A Novel Method for Detection of Phosphorylation in Single Cells by Surface Enhanced Raman Scattering (SERS) using Composite Organic-Inorganic Nanoparticles (COINs)

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Abstract

Background: Detection of single cell epitopes has been a mainstay of immunophenotyping for over three decades, primarily using fluorescence techniques for quantitation. Fluorescence has broad overlapping spectra, limiting multiplexing abilities.

Methodology/Principal Findings: To expand upon current detection systems, we developed a novel method for multi-color immuno-detection in single cells using “Composite Organic-Inorganic Nanoparticles” (COINs) Raman nanoparticles. COINs are Surface-Enhanced Raman Scattering (SERS) nanoparticles, with unique Raman spectra. To measure Raman spectra in single cells, we constructed an automated, compact, low noise and sensitive Raman microscopy device (Integrated Raman BioAnalyzer). Using this technology, we detected proteins expressed on the surface in single cells that distinguish T-cells among human blood cells. Finally, we measured intracellular phosphorylation of Stat1 (Y701) and Stat6 (Y641), with results comparable to flow cytometry.

Conclusions/Significance: Thus, we have demonstrated the practicality of applying COIN nanoparticles for measuring intracellular phosphorylation, offering new possibilities to expand on the current fluorescent technology used for immunoassays in single cells.

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Introduction

To better understand the processes occurring in abnormal cells compared to normal cells, there is an urgent need to improve the technology for simultaneous detection of multiple events in a single cell. When coupled with surface marker definitions of cell type, intracellular staining for phosphoproteins can be a powerful tool for understanding the biochemistry of primary cell samples. However, one rapidly reaches limits on the numbers of simultaneous measurements that can be deployed with fluorophore based approaches. To date, antibodies have been most commonly labeled with fluorescent molecules. The use of up to 17 different fluorescent molecules has been implemented by FACS [1], but as is well understood the often overlapping spectra of fluorophores requires compensation and becomes more difficult to carry out with each additional parameter added. Therefore, there is a need to develop molecules that overcome the limitations of fluorescence in multi-parameter detection. Raman scattering may allow the detection and specific attribution of a signal among several simultaneously measured signals and thereby exceed the limit of fluorescence emission overlap adjustment. A first step for implementing a Raman Spectral Flow Cytometer has recently been used for the detection and discrimination of several SER-tags [2,3] and the report detailed here is complementary to those efforts.

Spontaneous Raman scattering is typically very weak, and enhancement is required to improve the spatial resolution of the Raman scattering signal. Surface Enhanced Raman Scattering (SERS) has been successful in enhancing Raman signals using the elements silver, gold, or copper [4–11]. Particles composed of such elements are specifically useful as enhancers of Raman signals, since their surface plasmons (containing valence electrons) are easily excited by laser light, and generate an electric field that can be transferred to nearby Raman active molecules. This results in
an amplification of the Raman signal by $10^{3}$–$10^{14}$ fold [12–14]. By using a variety of Raman labels with distinct Raman spectral fingerprints, it is thus possible to generate a library of SERS molecules. With a carefully selected set of library members, it is possible to deconvolute the Raman spectra to determine the contribution of each individual signature in a combination of spectra. Thus the nanoparticles may be used as a tool for multiple signal detection.

Berlin and colleagues (Intel Corporation) created a clusters of highly active nanoparticles SERS nanoparticles with highly enhanced Raman scatters [9]. These nanoparticles were termed “Composite Organic-Inorganic Nanoparticles” (COINs). The composites are coalesced silver nanoparticles with entrapped organic Raman labels. The COINs are coated with BSA to be biocompatible [9]. COIN clusters enhance the Raman signal by $10^{3}$–$10^{14}$ fold compared to single silver particles coated with Raman biocompatible [9]. COIN clusters enhance the Raman signal by organic Raman labels. The COINs are coated with BSA to be “Composite Organic-Inorganic Nanoparticles” (COINs). The highly active nanoparticles SERS nanoparticles with highly signal detection.

To determine Raman activity related to COIN cluster size we generated COINs of increasing sizes. The nanoparticle size and polydispersity was determined using photon correlation spectroscopy (PCS: Zetasizer, Malvern). The crude COINs were scanned for their Raman spectra using IRBA (see following paragraph). We found that the intensity of the Raman spectra increases with the size of the COIN particles (Figure 1E). The trend is different for the different COINs. The Raman intensity for the AOH COINs increased abruptly when the mean size grew beyond 50 nm, and the intensity decreased when the particle size grew beyond 80 nm. The increase of the Raman signal for the BFU COIN was moderate but reached optimal intensity between 50–60 nm and decreased beyond that size. The COIN size suitable for bioassays was determined to be $60\pm 6$ nm for AOH and $52\pm 5$ nm for BFU, where the optimal intensity of the Raman peak was observed for each COIN. Thus, we have generated SERS based COIN nanoparticles that have specific and enhanced Raman shifts.

Raman microscopy

To reliably detect the Raman signal in a format appropriate for cellular analyses, we developed a automated Raman scanner (Intel Raman BioAnalyser – IRBA) that is suitable for detecting Raman signals (Figure 2A). The schema for the IRBA is illustrated in (Figure 2B). The key components of the microscope are the dichroic filter and notch filter. The dichroic filter allows the laser light to reach the sample, and reflect all other wavelengths. The notch filter blocks the laser light, and transmits all other light wavelengths. The Raman scattering is measured as spectral shifts as little as 30 nm from the excitation laser-light source, hence the slope of the notch filter is high (~90 degrees).

The IRBA scans 64-wells in a microtiter plate-like format. Biological specimens are immobilized on aldehyde glass slides, assembled into a FAST Frame slide holder adopting the 64-well footprint. The sample wells were filled with phosphate buffered saline (PBS), covered with cover glass and loaded into the sample tray holder of the IRBA (see arrow in Figure 2a). During the scan, samples were probed by a continuous wave, diode-pumped, solid-state laser. IRBA custom software is prompted to automatically focus the laser beam onto the sample using an aspheric objective lens with $f/0.5$ numerical aperture and a $20 \times$ magnification. The laser power at the sample stage is 100 mW, with a laser spot size $\sim 1 \mu m$ in diameter. A mechanical shutter reduces the sample exposure to laser light. A typical exposure time is 0.1 seconds per spot. The detector is a back-illuminated, thermoelectrically-cooled CCD camera. The IRBA custom software conducts automated data acquisition of the slide using a user-defined raster scan. The IRBA configuration is set up to collect a single Raman spectrum from a 1 micron spot at a distance of 10 microns with an acquisition time of 100 ms. The IRBA performs a raster scan of the sample containing wells, using a scan matrix of $2 \times 2$ up to $20 \times 20$ with $100 \mu m$ intervals. We tested the optimal raster scan using an AOH COIN solution. We found an increase in the Raman intensity signal with the increase in scan parameters. The
optimal results were obtained using a scan matrix of 17×17 matrices with 100 μm intervals (Figure 2C). Thus, the Raman scanner is able to scan a sample plated in a well chamber. This is applicable to further analysis of cells, as detailed below.

Detection of cell surface antigens using Raman COINs

We tested COINs in immunoassays. We first determined the ability to use COIN nanoparticles to detect surface antigens on single cells stained in suspension. Antibodies were conjugated to COINs. The ability for an antibody-conjugated COIN to function in a bioassay was first determined in an IL-8 ELISA sandwich assay (Figure S1A). The IL-8 antibody-COIN conjugate that shows a linear reactivity to IL-8 antigen concentration with a linear slope (r²>0.8) is considered suitable for use in additional bioassays. Both the AOH and BFU COINs, representing two different fabrication processes, passed the initial control and were considered suitable for use in other biomedical assays.

To further determine the utility of the COINs as detectors, we performed measurements of surface proteins expressed in the U937 cell line. The U937 cell line is a monocytic leukemia with high ICAM-1 (CD54) adhesion molecule expression (Figure 2D). The spectra are indicated (left) and the calculated peak heights are represented as histograms (right). The experiments were performed 3 times in duplicates. The peak heights for the 15×15 and 17×17 are significantly different from the 5×5, 10×10 and 20×20; **P<0.01. (d) Raman intensity of spectra from cells stained with different concentrations of αCD54-AOH-COIN (red - 0.5 mM, blue - 0.25 mM and yellow - 0.1 mM) and AOH-COIN (purple - 0.5 mM, green - 0.25 mM and orange - 0.1 mM), scanned by IRBA (left). Quantitation of the Raman peak height from the spectra observed illustrated as histograms *P<0.05 and **P<0.01. The experiment was performed three times in duplicates.

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Detection of intracellular phosphorylation signaling using Raman COINs

Next, we tested the potential of COIN nanoparticles for the detection of intracellular phosphorylation events. U937 cells activate intracellular signal transduction pathways when treated with IL-4 and IFNγ. Treatment with IL-4 induces the phosphorylation of Stat6, while treatment with IFNγ induces the phosphorylation of Stat1. We first confirmed the increase in phosphorylation of Stat1 and Stat6 by PhosphoFlow analysis (Figure 4A). We measured a 5.9 fold increase of the phosphorylation of pStat1 following IFNγ treatment and 3.3 fold increase in phosphorylation of pStat6 following IL-4 treatment. BFU and AOH COINs were conjugated to antibodies that recognize the Y701 phosphorylated epitope of the Stat1, and the Y641 epitope of the Stat6 proteins. The cells were then fixed and permeabilized as previously described [15].

Raman Tags for Cell Signaling
Raman Tags for Cell Signaling

(A) Graphs showing Raman shift (cm⁻¹) and Raman intensity (a.u.) for different samples.

(B) Graphs showing Raman peak height (a.u.) for different samples: Control, BFU, CD54, and CD8.

(C) Graphs showing Raman shift (cm⁻¹) and Raman intensity (a.u.) for different samples: Control, BFU, BFU-CD54.

(D) Images showing 5μm scale bars.

(E) Graphs showing Raman shift (cm⁻¹) and Raman intensity (a.u.) for different samples: Control, BFU, BFU-CD8.
To prevent non-specific binding of COIN to intracellular proteins, an additional fixation step was carried out. Non-treated and treated cells were stained with antibody-conjugated and non-conjugated COIN washed and scanned using IRBA. The average spectra for IFNγ and IL-4 treated and non-treated cells are shown for AOH-pStat6 (Figure 4D) and BFU-pStat1 (Figure 4C). To determine if the COIN itself affects the binding ability, the antibodies were alternated on each COIN. The changes in peak height were determined and the ratio of the Raman signal in treated cells was compared to non-treated cells (Figure 4C). We detected a 5.9 fold change in pStat1 phosphorylation using BFU-pStat1-BFU COIN and a 6.7 fold change using BFU-pStat1-AOH COIN. We detected a 2.9 fold change in pStat6 phosphorylation using BFU-pStat6-BFU COIN and a 2.7 fold change in using BFU-pStat6-AOH COIN. The detected changes in phosphorylation of the Stat1 and Stat6 molecules using the AOH or the BFU COINs was similar to what was observed by PhosFlow.

Thus we have demonstrated the utility of COINs for measuring intracellular phosphorylation events in single cells.

Detection of two simultaneous Raman signals using COINs

Ultimately, we determined the ability to conduct intracellular multiplex assays using COINs. A multi-parameter analysis was designed and simultaneous stained cells with AOH and BFU COINs, for detecting two phosphorylation events in a single cell. We co-treated U937 cells with IFNγ and IL-4. A simultaneous staining of the cells using BFU conjugated to pStat1 and AOH conjugated to pStat6 antibody. The samples were scanned using IRBA. The Raman spectra were deconvoluted using a software package designed for the dataset, termed MultiPlex (Intel Corporation). The Raman peak heights were calculated and represented as histograms (Figure S5A). The peak heights from cells stained with antibody conjugated COINs were normalized to the Raman signal from cells stained with non-conjugated COINs (Figure S5B). The Raman signal from cells treated with either IFNγ or IL-4 cytokine is statistically similar to the signal from cells stained with both cytokines simultaneously (p>0.2).

The data demonstrate it is possible to use COINs for the measurements of two simultaneous phosphorylation events in a cell staining assay.

Discussion

In this study we have demonstrated the ability to use COIN nanoparticles for multi-plex immuno-detection in single cells. We can generate multiple and distinct COIN Raman nanoparticles with resolvable signatures that can be used to detect surface antigens and to measure changes in intracellular phosphorylation events. Enhanced Raman signatures via SERS and COIN technology, offers possibilities exceeding fluorescent dye technology limits. COIN Raman spectra have several sharp peaks that define a “fingerprint” for each COIN. We can readily deconvolute multiple COIN spectra and may easily exceed the limit of fluorescence technology in multi-detection assays (we have already deconvoluted up to eight spectra using simulated complex spectra, unpublished). The detection of Raman signal of COINs, whose Raman detection is independent of fluorescence, may provide a dramatic increase in the multiplicity of simultaneous measurements. Another advantage of Raman COIN technology is versatility. The Raman spectra of COINs are measured as a shift relative to the excitation wavelength. The excitation of fluorophores is confined to a specific wavelength and re-emits energy at different (but very specific) wavelengths. COIN can be excited by different wavelengths depending on the available equipment.

Recently developed cadmium selenide-based quantum dots (Q-dots) are used to complement fluorescent dyes. Q-dots are physically uniform, may be excited by a wide band of light and emit a narrow band of light depending on its size. Since the emission spectra of the quantum dots are narrower than fluorescent molecules, the spectral overlap is less than with fluorescence molecules and thus require less manipulation than fluorescence when used for immunooassays [1]. To date, eight different quantum dots are available. The ability to use Q-dots as
fluorescent tags in combination with molecular dyes to tag antibodies has advanced our ability to measure several simultaneous events in a single cell setting the current high bar for flow cytometry at 17 simultaneous measurements per cell. The future goal of our work with COINs is to develop a technology for flow cytometry that can concurrently measure Raman spectra. We hope to integrate the Raman spectra detection with conventional flow cytometry to exceed the current limits of fluorescence technology.

For the purposes of this report, we developed an automatic scanner to acquire Raman spectra – the IRBA. The IRBA is a compact, bench-top instrument. It was formatted to scan samples in 64-well microtiter plate format with user-friendly custom software. The IRBA has a fully-enclosed laser system. The components were systematically integrated and optimized to meet the specifications of the intended experiments and target applications. The IRBA allowed the sampling of Raman spectra from single cells to generate data for further analysis and stands as a training platform for the development of future generations of machines, including those with true flow capabilities.

In conclusion, Raman COIN technology is emerging as a powerful tool that will be useful for multi-parameter simultaneous measurements of protein and protein modification occurring in a single cell. By enhancing our capacity to measure intracellular phospho specific events at the single cell level this will allow determinations of additional parameters in studies of cellular processes. Thus, studies that use intracellular potentiation as a marker of biochemical process [16–18], clinical outcome in primary patient materials [19–23], or for determinations of signaling networks by computational processes [24] can be enhanced.

Materials and Methods

Coin Fabrication

BFU and AOH COINs were fabricated at Intel and Stanford as previously described [9]. Briefly, for AOH COIN fabrication, 12 nm silver seeds were prepared with silver nitrate (AgNO$_3$) and reduced by sodium borohydride (NaBH$_4$). The silver seeds were heated at 95°C for 0.5 M AgNO$_3$ and 3–50 μM Acridine Orange Raman dye. The solution was heated at 95°C for 60 min during which seed particles grew with the adsorption of the Raman dye. The reaction was stopped by the addition of 0.5% BSA. The COIN clusters are encapsulated with BSA to stabilize and to introduce bio-functional groups on the surface.

COIN conjugation to antibodies

We conjugated the antibodies to the BSA encapsulation of the COINs as previously described [25]. The carboxylic groups on BSA are activated with N-(3-dimethylamino-propyl)-N/-ethyl-carbodiimide (EDC) (Sigma, #39391). Antibodies used for COIN conjugation are: CD54 (BD, #550302), CD8 (BD Bioscience, 554716), pStat1 (Y701) (BD Biosciences, #612596), pStat6 (Y641) (BD Biosciences, #612600).

IL8 antibody ELISA sandwich assay

We performed an ELISA immuno-quality control assay (iQC) using IL8 antibody to test the quality of COIN-antibody conjugate. We coated aldehyde treated slides (NUNC™, #23164) with IL8 capture antibody (BD Pharmingen, #554717), mounted on FAST® frames (Whitman Inc., #1048) with IL8 to the wells for 15 minutes and then washed with PBST (*2). We used BSA or AOH COINs conjugated to 3IL8 antibody (BD Pharmingen, #554717) to stain the wells for 1 hour at room temperature (RT). We washed the wells in PBST (PBS and 0.1% Tween 20) and 0.1 M NaCl. The wells were filled with PBS and covered with cover glass (VWR International, #48366 067). We measured the Raman spectra for each well, using the Integrated Raman BioAnalyzer (IRBA), and a 532 nm excitation laser. The COINs that pass the iQC criteria were used for further detection assays. The criteria are: 1) experimentally-derived linear relationship between IL-8 concentration and Raman intensity readings ($r^2 = 0.8–1$); 2) the COIN should not precipitate during antibody conjugation.

CD54 COIN ELISA direct binding assay

A CD54-COIN ELISA direct-binding assay was performed as described above using monoclonal αCD-54 antibody (BD Pharmingen, #555364) conjugated to AOH or BFU COINS. Wells were coated with 5 ng/ml–500 ng/ml recombinant human CD-54 (ICAM-1) protein (R&D, #ADP4-200). An experimentally-derived linear relationship between CD54 protein concentration and αCD54-COIN Raman intensity readings ($r^2 = 0.8–1$) was used to determine that the COINS passed iQC. These antibody-COIN conjugates were used for further cell staining procedures.

pStat-1 COIN ELISA sandwich assay

We performed the pStat1 COIN sandwich assay as described above. Rabbit monoclonal αStat-1 antibody (Cell Signaling Technologies, #9175) was used as the capture antibody. We incubated 0–10 μg pStat1 blocking peptide (Cell Signaling Technologies, #1038) in the antibody coated wells. We purified pStat1 (pY701) mouse monoclonal antibody (BD BioScience, #612233) using Protein G and Protein A orientation kits (PIERCE, #44990), then conjugated the antibody to the AOH or BFU COINs. An experimentally-derived linear relationship between pStat1 peptide concentration and αpStat1-COIN Raman intensity readings ($r^2 = 0.8–1$) was used to determine that the COINS passed iQC. These antibody-COIN conjugates were used for further cell staining procedures.

Measurement of Raman signal using IRBA

We measured COIN Raman spectra using the IRBA and a 532 nm excitation laser. We used raster scan matrices: 5×5,
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A

![Graph A](image)

Raman shift (cm⁻¹)

B

![Graph B](image)

C

![Graph C](image)

D

![Graph D](image)

E

![Graph E](image)
Figure 5. Detection of two intracellular phosphorylation events using two different COINs simultaneously. a) Raman spectra of the U937 cells treated with IFNγ and IL-4 simultaneously. The cells were stained with pStat1-BFU and pStat6-AOH simultaneously and separately. Cells were also stained with non-conjugated AOH and BFU COINs. The spectra are representative for five independent experiments. Cells were also stained with BFU and AOH that were not conjugated to antibodies. b) Extrapolated COIN spectra for treated (IFNγ+IL-4) and untreated cells (Non stim) stained with pStat1-BFU and BFU. c) Extrapolated COIN spectra for treated and untreated cells stained with pStat6-AOH and AOH. d) The Raman intensity of the Raman spectra for pStat1-BFU and pStat6-AOH COINs were calculated using the “MultiPlex” program (© Intel Corporation). The results are presented as histograms for single and double stain procedures. e) The fold change is the identified intensity of the spectra of the pStat1 and pStat6 conjugated COINs from treated and non-treated cells normalized to non-conjugated BFU and AOH COINs that were not conjugated to antibody. The results are the average of five independent experiments. doi:10.1371/journal.pone.0005206.g005

Supporting Information

Figure S1 ELISA sandwich assay for the detection of surface antigens using COIN based Raman spectroscopy. a) ELISA sandwich assay of zIL-8-AOH (left) and zIL-8-BFU (right). b) ELISA assay of zCD54-AOH (left) and zCD54-BFU (right). c) Comparison of concentration dependence and staining performance of AOH and BFU COIN. Found at: doi:10.1371/journal.pone.0005206.s001 (1.53 MB TIF)

Figure S2 Peak height analysis. A representative peak is selected and the peak start, top and end are determined. The Raman peak is identified and projected to all spectra in the scans performed by IRBA. The area under the peak is determined for each sample and determined as peak height. Found at: doi:10.1371/journal.pone.0005206.s002 (1.00 MB DOC)

Figure S3 Optimization of COIN concentration in staining protocol. a) Quantitation of the Raman peak height from the spectra observed for cells stained with different zCD54-BFU- COIN and BFU-COIN concentrations, scanned using IRBA illustrated as histograms *p<0.05 and **p<0.01. b) Comparison of concentration dependence of BFU and AOH COINs conjugated to zCD54. The fold change is the average of five independent experiments. There is no statistical difference between the BFU and AOH COINs (p>0.2). Found at: doi:10.1371/journal.pone.0005206.s003 (1.18 MB DOC)

Figure S4 Correlation between BFU and AOH COINs for surface antigen detection. a) Antigen specific detection of CD54 with COIN. Raman spectra quantitation of cells stained with zCD54-AOH and zCD8- AOH COINs represented as histograms (left) and is the average of five independent experiments. Specificity of zCD54-AOH in U937 cells is indicated (**p<0.01). Comparison of the Raman peak height ratio detected for the BFU and AOH COINs of CD54 and CD8 expression in U937 cells (right). b) Cell specific detection of CD54 surface antigen with AOH COIN. Raman spectra peak height quantitation of CD54 expressing U937 cells and non-expressing HB2 cells stained with zCD54-AOH COIN is represented as histograms (left) and is the average of five independent experiments. Specificity of zCD54-AOH in U937 cells is indicated (**p<0.01). Comparison of
the Raman peak height ratio detected for the BFU and AOH COINs of CD54 in U937 and H82 cells (right). c) Raman spectra peak height quantitation of human PBMC, H82 and U937 cells stained with αCD8-AOH COIN represented as histograms (left) and is the average of five independent experiments. Specificity of quantitation of human PBMC, H82 and U937 cells stained with αCD8-AOH COIN is indicated (**p < 0.01). Comparison of the Raman peak height ratio detected for the BFU and AOH COINs of CD8 expression in U937, H82 and human PBMC cells (right). The detection efficacy with BFU and AOH COINs are similar and not statistically different (p > 0.2) (right).

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Author Contributions

Conceived and designed the experiments: CMS JZ. Performed the experiments: CMS SME JZ LN DJM ALK. Analyzed the data: CMS SME JZ. Contributed reagents/materials/analysis tools: LN JZ KBS RS LS SC. Wrote the paper: CMS GPN.

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Figure S5

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