoparticles produce greater SERS enhancements than individual metal spheres. An early example of this approach (Su et al., 2005) employed organic thiols and heat to trigger the aggregation of silver spheres. The aggregation was slowed by cooling and the addition of protein (bovine serum albumin, BSA), which encapsulated and stabilized the aggregates. The BSA coating also provided a source of amine groups for bioconjugation. Later variants employed resonant dye molecules and a silica (Brown and Doorn, 2008), rather than protein, coating. SERS tags based on aggregated colloids have resonance wavelengths that are red shifted relative to isolated spheres because of the overall larger size of the aggregates, and because the LSPRs of individual nanoparticles couple, shifting the resonance to a lower energy state. They also can be significantly brighter than SERS tags based on single spheres because of hot spots formed at the interfaces of the aggregated spheres, and there are examples of these types of SERS tags being used in image cytometry (Sun et al., 2007a)(Shachaf et al., 2009)(Lutz et al., 2008a) and flow cytometry (Watson et al., 2008)(Watson et al., 2009). However, the aggregation process is difficult to control, and typically results in highly polydisperse aggregates with low uniformity, that have wide range of sizes, resonant wavelengths, and brightness. This makes the scalable and reproducible preparation of aggregate-based SERS tags a significant challenge.

3. Nanoshell-based SERS Tags—Thin shells of metal over a core of dielectric material such as silica provide a plasmonic particle in which the LSPRs of two edges of the shell can couple providing a strong SERS enhancement (Oldenburg et al., 1999). The resonant wavelength can be tuned by adjusting the thickness of the metal shell and size of the dielectric core, with decreasing shell:core ratios shifting the resonance to longer wavelengths. By building the shell over monodisperse silica cores, very uniform plasmonic particles with high SERS enhancements can be prepared. Silver nanoshells have been labeled with organic thiols and coated in silica for subsequent conjugation and use as an imaging probe (Yu et al., 2007a). Gold nanoshells have been labeled with resonant dye, and coated with a thin layer of silver, which provides additional signal enhancement (Sebba et al., 2009) that allows the Raman scattering from individual tags to be detected with sub-millisecond integration times. These particles can be effectively stabilized and functionalized with a sulfhydryl-PEG-based coating. Nanoshell-based SERS tags represent a promising approach to the scalable manufacturing of bright, uniform SERS tags for high speed imaging and flow cytometry applications

4. Nanorod-based SERS Tags—Metal nanorods exhibit a LSPR of that can be tuned by varying their aspect ratio (length to width) and have strong electric field enhancements at the tips that leads to strong SERS when illuminated by the appropriate wavelength of light (Orendorff et al., 2006). Gold nanorods labeled with an organic thiol and coated in polymer served as antibody label for the detection of surface markers on cultured cells (Park et al., 2009b). Gold rods labeled with resonant compounds were coated with polymer and injected into mice, where their distribution was determined using in vivo imaging (von Maltzahn et al., 2009). SERS tags based on nanorods also appear as promising reagents for high speed imaging and flow cytometry applications.

C. SERS Cytometry Data Acquisition and Analysis

1. General Considerations—A distinguishing feature of Raman scattering compared to fluorescence is its narrow spectral features, which requires higher spectral resolution to resolve than is commonly employed in image or flow cytometry. The approaches to cytometry spectral imaging hardware have been recently reviewed (Lerner et al., 2010). In general, Raman and SERS detection demands the high spectral resolution provided by

grating-based dispersive optics and array-type detectors. In addition, because Raman scattering spectral features occur at a fixed frequency shift from the exciting light, the resolution of SERS spectrum also depends on the spectral width of the excitation laser. Many diode laser sources suitable for fluorescence cytometry have spectral widths of 1 nm or more and will produce unacceptably broad Raman peaks. Once continuous spectral data are collected they are subjected to spectral unmixing to extract the contributions of individual tags and background sources to the spectra. Common approaches include linear least squares unmixing (Lutz et al., 2008b) or multivariate analysis such as principal components analysis (PCA) (Watson et al., 2008) or multiple curve resolution (MCR) (Haaland et al., 2009) to resolve individual components in mixed spectra.

2. SERS Imaging—Raman imaging systems are commercially available, however most are not designed for high speed biological imaging. In fluorescence imaging, relatively broad spectral bands are selected with dichroic mirrors and bandpass filters, and the system magnification and pixel size of the CCD camera determine the spatial resolution. Alternatively, laser scanning cytometry couples high speed scanning optics to render high resolution information using point detectors such as PMTs. Such approaches have enabled the development of very high speed imaging systems, as employed in the high content analysis field. In Raman imaging, the requirement for high resolution spectra necessitates the use of a high performance spectrograph and CCD array detector to collect this spectral information. Making such spectral measurements point-by-point at high spatial resolution from cells on a microscope slide, for example, is very slow by comparison to conventional fluorescence imaging. A more efficient approach is to use line focus excitation to collect one dimensional spatial information and spectral information simultaneously on a CCD array. This approach is taken by a few commercial systems, and can offer significantly higher imaging throughput.

3. SERS Flow Cytometry—There are no commercial Raman flow cytometers, but recent years have seen a renewed interest in developing high resolution spectral capabilities in flow cytometry. In contrast to imaging, where recent hardware developments have increased the speed of imaging systems, flow cytometry is intrinsically high speed. Conventional flow cytometers analyzers employ measurement integration times on the order of ten microseconds to measure hundreds to thousands of cell per second. High speed cell sorters may use integration times ten-fold faster to reach ten times higher analysis rates. Specially constructed laboratory instruments have used slower measurement times up to a msec, to increase measurement sensitivity. For high resolution spectral flow cytometry, as for high resolution spectral imaging, dichroic mirrors and bandpass filters are replaced with a spectrograph. Modern CCD arrays provide high resolution and sensitivity at exposure times as fast as 10 usec, however they have slower readout times compared to PMTs which can limit the analysis rates to a few hundred cells per second. Lab built Raman flow cytometers have demonstrated performance suitable for most popular flow cytometry applications, and the addition of spectral data acquisition capabilities to a commercial flow cytometer has also been demonstrated. As for SERS imaging, a limiting factor for the future development of SERS flow cytometry is the availability of bright and uniform SERS tags.

V. SERS Cytometry Applications

While the phenomenon of SERS has been known for more than 30 years, and its potential as a bioanalytical tool recognized for some time, it is only in the past decade that techniques for controlled nanoparticle fabrication have become widely disseminated. Thus, from an applications standpoint, SERS is a still young field. As discussed earlier, there have been several implementations of SERS in nucleic acid and protein analysis, and SERS tags have been used as labels in bioassays in a manner analogous to chromogenic or fluorescent labels

on a variety of measurement platforms including ELISA plates, planar substrates including microarrays, and lateral flow assays. Our focus in this Chapter is on the potential for SERS to extend the multiparameter analysis of cells and cell systems.

A. In vitro Measurements

At present, cytometry generally focuses on the study of cells cultured in vitro, or ex vivo analysis of primary cells or tissues, and most cytometry-related SERS applications have focused on these classes of applications as well. Several groups have used SERS tagged antibodies or other ligands to detect cell surface markers (Yu et al., 2007b)(Kim et al., 2006) (Noh et al., 2009)(Park et al., 2009a)(Nguyen et al., 2010) or intracellular antigens (Shachaf et al., 2009)(Lee et al., 2007) on cultured cell lines. SERS tags have also been used as antibody labels to stain tissue sections (Sun et al., 2007b)(Lutz et al., 2008a)(Lutz et al., 2008b). In many cases, these examples were qualitative demonstrations involving single SERS tags, but in a few cases efforts were made to extract quantitative information in multiplexed measurements (Shachaf et al., 2009)(Lutz et al., 2008a)(Lutz et al., 2008b).

B. In vivo Measurements

There is significant interest in adapting quantitative cytometry approaches to in vivo measurements, and SERS has some potential advantages for enabling this. The tunability of SERS tag resonance allows tags to be developed that are excitable in the far red, allowing greater light penetration into tissue and minimizing many sources of autofluorescence. Several groups have injected untargeted SERS tags into animals and used in vivo SERS imaging to determine their distribution and fate (Keren et al., 2008)(Zavaleta et al., 2009) (von Maltzahn et al., 2009) (Wang et al., 2010)(Matschulat et al., 2010).

More specific biological information can be obtained when SERS tags are functionalized to allow targeting, for instance with an antibody. Qian et al used anti-EGFR SERS tags to localize tumors in live mice using in vivo SERS imaging (Qian et al., 2008). An exciting aspect of this approach to in vivo imaging is the use of plasmonic nanoparticles to thermally ablate diseased tissues (O'Neal et al., 2004)(El-Sayed et al., 2006)(Cobley et al., 2010) presenting the prospect for coordinated cancer diagnosis and therapy using targeted nanoparticle probes (Hirsch et al., 2003)(Lal et al., 2008).

VI. Summary and Prospects

The past decade has seen significant advances in the preparation and applications of SERS-active materials for biomolecular analysis. Bright signals, photostability, and narrow spectral features offer attractive advantages for cytometric analyses. Following the path of semiconductor quantum dots, improvements in nanoparticle synthesis methods and surface chemistry have led to plausible schemes for the scalable and reproducible production of uniform SERS tags, a prerequisite for commercial application. In parallel, developments in high speed multispectral image and flow cytometry have laid the groundwork for the application of Raman tags application in high speed cell analysis. However, SERS cytometry is still in an early stage of development, and advances in both instrumentation and reagents will be necessary to realize its full potential.

Reagents will be critical to the further development of SERS cytometry. In order to allow researchers to focus on the development of the biological applications of SERS, stable and uniform tags that are readily functionalized with an antibody or other targeting molecule must be available, ideally from commercial sources. Tags must be reproducibly bright and uniform from batch to batch to allow comparison of results over time and between instruments. The status of SERS tags might be compared to that of quantum dots 5 or 10 years ago, with a handful of lab having extensive experience in making tags and with a

limited number of commercial products making their way towards the market, but with further development and optimization being pursued in both academic and commercial labs.

Finally, it is clear that SERS complements, rather than replaces fluorescence as a biological detection tool. The chief advantages of narrow spectral features and photostability make them very attractive as tags for antibodies or other targeting molecules. However, there are many applications, including cell viability, ion and pH fluxes, and the measurement of enzyme activities, for which fluorescence is currently clearly better suited. Thus, future applications will likely involve the simultaneous measurement of fluorescence and SERS tags. The wavelength tunability of SERS tags makes it possible to engineer SERS tags to be excited in the NIR, leaving the visible wavelengths for the wide variety of visible wavelength-excited currently available. In the future, we may see instruments designed for the simultaneous high speed measurement of both fluorescence and Raman signals

V. References

- Brown L, Doorn S. A controlled and reproducible pathway to dye-tagged, encapsulated silver nanoparticles as substrates for SERS multiplexing. *Langmuir*. 2008; 24:2277–2280. [PubMed: 18278969]
- Camden JP, Dieringer JA, Wang Y, Masiello DJ, Marks LD, Schatz GC, Van Duyne RP. Probing the Structure of Single-Molecule Surface-Enhanced Raman Scattering Hot Spots. *Journal of the American Chemical Society*. 2008; 130:12616–12617. [PubMed: 18761451]
- Cao YC, Jin R, Mirkin CA. Nanoparticles with Raman spectroscopic fingerprints for DNA and RNA detection. *Science*. 2002; 297:1536–1540. [PubMed: 12202825]
- Cobley CM, Au L, Chen J, Xia Y. Targeting gold nanocages to cancer cells for photothermal destruction and drug delivery. *Expert Opin Drug Deliv*. 2010; 7:577–587. [PubMed: 20345327]
- Daniels JK, Caldwell TP, Christensen KA, Chumanov G. Monitoring the kinetics of *Bacillus subtilis* endospore germination via surface-enhanced Raman scattering spectroscopy. *Anal. Chem*. 2006; 78:1724–1729. [PubMed: 16503629]
- Doering WE, Nie S. Spectroscopic Tags Using Dye-Embedded Nanoparticles and Surface-Enhanced Raman Scattering. *Analytical Chemistry*. 2003; 75:6171–6176. [PubMed: 14615997]
- Driskell JD, Zhu Y, Kirkwood CD, Zhao Y, Dluhy RA, Tripp RA. Rapid and sensitive detection of rotavirus molecular signatures using surface enhanced Raman spectroscopy. *PLoS ONE*. 2010; 5:e10222. [PubMed: 20419101]
- Efrima S, Zeiri L. Understanding SERS of bacteria. *J. Raman Spectrosc*. 2009; 40:277–288.
- El-Sayed IH, Huang X, El-Sayed MA. Selective laser photo-thermal therapy of epithelial carcinoma using anti-EGFR antibody conjugated gold nanoparticles. *Cancer Lett*. 2006; 239:129–135. [PubMed: 16198049]
- Evanoff DD, Heckel J, Caldwell TP, Christensen KA, Chumanov G. Monitoring DPA release from a single germinating *Bacillus subtilis* endospore via surface-enhanced Raman scattering microscopy. *J. Am. Chem. Soc*. 2006; 128:12618–12619. [PubMed: 17002334]
- Evans C, Potma E, Puoris'haag M, Cote D, Lin C, Xie X. Chemical imaging of tissue in vivo with video-rate coherent anti-Stokes Raman scattering microscopy. *Proc.Natl.Acad.Sci.U.S.A*. 2005; 102:16807–16812. [PubMed: 16263923]
- Fabris L, Dante M, Braun G, Lee SJ, Reich NO, Moskovits M, Nguyen T, Bazan GC. A heterogeneous PNA-based SERS method for DNA detection. *J. Am. Chem. Soc*. 2007; 129:6086–6087. [PubMed: 17451246]
- Golightly RS, Doering WE, Natan MJ. Surface-enhanced Raman spectroscopy and homeland security: a perfect match? *ACS Nano*. 2009; 3:2859–2869. [PubMed: 19856975]
- Gong J, Jiang J, Liang Y, Shen G, Yu R. Synthesis and characterization of surface-enhanced Raman scattering tags with Ag/SiO₂ core-shell nanostructures using reverse micelle technology. *Journal of Colloid and Interface Science*. 2006; 298:752–756. [PubMed: 16457836]

- Grubisha D, Lipert R, Park H, Driskell J, Porter M. Femtomolar detection of prostate-specific antigen: an immunoassay based on surface-enhanced Raman scattering and immunogold labels. *Anal.Chem.* 2003; 75:5936–5943. [PubMed: 14588035]
- Haaland DM, Jones HDT, Van Benthem MH, Sinclair MB, Melgaard DK, Stork CL, Pedroso MC, Liu P, Brasier AR, Andrews NL, et al. Hyperspectral confocal fluorescence imaging: exploring alternative multivariate curve resolution approaches. *Appl Spectrosc.* 2009; 63:271–279. [PubMed: 19281642]
- Hao E, Schatz GC. Electromagnetic fields around silver nanoparticles and dimers. *J Chem Phys.* 2004; 120:357–366. [PubMed: 15267296]
- Hirsch LR, Stafford RJ, Bankson JA, Sershen SR, Rivera B, Price RE, Hazle JD, Halas NJ, West JL. Nanoshell-mediated near-infrared thermal therapy of tumors under magnetic resonance guidance. *Proc. Natl. Acad. Sci. U.S.A.* 2003; 100:13549–13554. [PubMed: 14597719]
- Hoang V, Tripp RA, Rota P, Dluhy RA. Identification of individual genotypes of measles virus using surface enhanced Raman spectroscopy. *Analyst.* 2010 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/20838669>.
- Isola NR, Stokes DL, Vo-Dinh T. Surface-enhanced Raman gene probe for HIV detection. *Anal. Chem.* 1998; 70:1352–1356. [PubMed: 9553492]
- Jarvis RM, Brooker A, Goodacre R. Surface-enhanced Raman scattering for the rapid discrimination of bacteria. *Faraday Discuss.* 2006; 132:281–292. discussion 309–319. [PubMed: 16833123]
- Jarvis RM, Goodacre R. Characterisation and identification of bacteria using SERS. *Chem Soc Rev.* 2008; 37:931–936. [PubMed: 18443678]
- Jeanmaire DL, Van Duyne RP. Surface raman spectroelectrochemistry: Part I. Heterocyclic, aromatic, and aliphatic amines adsorbed on the anodized silver electrode. *Journal of Electroanalytical Chemistry.* 1977; 84:1–20.
- Kendall C, Isabelle M, Bazant-Hegemark F, Hutchings J, Orr L, Babrah J, Baker R, Stone N. Vibrational spectroscopy: a clinical tool for cancer diagnostics. *Analyst.* 2009; 134:1029–1045. [PubMed: 19475128]
- Keren S, Zavaleta C, Cheng Z, de la Zerda A, Gheysens O, Gambhir S. Noninvasive molecular imaging of small living subjects using Raman spectroscopy. *Proceedings of the National Academy of Sciences.* 2008; 105:5844–5849.
- Khoury CG, Vo-Dinh T. Gold Nanostars For Surface-Enhanced Raman Scattering: Synthesis, Characterization and Optimization. *The Journal of Physical Chemistry C.* 2008; 112:18849–18859.
- Kim J, Kim J, Choi H, Lee S, Jun B, Yu K, Kuk E, Kim Y, Jeong D, Cho M, et al. Nanoparticle Probes with Surface Enhanced Raman Spectroscopic Tags for Cellular Cancer Targeting. *Anal.Chem.* 2006; 78:6967–6973. [PubMed: 17007522]
- Kneipp K, Wang Y, Kneipp H, Perelman LT, Itzkan I, Dasari RR, Feld MS. Single Molecule Detection Using Surface-Enhanced Raman Scattering (SERS). *Phys. Rev. Lett.* 1997; 78:1667.
- Krafft C, Dietzek B, Popp J. Raman and CARS microspectroscopy of cells and tissues. *Analyst.* 2009; 134:1046–1057. [PubMed: 19475129]
- Lal S, Clare SE, Halas NJ. Nanoshell-enabled photothermal cancer therapy: impending clinical impact. *Acc. Chem. Res.* 2008; 41:1842–1851. [PubMed: 19053240]
- Lee S, Kim S, Choo J, Shin SY, Lee YH, Choi HY, Ha S, Kang K, Oh CH. Biological imaging of HEK293 cells expressing PLCgamma1 using surface-enhanced Raman microscopy. *Anal. Chem.* 2007; 79:916–922. [PubMed: 17263316]
- Lerner JM, Gat N, Wachman E. Approaches to spectral imaging hardware. *Curr Protoc Cytom.* 2010; Chapter 12:Unit 12.20. [PubMed: 20578107]
- Lutz B, Dentinger C, Sun L, Nguyen L, Zhang J, Chmura A, Allen A, Chan S, Knudsen B. Raman nanoparticle probes for antibody-based protein detection in tissues. *J.Histochem.Cytochem.* 2008a; 56:371–379. [PubMed: 18071064]
- Lutz B, Dentinger C, Nguyen L, Sun L, Zhang J, Allen A, Chan S, Knudsen B. Spectral analysis of multiplex Raman probe signatures. *ACS Nano.* 2008b; 2:2306–2314. [PubMed: 19206397]

- von Maltzahn G, Centrone A, Park J, Ramanathan R, Sailor MJ, Hatton TA, Bhatia SN. SERS-Coded Gold Nanorods as a Multifunctional Platform for Densely Multiplexed Near-Infrared Imaging and Photothermal Heating. *Adv Mater.* 2009; 21:3175–3180. [PubMed: 20174478]
- Matschulat A, Drescher D, Kneipp J. Surface-enhanced Raman scattering hybrid nanoprobe multiplexing and imaging in biological systems. *ACS Nano.* 2010; 4:3259–3269. [PubMed: 20503969]
- McLellan JM, Siekkinen A, Chen J, Xia Y. Comparison of the surface-enhanced Raman scattering on sharp and truncated silver nanocubes. *Chemical Physics Letters.* 2006; 427:122–126.
- Merican Z, Schiller TL, Hawker CJ, Fredericks PM, Blakey I. Self-Assembly and Encoding of Polymer-Stabilized Gold Nanoparticles with Surface-Enhanced Raman Reporter Molecules. *Langmuir.* 2007; 23:10539–10545. [PubMed: 17824719]
- Moger J, Gribbon P, Sewing A, Winlove CP. Feasibility study using surface-enhanced Raman spectroscopy for the quantitative detection of tyrosine and serine phosphorylation. *Biochim. Biophys. Acta.* 2007; 1770:912–918. [PubMed: 17360121]
- Mourant J, Dominguez J, Carpenter S, Short K, Powers T, Michalczyk R, Kunapareddy N, Guerra A, Freyer J. Comparison of vibrational spectroscopy to biochemical and flow cytometry methods for analysis of the basic biochemical composition of mammalian cells. *J.Biomed.Opt.* 2006; 11:064024. [PubMed: 17212547]
- Mourant J, Short K, Carpenter S, Kunapareddy N, Coburn L, Powers T, Freyer J. Biochemical differences in tumorigenic and nontumorigenic cells measured by Raman and infrared spectroscopy. *J.Biomed.Opt.* 2005; 10:031106. [PubMed: 16229631]
- Mulvaney S, Musick M, Keating C, Natan M. Glass-coated, analyte-tagged nanoparticles: A new tagging system based on detection with surface-enhanced Raman scattering. *Langmuir.* 2003; 19:4784–4790.
- Murphy CJ, Gole AM, Hunyadi SE, Stone JW, Sisco PN, Alkilany A, Kinard BE, Hankins P. Chemical sensing and imaging with metallic nanorods. *Chem. Commun. (Camb.).* 2008:544–557. [PubMed: 18209787]
- Nan X, Cheng J, Xie X. Vibrational imaging of lipid droplets in live fibroblast cells with coherent anti-Stokes Raman scattering microscopy. *J.Lipid Res.* 2003; 44:2202–2208. [PubMed: 12923234]
- Nguyen C, Nguyen J, Rutledge S, Zhang J, Wang C, Walker G. Detection of chronic lymphocytic leukemia cell surface markers using surface enhanced Raman scattering gold nanoparticles. *Cancer Lett.* 2010; 292:91–97. [PubMed: 20042272]
- Nie S, Emory S. Probing Single Molecules and Single Nanoparticles by Surface-Enhanced Raman Scattering. *Science.* 1997; 275:1102–1106. [PubMed: 9027306]
- Nijssen A, Koljenović S, Bakker Schut TC, Caspers PJ, Puppels GJ. Towards oncological application of Raman spectroscopy. *J Biophotonics.* 2009; 2:29–36. [PubMed: 19343683]
- Noh M, Jun B, Kim S, Kang H, Woo M, Minai-Tehrani A, Kim J, Kim J, Park J, Lim H, et al. Magnetic surface-enhanced Raman spectroscopic (M-SERS) dots for the identification of bronchioalveolar stem cells in normal and lung cancer mice. *Biomaterials.* 2009; 30:3915–3925. [PubMed: 19411103]
- Oldenburg SJ, Westcott SL, Averitt RD, Halas NJ. Surface enhanced Raman scattering in the near infrared using metal nanoshell substrates. *J. Chem. Phys.* 1999; 111:4729.
- O'Neal DP, Hirsch LR, Halas NJ, Payne JD, West JL. Photo-thermal tumor ablation in mice using near infrared-absorbing nanoparticles. *Cancer Lett.* 2004; 209:171–176. [PubMed: 15159019]
- Orendorff CJ, Gearheart L, Jana NR, Murphy CJ. Aspect ratio dependence on surface enhanced Raman scattering using silver and gold nanorod substrates. *Phys. Chem. Chem. Phys.* 2006; 8:165. [PubMed: 16482257]
- Owen CA, Nottingher I, Hill R, Stevens M, Hench LL. Progress in Raman spectroscopy in the fields of tissue engineering, diagnostics and toxicological testing. *J Mater Sci Mater Med.* 2006; 17:1019–1023. [PubMed: 17122913]
- Park H, Lee S, Chen L, Lee E, Shin S, Lee Y, Son S, Oh C, Song J, Kang S, et al. SERS imaging of HER2-overexpressed MCF7 cells using antibody-conjugated gold nanorods. *Phys.Chem.Chem.Phys.* 2009a; 11:7444–7449. [PubMed: 19690717]

- Park H, Lee S, Chen L, Lee EK, Shin SY, Lee YH, Son SW, Oh CH, Song JM, Kang SH, et al. SERS imaging of HER2-overexpressed MCF7 cells using antibody-conjugated gold nanorods. *Phys Chem Chem Phys*. 2009b; 11:7444–7449. [PubMed: 19690717]
- Patel IS, Premasiri WR, Moir DT, Ziegler LD. Barcoding bacterial cells: A SERS based methodology for pathogen identification. *J Raman Spectrosc*. 2008; 39:1660–1672. [PubMed: 19714262]
- Qian X, Peng X, Ansari D, Yin-Goen Q, Chen G, Shin D, Yang L, Young A, Wang M, Nie S. In vivo tumor targeting and spectroscopic detection with surface-enhanced Raman nanoparticle tags. *Nat. Biotechnol*. 2008; 26:83–90. [PubMed: 18157119]
- Rodriguez LG, Lockett SJ, Holtom GR. Coherent anti-stokes Raman scattering microscopy: a biological review. *Cytometry A*. 2006; 69:779–791. [PubMed: 16752420]
- Sebba DS, Watson DA, Nolan JP. High throughput single nanoparticle spectroscopy. *ACS Nano*. 2009; 3:1477–1484. [PubMed: 19472989]
- Shachaf C, Elchuri S, Koh A, Zhu J, Nguyen L, Mitchell D, Zhang J, Swartz K, Sun L, Chan S, et al. A novel method for detection of phosphorylation in single cells by surface enhanced Raman scattering (SERS) using composite organic-inorganic nanoparticles (COINs). *PLoS. One*. 2009; 4:e5206. [PubMed: 19367337]
- Shanmukh S, Jones L, Zhao Y, Driskell JD, Tripp RA, Dluhy RA. Identification and classification of respiratory syncytial virus (RSV) strains by surface-enhanced Raman spectroscopy and multivariate statistical techniques. *Anal Bioanal Chem*. 2008; 390:1551–1555. [PubMed: 18236030]
- Shanmukh S, Jones L, Driskell J, Zhao Y, Dluhy R, Tripp RA. Rapid and sensitive detection of respiratory virus molecular signatures using a silver nanorod array SERS substrate. *Nano Lett*. 2006; 6:2630–2636. [PubMed: 17090104]
- Short K, Carpenter S, Freyer J, Mourant J. Raman spectroscopy detects biochemical changes due to proliferation in mammalian cell cultures. *Biophys. J*. 2005; 88:4274–4288. [PubMed: 15764662]
- Stiles PL, Dieringer JA, Shah NC, Van Duyne RP. Surface-enhanced Raman spectroscopy. *Annu Rev Anal Chem (Palo Alto Calif)*. 2008; 1:601–626. [PubMed: 20636091]
- Su X, Zhang J, Sun L, Koo T, Chan S, Sundararajan N, Yamakawa M, Berlin A. Composite organic-inorganic nanoparticles (COINs) with chemically encoded optical signatures. *Nano Lett*. 2005; 5:49–54. [PubMed: 15792411]
- Sun L, Sung K, Dentinger C, Lutz B, Nguyen L, Zhang J, Qin H, Yamakawa M, Cao M, Lu Y, et al. Composite Organic-Inorganic Nanoparticles as Raman Labels for Tissue Analysis. *Nano Lett*. 2007a; 7:351–356. [PubMed: 17298000]
- Sun L, Sung K, Dentinger C, Lutz B, Nguyen L, Zhang J, Qin H, Yamakawa M, Cao M, Lu Y, et al. Composite Organic-Inorganic Nanoparticles as Raman Labels for Tissue Analysis. *Nano Lett*. 2007b; 7:351–356. [PubMed: 17298000]
- Sundararajan N, Mao D, Chan S, Koo T, Su X, Sun L, Zhang J, Sung K, Yamakawa M, Gafken P, et al. Ultrasensitive Detection and Characterization of Posttranslational Modifications Using Surface-Enhanced Raman Spectroscopy. *Anal. Chem*. 2006; 78:3543–3550. [PubMed: 16737206]
- Vo-Dinh T, Houck K, Stokes DL. Surface-enhanced Raman gene probes. *Anal. Chem*. 1994; 66:3379–3383. [PubMed: 7978314]
- Wang Y, Seebald JL, Szeto DP, Irudayaraj J. Biocompatibility and Biodistribution of Surface-Enhanced Raman Scattering Nanoprobes in Zebrafish Embryos: In vivo and Multiplex Imaging. *ACS Nano*. 2010; 4:4039–4053. [PubMed: 20552995]
- Watson D, Brown L, Gaskill D, Naivar M, Graves S, Doorn S, Nolan J. A flow cytometer for the measurement of Raman spectra. *Cytometry A*. 2008; 73:119–128. [PubMed: 18189283]
- Watson D, Gaskill D, Brown L, Doorn S, Nolan J. Spectral measurements of large particles by flow cytometry. *Cytometry A*. 2009 Available at: PM:19199345.
- Wiley BJ, Chen Y, McLellan JM, Xiong Y, Li Z, Ginger D, Xia Y. Synthesis and Optical Properties of Silver Nanobars and Nanorice. *Nano Letters*. 2007; 7:1032–1036. [PubMed: 17343425]
- Xie J, Zhang Q, Lee JY, Wang DIC. The Synthesis of SERS-Active Gold Nanoflower Tags for In Vivo Applications. *ACS Nano*. 2008; 2:2473–2480. [PubMed: 19206281]

- Yu K, Lee S, Han J, Park H, Woo M, Noh M, Hwang S, Kwon J, Jin H, Kim Y, et al. Multiplex Targeting, Tracking, and Imaging of Apoptosis by Fluorescent Surface Enhanced Raman Spectroscopic Dots. *Bioconjugate Chem.* 2007a; 18:1155–1162.
- Yu K, Lee S, Han J, Park H, Woo M, Noh M, Hwang S, Kwon J, Jin H, Kim Y, et al. Multiplex Targeting, Tracking, and Imaging of Apoptosis by Fluorescent Surface Enhanced Raman Spectroscopic Dots. *Bioconjugate Chem.* 2007b; 18:1155–1162.
- Yue Z, Zhuang F, Kumar R, Wang I, Cronin SB, Liu Y. Cell kinase activity assay based on surface enhanced Raman spectroscopy. *Spectrochim Acta A Mol Biomol Spectrosc.* 2009; 73:226–230. [PubMed: 19299194]
- Zavaleta CL, Smith BR, Walton I, Doering W, Davis G, Shojaei B, Natan MJ, Gambhir SS. Multiplexed imaging of surface enhanced Raman scattering nanotags in living mice using noninvasive Raman spectroscopy. *Proc. Natl. Acad. Sci. U.S.A.* 2009; 106:13511–13516. [PubMed: 19666578]
- Zhang X, Young MA, Lyandres O, Van Duyne RP. Rapid detection of an anthrax biomarker by surface-enhanced Raman spectroscopy. *J. Am. Chem. Soc.* 2005; 127:4484–4489. [PubMed: 15783231]

Figure 1. Use of the Optical Spectrum in Cytometry

A–C) Conventional fluorescence-based cytometry uses multiple excitation sources to excite multiple dyes. As an example taken from conventional flow cytometry, a violet laser (A) can excite small organic dyes and a range of quantum dots with emission that span the visible spectrum, a blue laser (B) can excite small organic dyes or phycoerythrin and its tandem conjugates, and a red laser (C) can excite small organic dyes and allophycocyanin and its tandem conjugates. The number of dyes that can be detected for a given excitation wavelength is ultimately limited by the spectral width of the emission and the available spectral space. D) Laser-excited Raman scattering features much narrower spectral features, opening the possibility to use more labels in narrower regions of the spectrum.

Figure 2. Anatomy of a SERS tag

A SERS tag generally consists of a plasmonic nanoparticle with the desired resonant wavelength, a Raman active compound that confers a particular spectral signature, a coating to stabilize and protect the SERS tag, and a targeting molecule such as an antibody or nucleic acid that confers molecular specificity to the SERS tag.

Figure 3. Wavelength tunability of plasmonic nanoparticles

Gold and silver nanostructures have size and shape-dependent optical properties that can be predicted from electromagnetic theory or numerical simulations. A) Solid spheres of silver and gold have resonance wavelengths that shift to longer wavelengths with the sphere radius. B) Gold nanorod resonance wavelengths shift to longer wavelengths with aspect ratio. C) Shells of gold over a silica core have resonance wavelengths that shift to longer wavelengths as the ratio of core radius to shell thickness increases.