Quantitative ratiometric discrimination between noncancerous and cancerous prostate cells based on neuropilin-1 overexpression

Alessia Pallaoro, Gary B. Braun, and Martin Moskovits

Department of Chemistry and Biochemistry, University of California, Santa Barbara, CA 93106-9510

Edited by* Richard P. Van Duyne, Northwestern University, Evanston, IL, and approved August 15, 2011 (received for review June 13, 2011)

A multiplexed, ratiometric method is described that can confidently distinguish between cancerous and noncancerous epithelial prostate cells in vitro. The technique is based on bright surface-enhanced resonance Raman scattering (SERRS) biotags (SBTs) infused with unique Raman reporter molecules, and carrying cell-specific peptides. Two sets of SBTs were used. One targets the neuropilin-1 (NRP-1) receptors of cancer cells through the RPARPAR peptide. The other functions as a positive control (PC) and binds to both noncancerous and cancer cells through the HIV-derived TAT peptide. Point-by-point 2D Raman maps of the spatial distribution of the two tags were constructed with subcellular resolution from cells simultaneously incubated with the two sets of SBTs. Averaging the SERRS signal over a given cell yielded an NRP/PC ratio from which a robust quantitative measure of the overexpression of the NRP-1 by the cancer cell line was extracted. The use of a local, on-cell reference produces quantitative, statistically robust measures of overexpression independent of such sources of uncertainty as variations in the location of the focal plane, the local cell concentration, and turbidity.

surface-enhanced Raman spectroscopy biomarker | cancer cell identification | multiplexing | silver nanoparticles

Early and rapid identification of malignant cells [ideally free-flowing in biological fluids such as urine (1) or blood (2–4)] is a central goal in cancer research. Accordingly, seeking improvements in sensitivity and accuracy of detection and quantification through, for example, the development of cell-specific diagnostic tools and biomarkers is an active enterprise. Although it is known that such cells are present in body fluids, concentrating them to levels appropriate for confident identification and quantification is still a serious challenge, despite significant advances that have been made by, for example, using microfluidics (5). Once collected, the cells must be identified as either cancerous or noncancerous within tolerable confidence limits, which may be improved by, for example, using several nonredundant biomarkers. Numerous promising identification methods have been reported using antibodies. In this article, we report a technique based on surface-enhanced Raman spectroscopy (SERS) and biotags that produce bright surface-enhanced resonance Raman scattering (SERRS) signals, comparable to or exceeding the intensities of fluorescent tags. The SERRS biotags (SBTs) used in this study exploit peptides as recognition moieties. We show that using SBTs ratiometrically can provide highly statistically significant, quantitative measures of neuropilin-1 (NRP-1) overexpression insensitive to normal sources of uncertainty in optical measurements such as variations in focal plane, cell concentration, and turbidity.

Normal Raman signals from cells and their infrared (FTIR) absorbance patterns have been previously used to differentiate between cancer and normal cells (6–9). SERS has been used as an alternative immunohistochemistry (IHC) tool (10, 11) for the detection of biomarkers in biological fluids or in vivo (12), and for cancer detection from blood (13). However, Raman and FTIR signals measured directly from cells are typically much weaker than those that are measurable with bright labels. Moreover, sample identification is often based on slight differences between highly overlapping spectra. By contrast, numerous nonoverlapping SERS and SBTs can be routinely synthesized (14) and simultaneously excited with a single, very low intensity laser source, making the determination of the relative contribution of the individual SBTs to the overall spectrum tractable. SERS analysis is also, in general, less time consuming than IHC protocols, because cell labeling is carried out in a single step, without pre- or post-treatments like washes, fixation, and permeabilization.

The central feature of SERS is its facile multiplexing capability by using premade, encapsulated nanoparticle clusters that are then infused with one of several highly Raman-active reporter molecules. The SERS spectrum of an individual SBT acts as a unique barcode that is easily differentiable in a composite SERS spectrum originating from many tags. Several synthetic challenges have been overcome in the course of this study so that its synthesis is now prescriptive. The SERS intensities achieved are comparable to fluorescence (15). We have previously shown that forerunners of the SBTs reported here are capable of direct binding to, and uptake by, live cells (16).

Importantly for biomedical applications, SERS employs tissue-penetrating lasers in the red to near-infrared range resulting in low autofluorescence; the high signal enhancement and sharp peaks make it possible to distinguish SERS and especially SERRS in biological specimens at low laser powers (16). Finally, several versions of the biotags can be incubated simultaneously to probe a variety of cell-specific markers taking advantage of the multiplexing capability of SERS.

In this paper, we describe a SERRS-based approach to discriminate between prostate cancer cells (PPC-1) and noncancerous prostate epithelial cells (RWPE-1) in vitro using cell-specific peptides. Multiplexed SBTs consisting of polymer-encapsulated silver particles each labeled with a unique Raman reporter molecule (14) display either a receptor-specific peptide, or a general cell-penetrating peptide (CPP). The SBTs are incubated with cells, SERS mapped, and the signal from the various SBTs deconvoluted to identify the cells while in suspension, simulating their capture from blood. The central idea being that tags displaying the CPP act as a cell-resident calibrant against which the cancer-indicating tags can be ratioed, thereby greatly reducing the uncertainty in the quantitative measure of the cancer-indicative marker that can result from, for example, variability in focal plane, changes in turbidity, cell concentration, or abbreviated measurement time. The use of a cell-resident reference would be parti-

Author contributions: A.P., G.B.B., and M.M. designed research; A.P. and G.B.B. performed research; A.P. and G.B.B. analyzed data; and M.M. wrote the paper.

The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor.

†To whom correspondence should be addressed. E-mail: moskovits@chem.ucsb.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1109490108/-/DCSupplemental.
cularly useful when a cancerous cell is identified on the basis of the degree of overexpression of a biomarker that may also be present (but to a lesser extent) on the noncancerous cell. The ratioetric approach, in essence, provides a quantitative local measure of the extent of expression of biomarkers associated with the cancerous modifications of a specific cell, essentially providing a basis for quantifying overexpression.

Results and Discussion

Two types of SBTs functionalized with cell-targeting peptides were synthesized. The first carries Cys-TAT (sequence CGRKKRRQRRR, cys added simply for conjugation purposes), a peptide derived from the HIV-1 TAT protein and known CPP (17, 18). TAT was used as a general targeting peptide because it binds rapidly and uniformly on the surface of cells (Fig. S1), and also enters many types through endocytosis (19). The second SBT uses the peptide Cys-RPARPAR, which specifically binds to a pocket in the biomarker protein NRP-1. (20) A receptor expressed on the outer plasma membrane of certain types of cancer cells, which is involved in angiogenesis, a hallmark of cancer growth, and tumor cell migration (21). The binding ability of the RPARPAR peptide to the NRP-1 receptor is given by the amino acid sequence that follows the so-called C-end rule (CendR): a C-terminal arginine (or lysine) in a general sequence of the type R/KXXR/K (where X indicates a generic amino acid) is fundamental for the binding activity of the peptide (20).

Cys-TAT is small and highly cationic. The exact mechanism that allows this peptide to enter cells is still uncertain; however, it is generally agreed that it relies heavily on the initial attachment to the negatively charged moieties on most membranes. When TAT is used to transport nanoparticles in cells, the main uptake mechanism has been shown to be lipid-raft macropinocytosis (22). TAT also has a cryptic CendR R/KXXR/K motif and could compete with binding of RPARPAR particles. This, however, was not observed, likely because of the low concentrations of SBTs. Our group has previously achieved rapid cellular uptake of hollow gold nanoshells through the self-assembly of a TAT–lipid layer around them (23). For the present application, TAT is bound directly to a protein on the surface of the SBT.

The two SBT sets each carry a Raman reporter: methylene blue (MB) (Fig. 1A) on the FAM-Cys-TAT positive control set (called PC-SBT) and thionin (Fig. 1B) on the FAM-Cys-RPARPAR carrying particles (therefore called NRP-SBT). The absorption maxima of the tags are approximately 670 nm for MB, and approximately 600 nm for thionin. Although the SERS enhancement arises primarily on account of plasmonic excitation in the silver nanoparticles, resonance Raman enhancement due to the laser wavelength’s (633 nm) resonance with the two reporter molecules likely contributes to their high overall SERRS cross-section. The structure of the Raman reporter molecules and the SERRS spectra of the SBTs are shown in Fig. 1. The number of SBTs that gives rise to each of the spectra in Fig. 1C was estimated to be approximately 4, assuming approximately 1 nM colloid concentration and a focal volume of 7 μm^3 for the 10x objective. Signals arising from individual SBTs can be detected in deposited samples mapped using the 100x objective (Fig. S2). We note in passing that our purification step does leave some particles with Ag monomer cores rather than Ag dimers. These do not produce strong signals (14).

When a mixture of SBTs reside together in the laser’s focal volume a composite spectrum results, which can be deconvoluted into its individual components, allowing the relative concentrations of each contributing species to be determined (14, 24, 25) provided that reference spectra of the pure components are known. Spectra were deconvoluted by assuming the composite spectrum to be a linear combination of the individual components—a reasonable assumption for stable, noninteracting particles. Concentrations were derived from the individual spectroscopic contribution using a calibration plot, constructed by deconvoluting SERS spectra of solutions containing known concentrations of the two SBTs (PC-SBT and NRP-SBT). Spectra of each cell line suspended in DMEM supplemented with 10% FBS at room temperature for 60 min, cells were placed on a microscope slide and SERRS-mapped without fixing, by scanning the focused laser beam (100x objective) in steps of 1.5 μm and recording a full SERRS spectrum at each point (Fig. 3).

The SERRS maps [each point comprised of a full (270–1,700 cm^-1) spectrum] were analyzed using Mathematica.

---

Fig. 1. Structural description of SBT system and spectra. (A) Thionin. (B) MB. (C) Schematic of a typical silver SBT: Two Ag monomers are encapsulated in a thin polymer shell and functionalized with a modified BSA carrying several copies of a cell-specific or a universal peptide, then the SBTs are infused with a Raman reporter (either thionin or MB). (D) Corresponding SERS spectra obtained from an ensemble solution of SBTs (approximately 4 per focal volume). The spectra have been shifted along the ordinate axis for clarity, and the dotted lines identify the bands of thionin that are nonoverlapping with bands in the MB spectrum. The star indicates a common band. Laser 633 nm, 10x objective, power at sample approximately 0.11 mW, hole 400 μm, slit 400 μm, and exposure time 1 s x 3 accumulations.
The spectra were fit to a linear combination of the two tags’ individual reference spectra, and included sloping baseline corrections:

\[ S(x) = a^*A(x) + b^*B(x) + c + d^*x, \]

where \( a, b, c, \) and \( d \) are nonnegative adjustable fitting parameters; \( A(x) \) and \( B(x) \) are the reference spectra as a function of the wavenumber value \( x \). The \( a \) and \( b \) coefficients together with the calibration plot (Fig. 2B) were used to construct the composition maps.

Percent composition maps are depicted in Fig. 4 A and D and colorized on the basis of the ratio of thionin (cancer marker NRP-1) to MB (control). A high value of the NRP/PC ratio is colored red; a low value of the ratio is green; intermediate values are red-green color mixtures as shown in the bar at the right of Fig. 4. Points corresponding to intensities below 100 counts per second were assumed not to contain SBTs and set to black. This threshold was based on the consideration of noise and the observation that individual SBTs deposited out of a dilute solution onto a glass slide produced well-resolved signals above 100 counts per second (Fig. S2). Bright field images of the same cell groups are shown in Fig. 4 B and E. The SERRS and bright field images are overlaid in Fig. 4 C and F to show that signals arise from the cells and not from the glass substrate. It is gratifying to note that the PPC-1 cells, which are expected to bind both peptides, are indeed stained as a mosaic with a mean ratio of approximately 1.0. By contrast, the SERRS images of the noncancerous prostate cell RWPE-1 show very little red, indicating very low NRP/PC ratios with the few red points situated primarily outside the cells (mean of approximately 0.3 including outer rim), likely indicating nonspecifically bound SBTs. The maps and their histograms (Figs. S4 and S5) agree well with the histograms of average-cell signals discussed below (Fig. 5A).

To discriminate confidently between these different types of cells, we analyzed the average spectrum arising at both the entire cell level and in maps with subcellular resolution (Figs. S6 and S7), extracting ratios of NRP/PC. Sixteen PPC-1 cells and 24 control RWPE-1 cells were analyzed, and the resulting histograms of the NRP/PC ratio are shown in Fig. 5A. Ninety-six percent of the PPC-1 were found to have ratios above this value. Interestingly, the distribution for the PPC-1 cells is skewed toward high ratio values, likely reflecting the heterogeneity of cell expression of NRP-1 and particle affinity, binding/uptake rates, or receptor recycling during prolonged incubation.

The nonnormal distribution depicted by the histogram (Fig. 5A) was converted into a normal distribution through a logarithmic transformation. The statistical analyses are summarized in Fig. 5B. Student’s t test was also performed on transformed data.

![Fig. 2. Calibration plots for the mixed-SBT deconvolution series. (A) SERRS spectra of mixtures of the two SBTs: green trace, 100% PC-SBT; red trace, 100% NRP-SBT. (B) Calibration curve derived from the relative least-squares coefficients for the two components corrected for the known concentrations of MB or thionin SBT present in the solution. This calibration curve was used to calculate the relative quantity of each tag resident on a given cell.](image)

![Fig. 3. Schematic of the SERRS cell mapping experiment. NRP- and PC-SBTs are synthesized, combined, and added to either noncancerous or cancer cells suspended in DMEM supplemented with 10% FBS. These are incubated for 60 min at room temperature. Cells are placed on a microscope slide, and their SERRS spectrum is acquired using microRaman. The sample is mapped along the x and y axes in 1.5-μm steps, 5-μW power at sample, 1-s exposure time. The SERRS spectrum averaged over the whole cell area is deconvoluted into the individual components.](image)
In Fig. 5B, the boxes define the interquartile range (IQR) (i.e., the range that contains the middle 50% of the data in the distribution), the black square dots are the means of the distributions, and the whiskers determine the upper and lower inner fences outside which suspected outliers (extreme values) lie. These limits are located, respectively, at Q1 (lower quartile) = 1.5 × IQR and Q3 (upper quartile) + 1.5 × IQR. No extreme values were observed for the PPC-1 group, whereas the RWPE-1 group has two. One is in the upper fence at ln(NRP/PC) = −0.15 and the other in the lower fence at ln(NRP/PC) = −2.01; however, neither were rejected as outliers. The higher extreme value observed was likely due either to nonspecifically bound SBTs (Fig. S8) or glass-bound SBTs located coincidentally close to a cell. The lower extreme is likely caused by a few nonspecifically bound tags. This is clearly illustrated in Fig. S8, which shows that for a noncancerous cell the body is uniformly green (PC-dominated) with the few red points (indicating NRP contribution) at the periphery. The location of the outliers at the cell’s periphery suggests that the high degree of confidence in distinguishing between cancerous and noncancerous cells based on statistics can be further augmented through microscopic observation of the cells. RWPE-1 cells show weak but nonzero RPARPAR binding (resulting in a mean observed NRP/PC ratio of approximately 0.3, somewhat greater than zero—the expected value if all interactions were specific). This is likely due to nonspecific adsorption of NRP-SBT on the control cells’ membrane.

The statistics indicate that the ratiometric SERS multiplexing approach we used does an excellent job in distinguishing cancerous from noncancerous epithelial prostate cells. Referring to Fig. 5B, the two cell populations form distinct clusters along the ratiometric axis, well separated both visually and numerically. Applying the t test to the two populations also shows them to be highly distinct (p < 0.001), suggesting that with proper choice of tags and peptides the ratio (or rather, its natural logarithm) easily discriminates between noncancerous and cancerous cells.

Conclusions

The multiplexed, ratiometric method described was shown to successfully distinguish between cancerous and noncancerous epithelial prostate cells. The technique is based on deconvoluting SERS spectra arising from two sets of bright SBTs simultaneously bound to the cells. One of the two sets of SBTs was functionalized to target the NRP-1 receptors of cancer cells through the RPARPAR CendR peptide. The other functions as a positive control and binds to both noncancerous and cancer cells through the HIV-derived TAT peptide. Each was infused with a unique Raman reporter molecule. Averaging the SERS signal over a given cell yielded an NRP/PC ratio that functioned as a statistically robust quantitative measure of the overexpression of the NRP-1 by the cancer cell line. This 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 very low laser intensities (100 μW) needed for good signal to noise and the use of a single wavelength to excite all SBTs.

Materials and Methods

Materials. All chemicals for the synthesis of the SBTs were purchased from Sigma except where specified. Silver nitrate (AgNO₃) 99.9999%; trisodium citrate dihydrate (Fisher); hexamethylenediamine (HMD), 98%; polyvinylpyrrolidone (PVP), molecular weight 40,000; bovine serum albumin (BSA), powder; potassium chloride (Mallinckrodt Baker); thionin acetate salt (dye content approximately 90%); MB (dye content ≥82%); N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP), Thermo Fisher Pierce no. 21857; Fluorescein-C-X-GRPARPAR-OH (where X is an aminohexanoic linker) designated FAM-Cys-RPARPAR was a gift from Erkki Ruoslahti’s group at Sanford–Burnham Medical Research Institute, University of California, Santa Barbara, CA;
Cys-TAT (CGGRKQRQRQR-OOH), Anaspec; Fluorescein-NHS (#46410, Pierce); polyoxymethylene (20) sorbitan monolaurate solution 10% in H2O (TWEEN 20, T20); D-PS (Gibco), Syringe filters Millipore-GV, 0.22 μm, PVDVF, 13 mm, ethylene oxide sterilized.

Peptide Labeling. Cys-TAT was labeled with NHS-Fluorescein (FAM-NHS, Pierce). FAM-NHS first was dissolved at 5 mg/mL in ERK. RWPE-1 cells (ATCC) were grown in Keratinocyte serum free medium (Invitrogen) supplemented with bovine pituitary extract (0.05 ng/mL) and recombinant EGF (5 ng/mL). Both were incubated at 37°C in a 5% CO2 atmosphere. For SERRS mapping experiments, cells were plated in multwell plates and after either 24 or 48 h harvested using a nonezymatic cell dissociation buffer (Invitrogen) that does not disrupt the membrane receptors. Cells were then washed by centrifugation for 2 min, and the pellet was resuspended in the appropriate volume of DMEM – 10% FBS in order to obtain a concentration of 1.2–1.3 x 10^5 cells/100 μL. SBTs were then added to cell suspension (with PC versus NRP ratio of 1:2 or 1:7 vol/vol) and incubated for 60 min at room temperature; then, a 10-μL aliquot was placed on a microscopy glass slide, covered with a coverslip, sealed with nail polish, and mapped by SERS. For microscopy characterization, cells were plated in multwell plates and after either 24 or 48 h incubated with SBT for 120 min at 37°C in a 5% CO2 atmosphere, then imaged.

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 spectral deconvolution and fitting of single spectra for the composition calibration was done using a weighted least-squares algorithm in IgorPro (WaveMetrics Inc.), whereas the deconvolution and NRP/PIC ratio calculations for point-to-point maps were done using Mathematica’s FindFit, a nonlinear least-squares-fitting algorithm, and the SBT pure spectra, taken from Fig. 2, were processed by a customized notebook. The statistics on the whole cell average data were performed using OriginPro (Version 8.1). Student’s t test was performed, and p < 0.001 was considered highly significant.

ACKNOWLEDGMENTS. We are grateful to Professor Erkki Ruoslahti for providing us with advice and samples of the FAM-C-X-GRPARPAR-Oh-Peptide. We thank Norbert Norbert Rouslahti, University of California, Santa Barbara, and Dr. Tambet Teesalu in the Ruoslahti lab for many helpful discussions. This work was supported by the Institute for Collaborative Biotechnologies through Grant DAAD19-03-D-0004 from the US Army Research Office and microiontophoretic use of the Materials Research Laboratory Central Facilities at University of California, Santa Barbara, supported by the National Science Foundation under Awards DMR-0080034 and DMR-0216466 for the high-resolution transmission electron microscopy/scanning transmission electron microscopy. G.B.B. was also supported in part by a fellowship from the Santa Barbara Cancer Center.


Fig. S1. Epifluorescence, bright field, and surface-enhanced Raman spectroscopy (SERS) maps of prostate cancer cells (PPC-1) and noncancerous prostate epithelial cells (RWPE-1) incubated with surface-enhanced resonance Raman scattering biotags (SBTs) with or without cys-TAT. (A and B) Epifluorescence images overlayed with bright field of PPC-1 cells incubated with rhodamine labeled non cys-TAT SBTs (A) and with cys-TAT SBTs (B) for 120 min in complete growth medium, at 37 °C, 5% CO₂. Bright field (C and E) images and corresponding SERS maps (D and F) of PPC-1 cells incubated for 30–60 min at room temperature in DMEM supplemented with 10% FBS with either TAT (C and D) or non-TAT (E and F) SBTs. The SERS maps represent the intensity of the band at 1,620 cm⁻¹ of thionin across the mapped area. Positive control (PC) SERS biotags were also incubated with RWPE-1 cells in suspension at room temperature for 30–60 min in DMEM supplemented with 10% FBS. (G) Bright field image of the SERS mapped cells (the red rectangle represents the actual scanned area). (H) SERS map of the group of cells enclosed in the red rectangle in G. The map represents the intensity of the methylene blue band at 1,620 cm⁻¹ across the scanned area.
SBTs were deposited on a glass slide and mapped using the same settings as for the cells. The signals from bright SBT were typically 100 counts or more at the most intense bands. The star indicates where in the map the spectrum comes from.

Representative spectra and corresponding deconvolution for the calibration of the percentages of the two SBTs. Known volumes of the two SBTs were mixed in the following proportions: 90% neuropilin (NRP) (or PC)—10% PC (or NRP), 70% NRP (or PC)—30% PC (or NRP), and 50% NRP—PC. (A) Equal volumes of NRP and PC SERS biotags were mixed together, and the resulting composite spectrum is shown (black), together with the corresponding fit (red). (B) The same experimental spectrum with the deconvoluted single components calculated from the fit.

The NRP/PC ratio from each point in the two maps (from Fig. 4 A and D), plotted as probability histograms. The tail of the RWPE is attributed to the nonspecifically associated particles around the cell periphery. The shape of these two distributions is consistent with the histogram of individual cell values in Fig. 5A that were obtained by averaging areas within maps.

Pallaoro et al. www.pnas.org/cgi/doi/10.1073/pnas.1109490108
Fig. S5. Example fits from the Mathematica program for some points in the PPC-1 map Fig. 4A (Left), and for the RWPE-1 map Fig. 4D (Right), chosen across a range of intensity to demonstrate the quality of the fitting. For RWPE-1 the dominant signal is coming from the methylene blue PC-SBTs.

Fig. S6. Representative SERS map, whole cell spectrum, and deconvolution for RWPE-1 cells. (A) Bright field image of one RWPE-1 cell incubated with NRP and PC SERS biotags simultaneously for 60 min at room temperature in DMEM supplemented with 10% FBS. (B) SERS map of the area enclosed in the blue rectangle in A of the intensity of the 1,620 cm⁻¹ band of thionin and methylene blue. (C) Full SERS spectrum averaged over the whole cell, after baseline subtraction and normalization (black trace) and corresponding fit (red trace). (D) Experimental SERS spectrum from the whole cell (black) and fit components corresponding to NRP (red) and PC (green) SERS biotags. The weight coefficients obtained from the fit algorithm were used together with the calibration curve of Fig. 2 to calculate the percentage of PC and NRP present on each cell and their ratio.
Fig. S7. Representative SERS map, whole cell spectrum, and deconvolution for PPC-1 cells. (A) Bright field image of a group of PPC-1 cells incubated with NRP and PC SERS biotags simultaneously for 60 min at room temperature in DMEM supplemented with 10% FBS. (B) SERS map of the area enclosed in the blue rectangle in A of the intensity of the 1,620 cm\(^{-1}\) band of thionin and methylene blue. (C) Full SERS spectrum averaged over the one whole cell, after baseline subtraction and normalization (black trace) and corresponding fit (red trace). (D) Experimental SERS spectrum from the whole cell (black) and fit components corresponding to NRP (red) and PC (green) SERS biotags. The weight coefficients obtained from the fit algorithm were used together with the calibration curve of Fig. 2 to calculate the percentage of PC and NRP present on each cell and their ratio.

Fig. S8. Point-by-point SERS maps of the NRP/PC ratio on two RWPE-1 cells possible outliers. (Left) Map of the cell that caused the highest extreme value in which there are two spots very close to the cell boundary corresponding to high NRP signal, indicated by the asterisk (*). (Right) map of the cell that caused the lowest extreme value in which the positive control signal is very uniform and there is extremely low NRP signal, consistent with low nonspecific binding.