Combined SERS biotags (SBTs) and microfluidic platform for the quantitative ratiometric discrimination between noncancerous and cancerous cells in flow

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ABSTRACT

SERS biotags are made from polymer-encapsulated silver nanoparticle dimers infused with unique Raman reporter molecules, and carry peptides as cell recognition moieties. We demonstrate their potential use for early and rapid identification of malignant cells, a central goal in cancer research. SERS biotags (SBTs) can be routinely synthesized and simultaneously excited with a single, low intensity laser source, making the determination of the relative contribution of the individual SBTs to the overall spectrum tractable. Importantly for biomedical applications, SERS employs tissue-penetrating lasers in the red to near-infrared range resulting in low autofluorescence. We have previously described a multiplexed, ratiometric method that can confidently distinguish between cancerous and noncancerous epithelial prostate cells in vitro based on receptor overexpression. Here we present the progress towards the application of this quantitative methodology for the identification of cancer cells in a microfluidic flow-focusing device. Beads are used as cell mimics to characterize the devices. Cells (and beads) are simultaneously incubated with two sets of SBTs while in suspension (simulating cells' capture from blood), then injected into the device for laser interrogation under flow. Each cell event is characterized by a composite Raman spectrum, deconvoluted into its single components to ultimately determine their relative contribution. We show that using SBTs ratiometrically can provide cell identification insensitive to normal causes of uncertainty in optical measurements such as variations in focal plane, cell concentration, autofluorescence, and turbidity.

Keywords: Cancer detection, SERS, microfluidics, silver nanoparticles, flow-focusing.

1. INTRODUCTION

A central goal in cancer research is the early and rapid identification of malignant cells, ideally free-flowing in bio fluids such as urine[1] or blood[2]. Raman[3,4] and Surface-enhanced Raman spectroscopy (SERS) have been used as an immunohistochemistry tool[5,6] for the detection of biomarkers in biological fluids or in vivo[7], and for cancer detection from blood[8]. Raman and FTIR signals measured directly from cells are typically much weaker than those that are measurable with bright labels such as SERS biotags (SBTs), which can now be routinely synthesized[9]. All SBTs can be excited using a single, very low intensity laser source, making the feasible a direct determination of the relative contribution of individual SBTs to the overall spectrum. SERS tags are especially applicable to biomedical applications, since they are excited by tissue-penetrating lasers in the red to near-infrared range resulting in low autofluorescence and high signal enhancement[10,11]. The central feature of SERS is its multiplexing capability by using pre-made, encapsulated nanoparticle clusters that are then infused with one of several highly Raman-active reporter molecules. The multiple narrow bands in the SERS spectrum acts as a unique barcode that is spectrally differentiable from other tags[9,11]. The SERS intensities achieved are comparable to fluorescence[12]. As a demonstration of the technology's far reaching capabilities, we recently used these spectrally rich barcodes to detect the unique neuropilin-1 biomarker expression pattern of prostate cancer cell, contrasting them to healthy prostate cells[11]. Two sets of SBTs were used in combination to discriminate the cell types. One SBT targeted the neuropilin-1 (NRP) receptors of cancer cells, while the other functioned as a positive control (PC) by binding to both non-cancerous and cancer cells (via the HIV derived TAT peptide). Methylene blue (MB) and thionin (Figure 1C) were used as Raman reporters, respectively, on the PC-SBT and

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on the NRP-SBT. Point by point 2D Raman maps and average SERS signal per cell yielded a characteristic NRP/PC ratio from which cancer cells were identified[11].

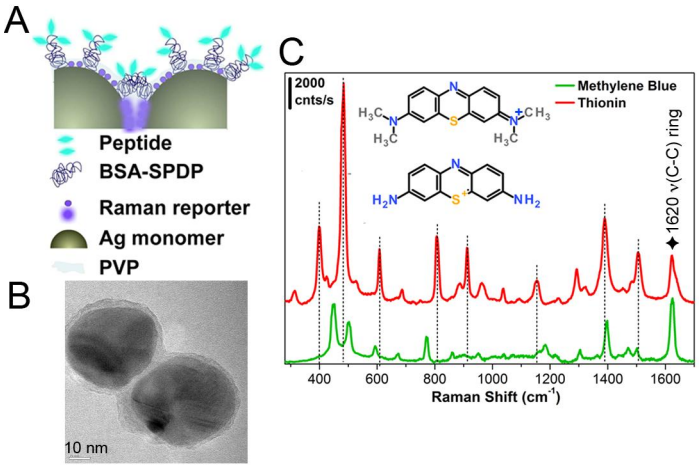

Figure 1. SBT SERS signals originate from closely spaced nanoparticles. A) SERS biotag design includes the dimer junction, a stabilizing coat with attached affinity agent, and the Raman active spectral tag. B) TEM of a silver dimer with coating visible. Plasmon resonance coupling between nanoparticle dimers form high field gradients, enhancing the Raman scattering from reporter molecules contained within the structure. C) Spectral signatures from the two encoded SBTs: thionin and methylene blue.

Here, we describe our progress towards the development of a powerful technique for identifying and sorting individual mammalian cells, such as individual cancer cells, by combining Raman spectroscopy with flow-focusing microfluidics. By labeling mammalian cells with SERS biotags (SBTs) and flowing them in a flow-focused microfluidic channel the cells can be individually and rapidly interrogated by laser Raman, with identification possible by spectral unmixing of the SERS signals.

The enhancement that SERS provides allows the biotags' entire Raman spectrum to be recorded, and, when multiple tags are used, to employ ratiometric methods as one or more of the on-board tags can act as a local intensity reference. A hydrodynamic flow focusing microfluidic system was designed to enable mammalian cell interrogation and identification at the single cell level using SBTs. Herein we report our progress in transferring this ratiometric method that we have previously used with 2 sets of SBTs to unequivocally distinguish between cancer and normal cells by at the ratio of the two SBTs[11], to the microfluidic environment (Figure 2). We have successfully built a microfluidic device in which we used streptavidin coated, ~6 µm diameter polystyrene beads as cell mimics to characterize the devices' and the SERS response. We show below that we are able to successfully detect flowing polystyrene beads loaded with SBTs via Raman, and detect two distinct populations based on our ratiometric approach. We will also present our preliminary results flowing live mammalian cancer cells labeled with SBTs.

2. METHODOLOGY

2.1 SBT synthesis

Silver colloid was synthesized according to the Lee and Meisel protocol[13]: 500 ml of deionized water (DI, resistivity 18 MΩ) with 1 mM silver nitrate (Sigma) 99.999%) were brought to a boil. Then, 10 ml of 1% trisodium citrate dihydrate (Fisher). The mixture was kept at boiling temperature for about 90 min until the color turned dark green/gray. Aliquots of the colloid were taken and centrifuged at 800×g to remove the smallest particles. The yellow supernatant was discarded and the pellet resuspended in deionized MilliQ water (DI) and diluted until the absorbance of the band at 406 nm was 0.3 at 0.1 mm path length. The resulting colloid will be called Ag03. SBTs were then prepared by adding to every 100 µl of Ag03 3.5 µl phosphate buffer (250 mM, pH 7.5), 4 µl hexamethylenediamine (Sigma, 98%, 0.4 mg/ml in DI, pH 4.0), waiting for 2 min, then adding 4 µl 1% polyvinylpyrrolidone 40kDa (Sigma) in DI and 100 µl DI, and, finally waiting for 5 min then adding 2 µl BSA-SPDP (6.5 mg/ml in 0.1x PBS. Bovine serum albumin, Sigma, modified

with N-succinimidyl 3-(2-pyridyldithio)-propionate, or SPDP, Pierce #21857 so that the modified protein interacts with the Ag surface strongly through the SPDP molecule with the pyridine group appearing in the SERS spectrum prior to Raman reporter being added. Each protein carries several free hanging SPDP groups that can interact with the cys-peptides thus holding peptides on the outside of the SBT coating for possible interaction with cell membranes. The particles resulting from this controlled aggregation stage were called Ag_{PHPB} and were then used to prepare either NRP and PC SBTs for use in cells, or biotinylated tags (b-SBTs) for use with beads. For PC-SBT, to every 200µl of Ag_{PHPB}, 4 µl MB (Sigma, dye content ≥82%, 600 µM in DI), 2 µl KCl (Mallinkrodt Baker Phillipsburg, NJ, 500 µM) were added and incubated at room temperature for 30 min, then we added 2 µl FAM-Cys-TAT (500 µM in DI), incubated for 15 min and backfilled with 1 µl BSA 5% (0.1xPBS). For the NRP-SBT, to every 200 µl of Ag_{PHPB}, we added 4 µl of thionin (Sigma, dye content ~90%, 0.2 mg/ml in DI), incubated for 30min at room temperature, added 2 µl BSA-SPDP (6.5 mg/ml in 0.1x PBS), wait for 15 min, added 1µl BSA 5% (in 0.1x PBS) and 5 µl FAM-CGRPARPAR-OH (provided by Erkki Ruoslahti's group at Sanford-Burnham Medical Research Institute, 200 µM in DI). For biotinylated SBTs (b-SBTs), the peptide step was replaced by the addition of SVA (succinimidyl valerate)-PEG_{5kDa}-biotin (LysanBio) at a final concentration of 1mg/ml of colloid, and let incubate at 4˚C overnight. After adding 0.005% final concentration of Tween-20 (T20 - polyoxyethylene (20) sorbitan monolaurate solution 10% in H_2O, Sigma), the SERRS biotags were washed by centrifugation (10 min @ 800×g), the supernatant was discarded (to remove most of the non-SERS bright single silver nanoparticle biotags) and the pellet resuspended in either $1/20^{th}$ (for beads) or $1/40^{th}$ (for cells) the initial volume in 0.1x PBS/0.1 % BSA/ 0.005% T20.

2.2 Device manufacturing

The microfluidic device (see Figure 2A for layout design) was fabricated using PDMS sandwiched between two glass slides. Inlets and outlets were drilled into the glass. Pipet tips were glued to the glass as reservoirs and the vacuum line interface at the inlets and outlet, respectively. To pattern the channels on the PDMS layer, a mold was fabricated first out of SU-8 photoresist on a silicon wafer using soft photolithography. PDMS was then mixed with cross-linker and poured on the mold. After an hour of heating at 80˚C the PDMS was peeled off the mold with channels facing the mold. Via holes were punched in the outlet and inlets and the PDMS layer was bonded to glass slides after the ozone treatment of the bonding side. Two types of devices, varying the channel dimensions, were used depending on whether cells or beads were used. Device 1 was used for beads, with a depth of 20 µm and a focal width (the width of the middle flow after the jucntion) of about 20 µm. Device 2 was used with cells, having a depth of 40 µm and a focal width of 10 µm.

2.3 Bead preparation

Streptavidin coated polystyrene beads of about 6 µm in diameter (Polysciences # 24158-1) were washed twice by centrifugation according to manufacturer's protocol to remove excess streptavidin. The pellet was resuspended in PBS with 1% BSA (Sigma) and 0.005% T20 and stored at 4˚C. For incubation with SBTs the beads stock was used at $1/100^{th}$ and 1 µl b-SBTs at the required ratio were added to every 100 µl of beads, and incubated for >30 min at room temperature. Beads were then either placed on a microscope slide for 2D SERS mapping or injected into the devices. This concentration of beads resulted in a throughput of ~12 beads/min after the junction in the device. The velocity of the beads at the interrogation spot were ~5mm/s which means that each bead was effectively exposed to the laser for 1-2ms.

2.4 Cell culture

PPC-1 cells, an epithelial cell line originating from a bone metastasis of a prostate cancer patient, expressing the biomarker NRP-1, were a generous gift from Erkki Rouslahti's group (Sanford-Burnham Medical Research Institute, UC Santa Barbara). They were grown in DMEM/high glucose (HyClone) supplemented with 10% FBS, at 37˚C in a 5% CO_2 atmosphere. Cells were plated in multiwell plates and after 24/48 hrs harvested using a non-enzymatic cell dissociation buffer (Invitrogen) that does not disrupt the membrane receptors. Cells were then washed by centrifugation for 2 min at 1000 × g and the pellet was resuspended in the appropriate volume of DMEM+10% FBS in order to obtain a concentration of $1x10^5$ cells/100 µl. SBT were then added to each cell suspension (with PC versus NRP ratio of 1:1 v/v) and incubated for 60 min at room temperature mixing every 15 minutes, then injected into the device's inlet.

2.5 SERS measurements

The SERS measurements were conducted using a Horiba Jobin-Yvon LabRAM Aramis spectrometer, equipped with a 633 nm excitation. The static measurements on beads deposited on glass were carried out by scanning each bead with the 100x objective (5 µW at sample), using a hole of 600 µm, slit of 300 µm and a 600 gr/mm grating. Each point in the 2D

map was collected at 1.8 µm step size. SERS time measurements were carried out using a 50x LWD objective (8 mW spread across the line) in line scan mode. In this modality the laser is rastered by oscillating mirror over a region of the sample at high speed, forming an excitation line, with the laser 'curtain' axis perpendicular to the flow (see Fig. 2B). The slit was set to 250 µm, the hole to 600 µm and a 600 gr/mm grating. The data were acquired with a point every 150-200ms, with laser exposure times of 20 ms for beads and a point every 550 ms with laser exposure time of 400 ms. A solution of SERS-tagged cells/beads was put in the inlet middle channel and the side channels were filled with buffer.

2.6 Data Analysis

Initial data analysis (mapping, whole cell averaging of signal, baseline correction, normalization) was performed with the help of the on-board program LabSpec. The deconvolution and NRP/PC ratio calculations for point-to-point maps were done using Mathematica's FindFit, a nonlinear least-squares-fitting algorithm, and the SBT pure spectra were processed by a customized Mathematica program. For microfluidics time series the sequential spectra are thresholded, based on the intensity (counts/s) of the band at 480cm^{-1} in the raw data, which was decreased iteratively until a significant number of events are collected. The unmixing by linear combination of basis spectra give ratios of the two tags (thionin and methylene blue SBTs). We observe that as the threshold is lowered to a level that approaches the average background intensity, the number of events counted increases dramatically. The threshold was, therefore, set at the point at which the background registers only a moderate number of events for the sample with beads or cells, and the blank sample has few events (>10-fold difference). A single threshold was applied to all samples (with and without the beads or cells).

3. RESULTS AND DISCUSSION

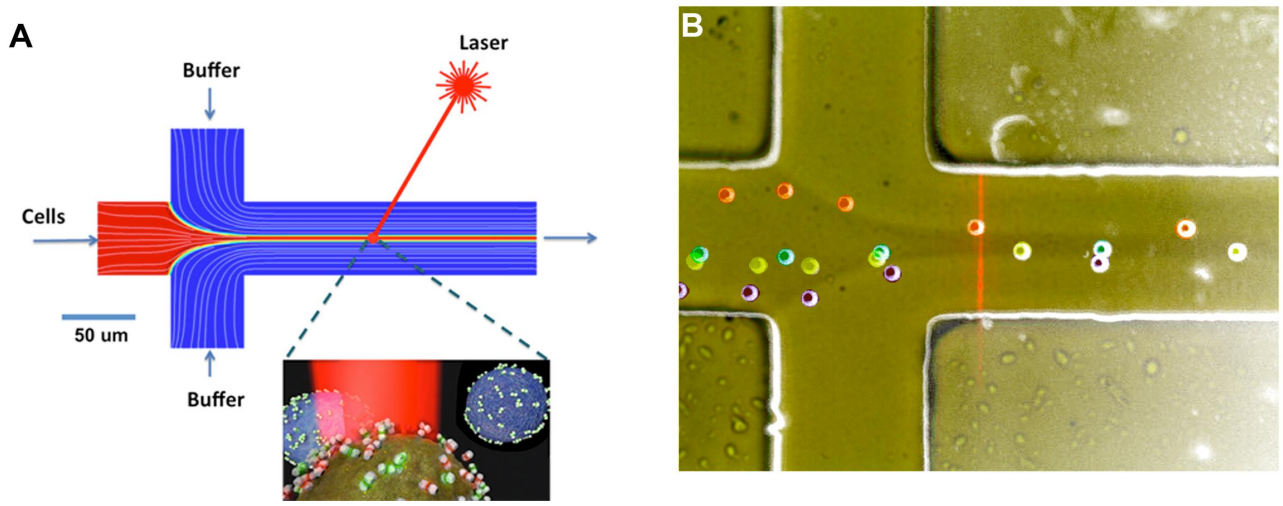

Figure 2. A) Cartoon illustrating Flow focusing that we propose for cell identification using two SERS Biotags. "Green" SBTs bind to all cells whereas "red" ones bind only to the target cancer (brown) cell. As each cell passes through the laser the ratio of tags is determined. B) Time lapse composite image of some beads flowing in the device. Each bead is colorized so that its path along the channel can be recognized in the picture. The image is overlaid with the picture of the device under the Raman microscope. The red laser line is visible to the right of the junction. The buffer flowing in the cell/bead inlet channel is colored so that the actual shape of the flow is also visible as a darker area that narrows after the junction where the two sheath flows meet with the cell/beads flow.

To combine the ratiometric analysis with the microfluidic platform, a sample of target cells (or beads), previously incubated with SBTs, were injected into the microfluidic device. The cells/beads are hydrodynamically-focused to the center of the channel, so that they can be interrogated, single file, by the laser (Figure 2B). The device developed for this purpose has three inlet flows that meet at a junction where the middle fluid, containing cells/particles, is sandwiched by two sheath flows of buffer. The channel lengths and flows are designed such that cells/particles advect in a single file after the junction and with a velocity that dictates residence time in the laser. The exposure time for each cell can be

calculated knowing the cell size and the flow velocity. The whole system is run with a single vacuum pump at the outlet [14].

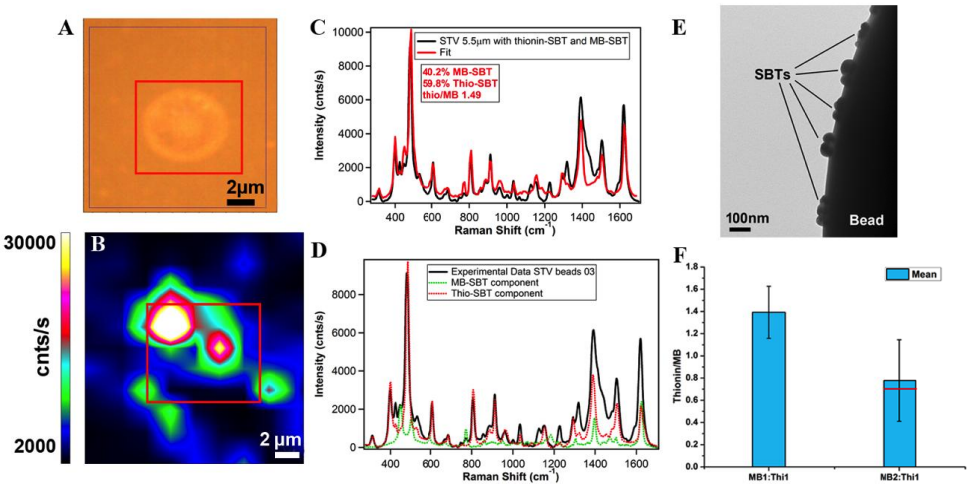

Figure 3. Panels A-D summarize the data collection and analysis done on each STV beads carrying b-SBTs. Firstly each bead is scanned by the laser (A), to obtain a 2D map of it that shows the variation of intensity of a certain band of interest (1620 cm-1 in our case) across the x and y directions (B). The average signal across each bead is then fit using a direct least squares algorithm (C), whose individual component can be seen in panel (D). From the fit coefficients and an appropriate calibration curve we then estimate the percent composition of each tag. Panel (E) shows a TEM image of a STV bead carrying several b-SBTs. In (F) we demonstrate the robustness of our calibration method. A sample prepared using a tag ratio of 1:1 produces a measured value of 1.4±0.2. Using this correction a 1:2 sample should yield an observed value of 0.7, close to the observed value of 0.8±0.4.

We obtained initial results using streptavidin functionalized beads with SBTs. We validated the detection and spectral unmixing first by placing on a glass slide, beads on which biotinylated SBTs (b-SBTs) were adsorbed at different ratios to replicate the scenario of differential binding and uptake rate by various cells. Figure 3 summarizes how beads carrying SBTs were analyzed on glass. The chosen bead is scanned with the laser across the area enclosed by the red square, then the average spectrum from the whole bead is fitted using a least squares algorithm to determine the relative fraction of each tag (Figure 3F).

Figure 3F shows the measured ratio from several beads incubated with either a 1:1 or a 1:2 v/v ratio of the two b-SBTs. When the NRP/PC ratio is 1:1, the actual measured value was 1.4. Knowing this we convert a second volume ratio 1:2, to an expected intensity ratio of 0.8. The observed value of 0.7 indicates that the spectra were accurately unmixed.

When beads incubated with b-SBTs at a NRP/PC ratio of 1:1 are flowed into the device we are able to detect their passage based on the Raman signature of the SBTs. After applying the threshold according to the procedure in section 2.6, 60 bead events were counted (Figure 4A, top panel). We expected 75 based on the integration time, duty cycle of the detector (10% at 20 ms integration), and flow rate. The average NRP/PC ratio (Figure 4A, bottom panel) for the 60 events was 1.8±1.5. The high standard deviation is due to the presence of 9 events which exhibit a ratio considerably higher than 2.5 and could be due either to beads with an unusually high number of bound NRP b-SBTs, or to random free flowing unusually bright SBTs or their aggregates. When the outliers are excluded, the mean NRP/PC ratio is 1.3±0.5, in accordance with the average value of 1.4±0.2 obtained from the static maps on glass (Figure 3F). We observe that in the background data, after applying the threshold, there are 8 events. All exhibit NRP/PC ratios >2.5, which would be consistent with the hypothesis that very high ratio are caused by abnormal clusters of NRP b-SBTs.

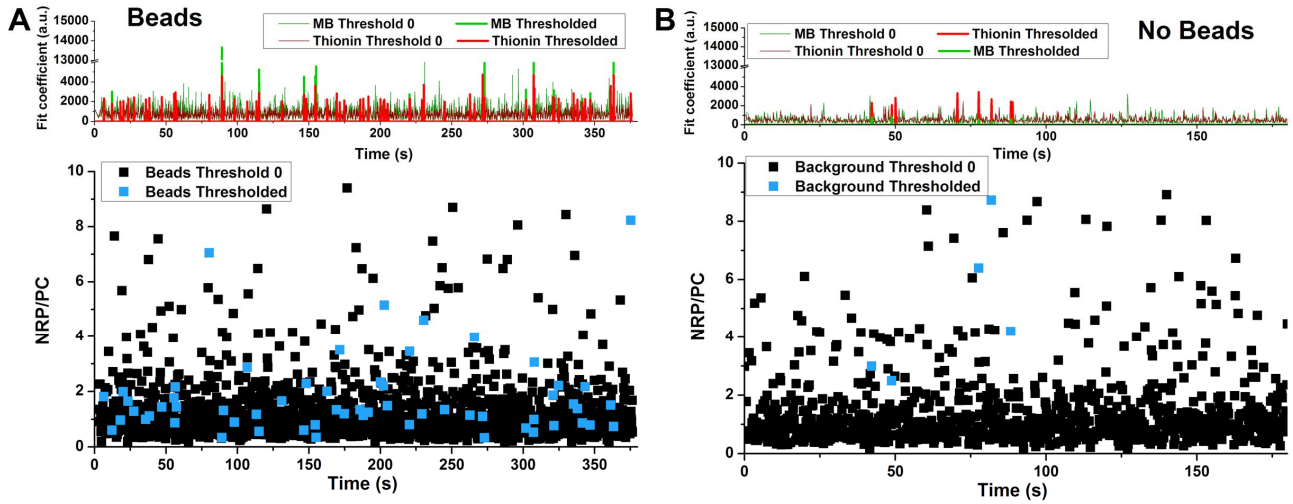

Figure 4. Thresholding and resulting NRP/PC ratios for beads carrying b-SBTs in Device 1. A) The upper panel show the values of the fit coefficients for both tags (thionin in red and MB in green) at each time-point before (dark color code) and after (bright color code) applying the threshold. The lower panel shows the NRP/PC ratios for each spectrum in the time series before (black dots) and after (blue dots) applying the threshold. B) Fit coefficients and NRP/PC ratios as in A), but without beads flowing in the device.

To test the device with living cancer cells we incubated PPC-1 cells with NRP and PC SBTs (Device 2). Although the cells ceased flowing after a few minutes, we were able to identify cell events using the threshold methodology employed for beads (Figure 5A and 5C). We confidently detected 30 cell events, yielding a mean NRP/PC ratio of 1.0±0.6, consistent with our previous reference[11] value of 1.1±0.1. We observe the presence of one event with abnormally high ratio of 3.9, once again possibly due to either a cell binding mostly NRP-SBTs or to presence of particularly bright free flowing NRP-SBTs. Figure 5B shows some examples of the fits obtained with the Mathematica algorithm for both cell events above threshold and below threshold. We are currently optimizing the device for collecting more events to analyze statistical populations of cancer cells, and mixtures of normal and cancer cells.

4. CONCLUSION

We successfully built a flow focusing microfluidic device for the ratiometric SERS analysis of beads and cells, tagged with two sets of SBTs. The beads/cells travel along a single file focused in the center of the device and when they cross the laser line a full Raman spectrum is acquired. The signal is a composite of the two SBTs and can be deconvoluted to ultimately determine the relative amount of each component. We show that we can detect beads/cells by SERS that travel across the laser at times in the order of a few ms. The indicative ratio is independent of variations in the location of the focal plane, the local cell concentration and turbidity. The technique also benefits from the low laser intensities (8 mW total, spread across the line) needed for good signal to noise and the use of a single wavelength to excite all SBTs.

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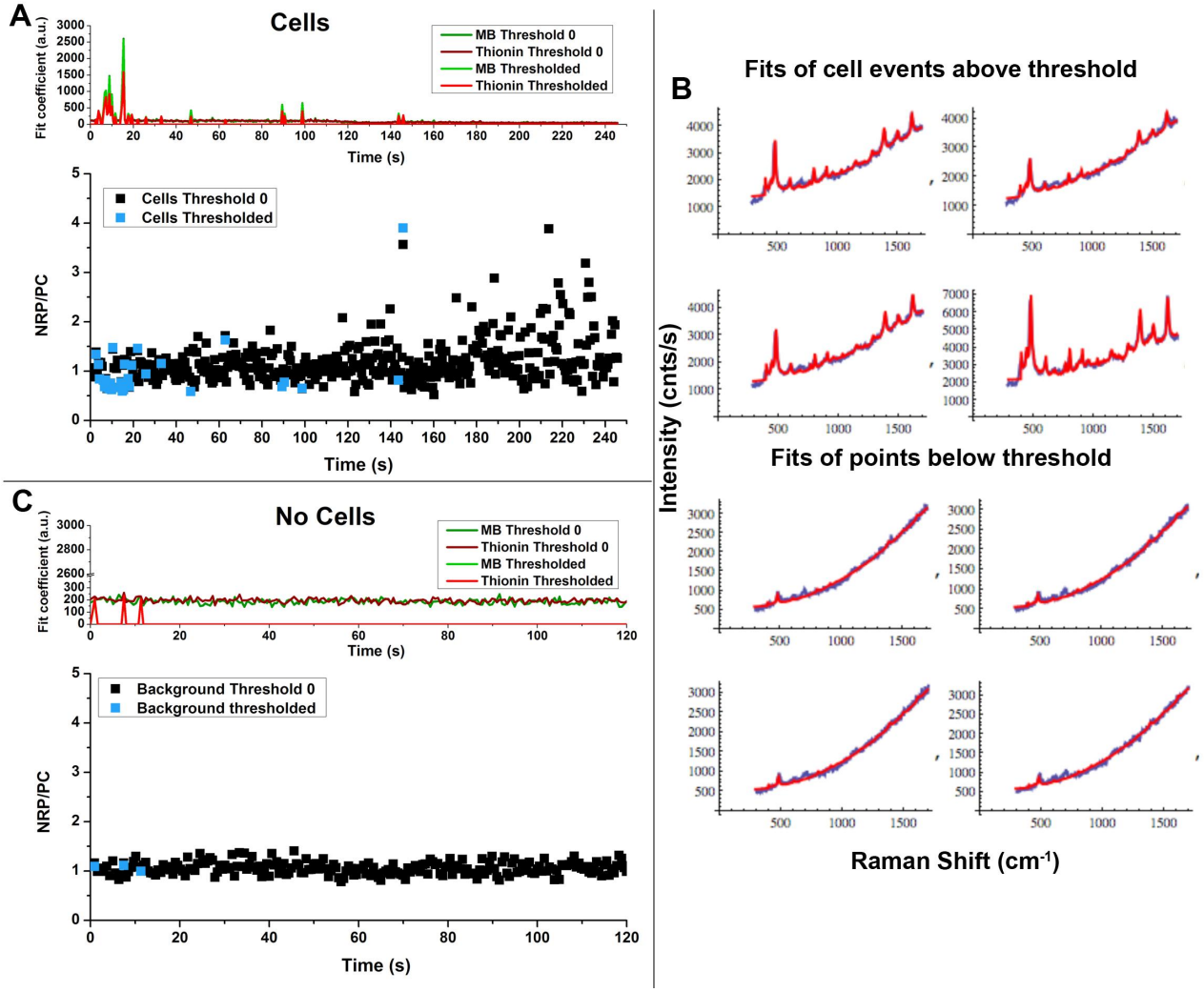

Figure 5. Thresholding and resulting NRP/PC ratios for cells carrying NRP and PC-SBTs in Device 2. A) The upper panel show the values of the fit coefficients for both tags (thionin in red and MB in green) at each time-point before (dark color code) and after (bright color code) applying the threshold. The lower panel shows the NRP/PC ratios for each spectrum in the time series before (black dots) and after (blue dots) applying the threshold. B) Fits (red trace) of events above and below threshold for Device 2 when cells are flown in it, overlaid with the experimental spectrum (blue trace). C) Fit coefficients and NRP/PC ratios as in A), but without cells flowing in the device.

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