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Multicolor multicycle molecular profiling (M3P) with quantum dots for single-cell analysis

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Abstract

Here we present a detailed protocol for molecular profiling of individual cultured mammalian cells using multicolor multicycle immunofluorescence with quantum dot probes. It includes instructions for cell culture growth and processing (2 h + 48–72 h for cell growth), preparation and characterization of universal quantum dot probes (4.5 h + overnight incubation), cyclic cell staining (~4.5 h per cycle), and image analysis (varies by application). Use of quantum dot fluorescent probes enables highly multiplexed, robust quantitative molecular imaging with a conventional fluorescence microscopy setup, whereas the probe preparation methodology employing self-assembly between Protein A-decorated universal quantum dots and intact primary antibodies offers fast, simple, and purification-free route for on-demand preparation of antibody-functionalized quantum dot libraries. As a result, this protocol can be employed by biomedical researchers for a variety of cell staining applications and, with further optimization, for staining of other biological specimens (e.g., clinical tissue sections).

Keywords

quantum dots; multifunctional nanoparticles; multicycle staining; molecular profiling; single-cell analysis; proteomics; molecular imaging; multicolor; immunofluorescence

INTRODUCTION

Molecular profiling is a powerful technique for the study of complex molecular networks underlying physiological and pathological processes through the comprehensive interrogation of individual molecular components comprising such networks. It promises to become a key tool for advancing biomedical research, clinical diagnostics, and targeted therapy. Gaining access to molecular profiles of individual cells, however, is technologically challenging¹. To this point, a variety of analytical approaches have been developed and

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AUTHOR CONTRIBUTIONS

P.Z., L.D.T., and X.H.G. contributed to the experiment design and data analysis. P.Z. performed the experiments. P.Z. and X.H.G. wrote the paper.

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routinely used for highly multiplexed genomics and proteomics studies, including two-dimensional gel electrophoresis, RT-qPCR, gene chips, and biomolecular mass spectrometry^{2–8}, but unique states and responses of individual cells are obscured, and specimen morphology is often not accessible through such methods. A number of single-cell proteomic technologies have been introduced in recent years to overcome these limitations (Table 1). In general, single-cell approaches generate complex data sets where each individual cell serves as an independent sample, enabling assessment of cell heterogeneity and study of intracellular processes at a mechanistic level. For example, flow cytometry techniques feature high multiplexing capacity for comprehensive molecular profiling of individual suspended cells^{9, 10}, whereas microfluidic-based proteomic assays utilize sequestration of individual cells within separate microchambers for subsequent analysis of secreted or intracellular proteins, addressing variability of cell-to-cell behavior^{11, 12}. Microscopy techniques, such as immunohistochemistry (IHC) and immunofluorescence (IF), feature an additional important functionality of assessing specimen morphology and cell microenvironment^{13, 14}. While conventional methods lack sufficient parallel multiplexing capacity for molecular profiling, application of IF in a multicycle format enables interrogation of substantially larger sets of antigens using organic fluorophores^{15, 16}. Yet, adoption of such technologies might be hampered by high instrumentation complexity and cost. Therefore, the current lack of easily accessible methods that can simultaneously address the need for highly multiplexed molecular and morphological detail leaves many fascinating research opportunities of single-cell proteomics awaiting the development of new technologies.

Quantum dots (QDots) represent a particularly interesting class of nanoscale probes well-suited for advanced fluorescence imaging applications due to a number of unique photo-physical and chemical properties^{17–25}. Importantly, QDots have the fundamental capacity for (i) simultaneous encoding of multiple individual molecular targets via distinct emission spectral signatures, essential for multiplexed profiling on the same specimen, and (ii) production of bright and photo-stable fluorescence signals, critical for reliable assessment of target expression levels in a quantitative manner, while also featuring small size and versatile surface chemistry for synthesis of compact biofunctional probes. Initial proof-of-concept studies have already demonstrated feasibility of the single-cell molecular profiling concept and utility of QDot probes as a platform for its practical implementation^{9, 26, 27}. However, multistep staining methodologies commonly employed with commercially-available QDot probes hamper simultaneous labeling of multiple molecular targets^{28–34}, whereas highly multiplexable direct target labeling with QDot-Antibody bioconjugates suffers from the requirement for time-consuming and prohibitively expensive synthesis of custom-designed probes^{26, 35} (e.g., one conjugation reaction takes over 5 h and costs over \$800 to complete), limiting their applicability for biomedical researchers.

Recently, we have developed a universal QDot-based nanoparticle platform that can be converted on-demand into functional QDot-Antibody probes by self-assembly with intact antibodies, thus dramatically reducing the complexity of QDot-Antibody probe preparation³⁶. Sufficient stability enables simple mixing of pre-assembled multicolor QDot-Antibody probes in a cocktail for highly multiplexed parallel staining, facilitating use of a full range of spectrally distinguishable QDots without antibody species or buffer

composition limitations. Notably, this procedure requires no chemical modification of antibodies, eliminates the need for QDot-Antibody probe purification, streamlines assay development, significantly lowers cost of probe preparation, and makes QDot platform accessible to a wide range of biomedical researchers (Table 2). To access single-cell molecular information, we have developed a complementary multicolor multicycle molecular profiling (M3P) technology that employs unique features of self-assembled QDot-Antibody probes for direct multiplexed labeling of 5–10 molecular targets, and a cyclic staining/imaging/de-staining methodology for interrogation of expanded target sets³⁶. In comparison to organic dye-based methods, the QDot imaging platform significantly expands the amount of molecular information that can be obtained during each staining cycle, both in terms of target number and quantitative content, dramatically reducing specimen processing time and enabling the collection of comprehensive molecular profiles within just few cycles (Supplementary Fig. 1).

Here we present a detailed protocol for the M3P technology and offer the main design criteria that should be considered when optimizing this technology for molecular profiling applications.

Description of the method

The method for single-cell molecular profiling with M3P technology consists of three main components: (i) preparation of biological specimens for analysis (using cultured mammalian cells as an example), (ii) preparation of multicolor universal QDots, and (iii) multicolor multicycle staining, imaging, and image analysis (Fig. 1). Preparation of biological specimens involves cell culture on a coverglass surface and pre-staining processing optimized for labeling of most targets with QDot probes (Fig. 1, Steps 1–14 of the PROCEDURE). We also recommend performing reference staining of new specimens using conventional IF with commercially-available secondary Antibody-QDot bioconjugates (Box 1) to confirm proper preservation and labeling of all molecular targets of interest. Preparation of multicolor universal QDots involves a bioconjugation reaction between amine-functionalized PEG-coated QDot scaffolds and adaptor proteins, along with QDot characterization (Fig. 1, Steps 15–30 of the PROCEDURE). Epifluorescence microscope with a hyperspectral imaging (HSI) camera is used to collect reference QDot spectra and measure differential brightness, which is necessary for accurate quantitative analysis of multicolor images. An alternative procedure for direct measurement of QDot differential brightness might be employed in cases when precise QDot concentration cannot be determined (Box 2). Multicolor multicycle staining, in turn, consists of several discrete steps that are repeated in a cyclic manner (Fig. 1, Steps 31–44 of the PROCEDURE): (i) self-assembly of target-specific QDot-Antibody probes, (ii) single-step multicolor cell staining, (iii) hyperspectral imaging and image analysis, and (iv) specimen regeneration (or de-staining). The number of molecular targets labeled simultaneously and the number of cycles performed should be determined by application-specific requirements. Raw data obtained with M3P technology comprise a set of fluorescence signal intensities (for each QDot color and staining cycle) for each image pixel. The type of analysis applied to such datasets depends on the desired specific applications.

Applications and limitations of the M3P technology

The main purpose of employing the M3P technology is to achieve molecular characterization of biological specimens in a comprehensive manner with a technologically simple setup and straightforward procedure. The primary application described in this protocol focuses on single-cell molecular profiling within monolayer cell cultures, which represent a useful model for the study of intracellular signaling pathways, identification of disease-specific molecular signatures, and evaluation of cell response to therapeutic intervention. Examination of cultured cells with M3P technology should provide access to understanding the molecular composition of individual cells, along with valuable 3D morphological information for addressing phenotypic heterogeneity within large cell populations^{37, 38}. Additional analysis should enable target colocalization and intracellular translocation studies, cell morphometric analyses, and assessment of specimen morphology, further enriching single-cell molecular information.

The versatile design of the M3P technology features a great degree of flexibility for optimization of individual components and steps towards specific applications. As a result, we expect this technology to become applicable to molecular characterization of variety of targets on solid supports, including multiplexed immunoassays, Western blots, flow cytometry, and IF on clinical tissue sections. For example, in an unpublished preliminary study, we have achieved 3-color labeling of formalin-fixed paraffin-embedded tissue sections using self-assembled QDot-SpA-Antibody probes and a 1-step staining methodology (Fig. 2).

It is important to consider, however, that QDot probes differ substantially from conventional fluorophores, featuring unique beneficial optical properties, but also imposing a number of limitations on specimen processing and staining conditions^{25, 35, 39}. In addition, parallel labeling of multiple targets requires complete preservation of antigenicity and sufficient QDot accessibility for the whole set of targets, thus further restricting specimen processing options. Relatively large size of QDot-Antibody probes might hamper labeling of tightly packed molecular targets or targets sequestered within intracellular compartments. Therefore, careful optimization of specimen processing and staining conditions tailored to QDot probes should be used. The multicycle staining methodology, in turn, might be incompatible with certain fragile molecular targets. We have identified one set of conditions that offers robust staining of various targets with QDots in fixed adherent cell lines; yet modifications to this procedure might be necessary to satisfy unique criteria of specific molecular profiling applications.

Fairly lengthy staining and imaging steps impose a limitation on the number of cycles that can be performed in a timely manner. Further development of the M3P technology should benefit from integrating QDot-based labeling with automated staining-imaging instruments. For example, an automated system consisting of a fluorescence microscope and a pump-driven flow chamber can be engineered to achieve easy alignment of images obtained from different staining cycles and substantially reduce the labor requirement. It is also worth mentioning that this protocol features the use of standard devices and equipment that are widely accessible in research labs. Specialized instruments and devices capable of shortening the immunostaining process from hours to minutes also started to appear^{40, 41},

which should dramatically enhance the capability of M3P technology for multi-cycle staining.

Experimental design

Processing of cultured cells—In general, M3P technology should be applicable to all adherent cells cultured in a monolayer (the prostate cancer cell line LNCaP, and cervical cancer cell line HeLa, have been tested in our laboratory). Strong attachment of cells to glass surfaces is required to facilitate high-magnification fluorescence imaging of labeled cells without applying a coverslide and experiencing high levels of autofluorescence (typical for plastic surfaces). Proper pre-staining processing, including cell fixation, permeabilization, and blocking, is critical for accurate labeling of molecular targets with QDot probes. In particular, pre-staining processing should preserve specimen morphology and target antigenicity, while providing sufficient access to intracellular compartments and precluding non-specific interactions between QDot probes and cell components. In this regard, access to intranuclear targets is particularly hard to achieve due to the relatively large size of QDots and antibodies. Use of ionic surfactants typically improves staining of intranuclear targets, whereas brief treatment with non-ionic surfactants (such as Triton X-100 and Tween-20) helps to reduce non-specific QDot binding to the specimen. In this protocol, we offer one cell fixation/permeabilization procedure that features a good balance between specimen preservation and intracellular QDot penetration (Steps 7–14 of the PROCEDURE). Should deeper QDot penetration be required, cells can be treated with proteinases (e.g., Proteinase K). However, along with improving intranuclear access, protein digestion might result in degradation of cell-surface and cytoplasmic targets (Supplementary Fig. 2). Alternative methods of cell fixation with ice-cold methanol or acetone, in our experience, deteriorate cell morphology and lead to enhanced non-specific binding by QDot probes. Therefore, we recommend confirming proper preservation and staining of each target of interest using conventional immunofluorescence with secondary QDot probes (Box 1). It should also be noted that commercial PEG-coated QDots might exhibit disparity in surface coverage with PEG (and thus in levels of non-specific staining) between different lots. If exchange to a better lot is not an option, commonly used BSA should be supplemented by 0.1% alkali-soluble casein in a blocking step to compensate for this effect. Being more negatively charged and hydrophobic than BSA, casein serves as a more stable blocking reagent for QDots (which also carry a net negative charge despite the PEG coating). However, use of high casein content during blocking step or its incorporation in staining buffer often results in decreased staining intensity, thus requiring optimization for use in specific specimens and applications.

Universal QDot platform design—A universal QDot platform should offer a straightforward method for on-demand QDot-Antibody probe preparation, requiring no specialized expertise or instrumentation. Specifically, such a platform should (i) bind a wide range of primary antibody types; (ii) require no chemical modification to the antibody; (iii) require no probe purification, conserving precious antibodies; (iv) provide control over the antibody orientation on the QDot surface; (v) remain stable for the duration of the staining procedure, resisting probe disassembly and/or aggregation; and (vi) feature high labeling specificity while suppressing non-specific interactions with biological specimens. A

universal QDot platform employed in the M3P technology satisfies these criteria, in part, by using self-assembly between an antibody and an adaptor protein (Staphylococcal protein A, SpA). Adaptor proteins have been extensively studied and used for a variety of applications, including routine antibody isolation and purification as well as novel immunoassays and live cell imaging methods utilizing QDot-adaptor protein conjugates^{42–45}. Labeling of cell surface targets on intact cells is also easily achievable with variety of QDot probes. Staining of intracellular molecular targets, in contrast, requires permeabilization of fixed cells with detergents, which removes natural lipid barriers, eliminates the negative charge on the cell surface, and opens access to a variety of electrostatic and hydrophobic interactions. As a result, many QDot probes, commonly featuring negatively-charged surface and stabilized via electrostatic repulsion, become inapplicable for staining of fully processed specimens. To overcome this limitation, QDot surface is often decorated with a highly hydrophilic and non-fouling PEG layer, which shields charged and hydrophobic regions on the QDot surface. Despite increasing the nanoparticle size and impeding bio-conjugation efficiency, PEG modification remains the most robust and popular approach for producing stable QDots for a variety of biological assays (including majority of commercially-available QDot probes). Therefore, we recommend the use of PEG-coated QDots for preparation of universal QDot-SpA bioconjugates. At the same time, alternative non-fouling coatings might be explored for this application, as long as non-specific interactions are efficiently suppressed. It should also be noted that, while variety of adaptor proteins, such as Protein A, Protein G, Protein A/G, and streptavidin, may be used for preparation of a universal QDot platform, it must be assured that there are no sterically accessible binding sites left on antibodies after QDot-Antibody complex formation to prevent cross-linking of different QDots via antibodies.

Use of hyperspectral imaging for multiplexed QDot analysis—Accurate quantitative analysis of multiple targets based on QDot labeling demands standardization of image acquisition and processing algorithms. Narrow symmetrical emission profiles facilitate spectral isolation of individual QDot signals from a multiplexed specimen. For example, careful choice of band-pass emission filters might enable imaging of 3–4 QDot colors with conventional fluorescence microscope equipped with CCD camera. Quantitative analysis of a larger number of targets, nonetheless, might be compromised by spectral cross-talk between probes with closely spaced emission peaks and non-linear response of the detector. HSI overcomes these limitations to a great extent, enabling extraction of high-content information from a narrow visible spectral window^{46–48}. Generally, HSI systems incrementally apply narrow band-pass filters and collect a series of images for each wavelength band over a specified spectrum, thus producing an “image cube” providing spectral information for each pixel of an image. Deconvolution of known emission profiles from the resulting image cube separates different probe signals from each other and from the background fluorescence, enabling qualitative target colocalization studies and quantitative analysis of molecular expression profiles. HSI camera used for the exemplar microscope setup (Nuance from Advanced Molecular Vision) is able to identify multiple fluorescent tags based on small but meaningful spectral differences by scanning a wavelength range of 420–720 nm with a built-in liquid crystal tunable filter at step increments as small as 1 nm. QDots with fluorescence emission peaks spaced as close as 20 nm apart can be accurately

distinguished with such a setup (Supplementary Fig. 3). Furthermore, HSI camera captures images at each wavelength with constant exposure, and the software mathematically separates the color components based on reference spectra, thus enabling accurate quantitative analysis. Since HSI camera can be mounted on any fluorescence microscope and controlled by a standard PC with Nuance Image Analysis software, it offers a straightforward and cost-effective solution to high-resolution hyperspectral imaging of QDot-labeled specimens that can be easily adopted by a range of research and clinical laboratories. At the same time, it is expected that alternative imaging techniques capable of measuring fluorescence of spectrally-distinct QDot probes might be successfully employed with M3P technology.

It is important to note that fluorescence signals measured with HSI cannot be directly compared to each other due to differential brightness exhibited by multicolor QDot probes. In fact, sensitivity of detection achieved with larger (red) QDots is often greater compared to smaller (green-blue) QDots^{26, 49}. To account for this effect, differences in photo-physical properties of individual probes should be characterized in advance and incorporated into signal analysis algorithms (Steps 27–30 of the PROCEDURE and Box 2). At the same time, imaging instrumentation parameters might also introduce bias in signal recording. Therefore, differential QDot brightness should be evaluated with exactly the same imaging setup and parameters as used for M3P. Differential QDot brightness is also important to take into account when performing multiplexed staining of targets with varying abundance levels and intracellular distribution. We suggest that brighter red QDot probes are used for less abundant (or more diffusely distributed) targets, while dimmer green QDots are reserved for more abundant (or more densely packed) targets to achieve a relatively uniform apparent staining intensity throughout all targets and avoid camera saturation by any one exceptionally bright signal.

Criteria for multicycle staining—Cyclic IF critically depends on complete specimen regeneration after each cycle, including removal or blocking of all the probe components and elimination of fluorescence signal. In particular, successful regeneration should achieve (i) complete de-staining after each IF cycle to avoid false-positive detection due to signals carried over from previous cycles, (ii) complete removal or blocking of target-bound primary antibodies to preclude binding of probes during further cycles, and (iii) preservation of specimen morphology and target antigenicity to gain consistent staining intensity on every cycle. Microwave treatment⁵⁰, strong acidic conditions^{51–53}, and specimen dehydration⁵¹ developed for cyclic staining with conventional IF and IHC often lead to specimen degradation. In contrast, self-assembled QDot-SpA-Antibody probes employed for M3P technology are uniquely suited for quick and efficient specimen regeneration via chemical stripping, directly benefiting from the non-covalent semi-stable nature of the QDot-SpA-Antibody probe assembly and direct biomarker-QDot binding. Regeneration buffer used in this protocol is compatible with all the model targets tested (Ki-67, HSP90, Lamin A, Cox-4, and β -tubulin). However, buffer optimization might be required for some fragile molecular targets. A suitable regeneration buffer should satisfy the following criteria: (i) all probe components should be completely (or nearly completely) removed from the specimen, (ii) trace amounts of QDots remaining in the specimen should be completely

quenched, (iii) trace amounts of primary antibodies should remain inaccessible to unoccupied QDot-SpA probes, (iv) probe components remaining on the specimen should not interfere with subsequent staining cycles, and (v) specimen antigenicity should be preserved through multiple regeneration cycles. It is advisable to perform evaluation of specimen degradation for all targets of interest as well as for alternative pre-staining specimen processing conditions, as some antigens might demonstrate greater susceptibility to degradation. For example, we have found that incomplete cell fixation achieved by incubation with formaldehyde in TBS for 10 minutes (in contrast to the optimized procedure outlined in Steps 7–14) results in over 40% staining signal drop in LNCaP cells after 10 regeneration cycles, while fixation with formaldehyde in PBS followed by Triton X-100 permeabilization fails to preserve target antigenicity even after one regeneration treatment.

MATERIALS

REAGENTS

General reagents

- Deionized water ($> 18 \text{ M}\Omega\text{-cm}$)
- Tris Buffered Saline (TBS), 10x solution (250 mM Tris-HCl, 1.3 M NaCl, 27 mM KCl, pH 7.4, Fisher scientific, cat. no. BP2471-1)
- Phosphate Buffered Saline (PBS), 10x solution (30 mM $\text{Na}_2\text{HPO}_4\cdot 7\text{H}_2\text{O}$, 1.55 M NaCl, 10 mM KH_2PO_4 , pH 7.4, Gibco, cat. no. 70011)

Cell culture

- HeLa cells (ATCC, cat. no. CCL-2). **CAUTION** Human cell cultures are biohazardous and potentially infectious materials. Handling of human cell cultures must be done in a BSL-2 facility by trained certified personnel. Proper personal protective equipment (PPE) should be used. Refer to local biosafety regulations for specific requirements
- MEM culture medium with Earle's salts and L-Glutamine (Gibco, cat. no. 11095-080)
- Fetal Bovine Serum (FBS) (PAA Laboratories, cat. no. A15–201)
- Penicillin-Streptomycin (10,000 U/mL, Gibco, cat. no. 15140-122)
- Dulbecco's PBS (DPBS), modified, without Ca^{2+} and Mg^{2+} (HyClone, cat. no. SH30028.02)
- Trypsin-EDTA, 0.25% solution (Gibco, cat. no. 25300-054)
- Bleach. **CAUTION** Bleach is toxic and corrosive. Proper PPE should be used
- Ethyl alcohol, 200 proof (Decon Labs, cat. no. 2701). **CAUTION** Ethanol is highly flammable. Keep away from ignition source

Cell fixation and permeabilization

- 16% (wt/vol) formaldehyde, methanol-free solution (Thermo Scientific, cat. no. 28908). **CAUTION** Formaldehyde is toxic and flammable. Proper PPE should be used. Keep away from ignition source
- Dodecyltrimethylammonium chloride (DTAC) (Sigma-Aldrich, cat. no. 44242-25G). **CAUTION** DTAC is an irritant. Proper PPE should be used
- 10% (wt/vol) Triton X-100 in H₂O (Thermo Scientific, cat. no. 28314)
- Sodium azide (Sigma-Aldrich, cat. no. 438456-5G). **CAUTION** Sodium azide is highly toxic. Proper PPE should be used

QDot-SpA bioconjugation

- Qdot ITK amino (PEG) quantum dots with emission peaks centered at 525, 545, 565, 585, and 605 nm, 250 µL, 8 µM (Invitrogen, cat. no. Q21541MP, Q21591MP, Q21531MP, Q21511MP, and Q21501MP, respectively)
- Bis[sulfosuccinimidyl] suberate (BS3), 8×2 mg no-weigh vials (Thermo Scientific, cat. no. 21585)
- Protein A from *Staphylococcus aureus* (SpA), lyophilized (Sigma-Aldrich, cat. no. P6031-5MG)

Cell staining

- Bovine serum albumin (BSA) (Sigma-Aldrich, A7906-100G)
- 5% (wt/vol) Casein, alkali-soluble (Novagen, cat. no. 70955-225ML)
- Sodium dodecyl sulfate (SDS) (Sigma Aldrich, cat. no. L4390-100G)
- IgG Elution buffer, pH2.8, 1 L (Thermo Scientific, cat. no. 21004)
- SpA-compatible primary antibodies, whole IgG, affinity purified, 0.2 mg/mL stock concentration. **CRITICAL** Only whole IgG antibodies that exhibit strong binding between Fc region and SpA can be used. **CRITICAL** Avoid using IgG antibodies that also exhibit binding between Fab region and SpA, as it might lead to QDot cross-linking and aggregation. **CRITICAL** Antibody stocks with carrier proteins and other stabilizers can be used, but they should be free of non-targeting IgG. **CRITICAL** Typically, one staining experiment requires 1.5 µL of primary antibodies

Microscopy

- Immersion oil for fluorescence microscopy, type FF (Cargille Laboratories, cat. no. 16212)
- Optical lens cleaner (Fisher Scientific, cat. no. 22-143974)
- Glycerol, 1L (Fisher Scientific, cat. no. BP2291)

EQUIPMENT

- 500-mL glass or plastic bottles
- Tissue culture dish, 100 mm (Becton Dickinson Labware, cat. no. 353003)
- Glass-bottom 24-well plates (Greiner Bio-One, cat. no. 662892)
- Vacuum aspirator (e.g., Bel-Art Scienceware, cat. no. F199170002)
- 15-mL centrifuge tubes (Corning, cat. no. 430790)
- 50-mL centrifuge tubes (Corning, cat. no. 430828)
- 0.65-mL microcentrifuge tubes (Corning, cat. no. 3208)
- 1.7-mL microcentrifuge tubes (Corning, cat. no. 3620)
- Illustra NAP-5 desalting columns (GE Healthcare, cat. no. 17-0853-02)
- Test tube clamp/holder on a stand
- Handheld UV lamp (e.g., UVP, cat. no. 95-0006-02)
- Vivaspin 500 100KDa MWCO concentrators (GE Healthcare, cat. no. 28-9322-37)
- Amicon Ultra 0.5 mL 100KDa MWCO centrifugal filters (Millipore, cat. no. UFC510024)
- Spectrophotometry cuvettes, 100 μ L sample volume (e.g., BrandTech Scientific, cat. no. 759220)
- Clear-bottom 96-well plate, for fluorescent assays (Corning, cat. no. 3631)
- Lens paper (Fisher Scientific, cat. no. 11-996)
- Microscope cover glasses, 50 $\times$ 22 mm, no. 1.5 (Fisher Scientific, cat. no. 12-544D)
- Spectrophotometer (e.g., UV-2450, Shimadzu)
- Centrifuge for 50-mL centrifuge tubes
- Microcentrifuge for 1.7 mL and 0.65 mL microcentrifuge tubes
- Inverted fluorescence microscope (e.g., IX-71, Olympus)
- Hyperspectral imaging (HSI) camera (e.g., Nuance, 420–720 nm spectral range, Advanced Molecular Vision)

REAGENT SETUP

Cell culture—Culture medium (for HeLa cells). To 500 mL of MEM medium add 50 mL of FBS and 3 mL of penicillin-streptomycin. Culture medium can be prepared in advance and stored at 4°C for several weeks. **CRITICAL** This step must be performed inside the cell culture hood following aseptic cell culture techniques.

70% (vol/vol) Ethanol. Combine 300 mL water and 700 mL 200 proof ethyl alcohol in a 1 L glass bottle. Cap tightly to prevent evaporation. 70% Ethanol can be stored capped at

room temperature for several weeks. **CAUTION** Ethanol is highly flammable. Keep away from ignition source.

Cell fixation and permeabilization—CRITICAL Reagent amounts stated below are sufficient for processing all cells in one 24-well plate.

Fixation buffer. Combine 7.8 mL water with 3 mL 16% (wt/vol) formaldehyde and 1.2 mL 10x TBS in a 15-mL centrifuge tube to obtain 12 mL of 4% formaldehyde/TBS. Optionally, 60 μ L 10% (wt/vol) Triton X-100 can be added for improved intracellular access.

CAUTION Formaldehyde is toxic and flammable. Proper PPE should be used. Keep away from ignition source.

Permeabilization buffer A. Combine 10.8 mL water with 240 mg DTAC and 1.2 mL 10x TBS in a 15-mL centrifuge tube to obtain 12 mL of 2% (wt/vol) DTAC/TBS. **CAUTION** DTAC is irritant. Proper PPE should be used.

Permeabilization buffer B. Combine 10.5 mL water with 0.3 mL 10% Triton X-100 and 1.2 mL 10x TBS in a 15-mL centrifuge tube to obtain 12 mL of 0.25% (wt/vol) Triton X-100/TBS.

QDot-SpA bioconjugation—SpA stock. Add 900 μ L water and 100 μ L 10x PBS directly into a vial with 5 mg SpA. Allow powder to completely dissolve in PBS. Vortex the vial to collect powder from the walls and cap and mix the solution. Optionally, the vial can be placed in a 50-mL centrifuge tube and centrifuged at 1,000g for 1 minute to collect solution from the walls and cap. Aliquot SpA/PBS solution into ten 0.65 mL microcentrifuge tubes with 100 μ L each. Freeze these aliquots at -20°C and keep frozen until needed for bioconjugation reaction. Frozen aliquots can be stored for several months. **CRITICAL** As SpA solution is added directly to reaction, it is important to avoid contamination with primary amines (e.g., from amine-containing buffers or carrier proteins). **CRITICAL** One 100 μ L aliquot is sufficient for 1 bioconjugation reaction.

Cell staining—CRITICAL Reagent amounts stated below are sufficient for performing 10 staining cycles in one well of a 24-well plate.

Regeneration buffer. Combine 15 mL IgG Elution buffer and 75 mg SDS in a 50-mL centrifuge tube to get Elution buffer with 0.5% (wt/vol) SDS. Mix by brief vortexing. **CAUTION** SDS is irritant. Proper PPE should be used.

Blocking buffer. Combine 13.2 mL water, 1.5 mL 10x TBS, 300 mg BSA, and 300 μ L 5% casein in a 50-mL centrifuge tube to obtain 15 mL of 2% (wt/vol) BSA, 0.1% (wt/vol) casein, 1x TBS. Allow BSA to completely dissolve. Mix by brief vortexing.

Staining buffer. Combine 2.7 mL water, 0.3 mL 10x TBS, and 180 mg BSA in a 15-mL centrifuge tube to obtain 3 mL of 6% (wt/vol) BSA in 1x TBS. Allow BSA to completely dissolve. Mix by brief vortexing.

Washing buffer. Combine 17.6 mL water, 2 mL 10x TBS, 200 mg BSA, and 400 μ L 5% casein in a 50-mL centrifuge tube to obtain 20 mL of 1% (wt/vol) BSA, 0.1% (wt/vol) casein, 1x TBS. Allow BSA to completely dissolve. Mix by brief vortexing.

80% (vol/vol) Glycerol/TBS. Combine 0.5 mL water, 0.5 mL 10x TBS, and 4 mL glycerol in a 15-mL centrifuge tube to obtain 5 mL of 80% (vol/vol) glycerol in 1x TBS. Mix well by vortexing.

EQUIPMENT SETUP

NAP-5 desalting column. Fix NAP-5 column in a test tube clamp on a stand. Place aqueous waste collection container under the column. Remove the top and bottom caps of the column and let storage solution to completely pass through the column. Follow NAP-5 column instructions provided by the manufacturer to equilibrate the column with 1x PBS buffer.

CRITICAL Do not allow the NAP-5 column to dry; refill with PBS in a timely manner.

Fluorescence microscope. Inverted fluorescence microscope must be used in order to examine cells attached to the cover-glass bottom of a 24-well plate. Single source producing light in UV-violet spectral range (300–400 nm) should be used for simultaneous excitation of all QDot colors. Long-pass emission filter covering full spectral range of the HSI camera should be used for simultaneous registration of multicolor QDot fluorescence. Microscope should be equipped with high-magnification 100x oil-immersion objective and low-magnification 10x or 20x objective. HSI camera should be mounted on one of the optical ports of the microscope. Exemplar microscope setup: IX-71 inverted fluorescence microscope (Olympus) equipped with 20x dry objective (NA 0.75, Olympus), 100x oil-immersion objective (NA 1.40, Olympus), mercury lamp light source, Wide-UV filter cube (330–385 nm band-pass excitation, 420 nm long-pass emission, Olympus), and Nuance HSI camera (420–720 nm spectral range, Advanced Molecular Vision). HSI camera is controlled by a PC running Nuance Image Analysis software.

PROCEDURE

Growing cell culture in a glass-bottom 24-well plate

TIMING 30 min plus 48–72 h for cell growth

CAUTION Human cell cultures are biohazardous and potentially infectious materials. Handling of human cell cultures must be done in a BSL-2 facility by trained certified personnel. Proper PPE should be used. Refer to local biosafety regulations for specific requirements.

- 1 Grow cells in a 100-mm tissue culture dish to 90–100% confluence.
CRITICAL STEP Amount of cells obtained from one 100-mm dish is sufficient for seeding cells in up to four 24-well plates.
- 2 Prepare biosafety cabinet for cell culture. Pre-warm cell culture medium, trypsin, and DPBS to 37°C in a water bath.

- 3 Aspirate old cell culture medium from the dish and gently add 5 mL of DPBS onto the wall of the dish. Aspirate DPBS and repeat DPBS wash. **CRITICAL STEP** Do not add DPBS directly onto cells, as it might result in cells being detached from the dish surface and aspirated.
- 4 Aspirate DPBS and add 1 mL of trypsin directly onto cells. Place the dish in an incubator for 2 min and then transfer it back to the cell culture hood. Cells should easily roll off the surface upon tilting of the dish. Add 10 mL of cell culture medium directly to cells using 10-mL sterile serological pipet. Wash cells off the surface of the dish completely. Optionally, vigorously pipet solution against the surface of the dish to break apart cell aggregates and obtain a homogeneous cell suspension. **CRITICAL STEP** The duration of trypsin treatment should be optimized for cell lines of interest.
- 5 Transfer 22.5 mL cell culture medium to a sterile 50-mL centrifuge tube. Then transfer 2.5 mL cell suspension (~1,000,000 cells) from the dish into the same 50-mL centrifuge tube. Cap the tube and shake to obtain a homogeneous cell suspension. Transfer 1 mL of cell suspension from the 50-mL tube to each well of a 24-well glass-bottom plate to seed ~40,000 cells/well. Cover the plate and place it back into a cell culture incubator. **CRITICAL STEP** Typically, cells grow to ~80% confluence in 48–72 h, at which point cells must be fixed.

TROUBLESHOOTING

- 6 Discard the rest of the cell suspensions and clean the cell culture hood.
CAUTION Cell suspensions and materials containing cells are biohazards. Follow local biosafety guidelines for details on handling biohazard waste.

Cell fixation and permeabilization

TIMING 1.5 h

CRITICAL This procedure is optimized for proper labeling of common intracellular targets in adherent cells. However, preservation of antigenicity and accessibility varies for different molecular targets, which might require further optimization of fixation (Step 10) and permeabilization (Steps 12 and 13) conditions. It is recommended to confirm adequate staining of targets of interest using conventional immunofluorescence procedure with secondary QDot probes (see Box 1).

CRITICAL All buffers should be prepared immediately before performing cell fixation.

CRITICAL Do not let cells dry throughout this procedure; do not aspirate solution from more than 4 wells at a time; refill aspirated wells with appropriate buffers immediately.

CAUTION Human cell cultures are biohazardous and potentially infectious materials. Handling of human cell cultures must be done in a BSL-2 facility by trained certified personnel. Proper PPE should be used. Refer to local biosafety regulations for specific requirements.

- 7 Prepare 1x TBS in a 50-mL tube and place it in a water bath set to 37°C. Prepare Fixation buffer, Permeabilization buffer A, and Permeabilization buffer B (see Reagent Setup).
- 8 Transfer tubes with pre-warmed 1x TBS and the fixation buffer into the cell culture hood. Transfer 24-well glass-bottom plate with cell culture from the incubator to the cell culture hood.
- 9 Replace old cell culture medium with 1 mL/well pre-warmed 1x TBS in groups of 4 wells at a time. Gently aspirate medium using vacuum aspirator. Gently add 1 mL of pre-warmed 1x TBS onto the wall of each aspirated well. **CRITICAL STEP** During aspiration, tilt the plate and place the aspirator tip to a corner of the well to avoid damage to and/or aspiration of cells. **CRITICAL STEP** Do not add TBS directly onto cells, as it might result in cells being detached from the glass surface and aspirated.
- 10 Fixation of cells with formaldehyde. Replace TBS solution with 500 µL/well Fixation buffer in groups of 4 wells at a time. Gently aspirate TBS from the corner of the well. Gently add fixation buffer onto the wall of each aspirated well. Cover the plate and let cells incubate with Fixation buffer for 20 min at 37°C.
- 11 Replace Fixation buffer with 1 mL/well of pre-warmed 1x TBS in groups of 4 wells at a time. Gently aspirate Fixation buffer from the corner of the well using a 1,000-µL pipetter and transfer it into a waste container. Gently add TBS onto the wall of each aspirated well.

CAUTION Refer to local safety guidelines for proper disposal of formaldehyde-containing chemical waste.

CAUTION Fixed cells do not represent a biohazard. The rest of procedures can be safely performed in a general laboratory setting.

- 12 Permeabilization of cells with buffer A. Replace TBS solution with 500 µL/well Permeabilization buffer A in groups of 4 wells at a time. Gently aspirate TBS from the corner of the well using a 1,000-µL pipetter and transfer it into a waste container. Gently add Permeabilization buffer A onto the wall of each aspirated well. Cover the plate and incubate for 20 min at room temperature. Then rinse cells with 1 mL/well 1x TBS.
- 13 Permeabilization of cells with buffer B. Replace TBS solution with 500 µL/well Permeabilization buffer B in groups of 4 wells at a time. Gently aspirate TBS from the corner of the well using 1,000-µL pipetter and transfer it into a waste container. Gently add Permeabilization buffer B onto the wall of each aspirated well. Cover the plate and incubate for 5 min at room temperature. Then rinse cells with 1 mL/well 1x TBS.
- 14 Wash cells for 5 min 3 times with 1 mL/well 1x TBS at room temperature. Then refill with 1 mL/well 1x TBS for storage. Optionally, sodium azide can be added

to TBS at a final concentration of 0.05% (wt/vol) to prevent microbial contamination over long-term storage.

CAUTION Sodium azide is highly toxic. Proper PPE should be used.

PAUSE POINT Fixed cells can be stored at 4°C for several weeks. Seal plates with parafilm to prevent evaporation of the storage buffer.

QDot-SpA bioconjugation

TIMING 2.5 h plus 4 h (or overnight) incubation

CRITICAL The following procedure can be used to prepare 100 μ L of $\sim$ 1 μ M QDot-SpA bioconjugates of one color. Several bioconjugation procedures (no more than 3 are recommended) can be performed in parallel for preparation of multicolor QDot-SpA stocks.

- 15** Equilibrate tubes with Qdot ITK amino (PEG) quantum dots, BS3, and SpA (see Reagent Setup) at room temperature ($\sim$ 21°C). Equilibrate NAP-5 column with PBS (see Equipment Setup).
- 16** Activation of QDots with BS3. Add 70 μ L water directly to a no-weigh tube with BS3 powder in it and vortex to obtain 50 mM solution of BS3. In a 1.7-mL microcentrifuge tube combine 25 μ L 8 μ M QDot stock, 61 μ L water, 10 μ L 10x PBS, and 4 μ L 50 mM BS3 solution to obtain 100 μ L 2 μ M QDots in PBS with 1,000x molar excess of BS3. Vortex briefly. Optionally, reaction tube can be centrifuged at 1,000g for 1 min to collect the solution from tube walls and cap. Incubate for 30 min at room temperature. **CRITICAL STEP** BS3 solution must be prepared immediately prior to its addition to QDots to avoid hydrolysis of reactive groups in the water. **CRITICAL STEP** Do not vortex or otherwise agitate the reaction tube during incubation, as it might result in partial QDot aggregation.
- 17** Purification of activated QDots with NAP-5 columns. Let PBS completely pass through the column, leaving no solution above the frit (Fig. 3a). Carefully transfer QDot/BS3 reaction solution onto the top frit of NAP-5 column (Fig. 3b). Let QDot solution enter the frit completely (Fig. 3c), then add 1.5 mL 1x PBS. Note that QDots might not travel at a rate indicated by the NAP-5 manufacturer's instructions; use a handheld UV lamp to trace the QDots' position inside the column (Fig. 3d). Collect $\sim$ 500 μ L colored QDot solution, as it elutes from the NAP-5 column, into a 1.7-mL microcentrifuge tube (Fig. 3e). **CRITICAL STEP** This Step must be performed as rapidly as possible to minimize hydrolysis and de-activation of reactive groups on QDots. **CAUTION** UV light is hazardous to eyes and skin. Wear gloves and safety glasses rated to protect against UV light when using handheld UV lamp.
- 18** Concentration of activated QDots and reaction with SpA. Transfer the purified QDot solution to a Vivaspin 500 100KDa MWCO concentrator (Fig. 3f). Centrifuge at 6,000g for 7 min to concentrate QDots down to $\sim$ 20 μ L (Fig. 3g). Add 100 μ L 5 mg/mL SpA solution directly to concentrated QDots and transfer

this reaction mixture to a new 1.7-mL microcentrifuge tube. Incubate for at least 4 h at room temperature. **CRITICAL STEP** This Step must be performed as rapidly as possible to minimize hydrolysis and de-activation of reactive groups on QDots. **CRITICAL STEP** Do not use dilute QDot solution for reaction, as it results in poor conjugation yield. **CRITICAL STEP** Do not vortex or otherwise agitate the reaction tube during incubation, as it might result in QDot aggregation.

TROUBLESHOOTING

PAUSE POINT Reaction mixture can be left overnight at room temperature for incubation; procedure can be resumed on the next day.

- 19 Purification of QDot-SpA bioconjugates. Dilute reaction mixture up to 500 μ L with 1x PBS and transfer it to a new Amicon Ultra 0.5 mL 100KDa MWCO centrifugal filter. Centrifuge at 6,000g for 7 min to concentrate the QDot-SpA solution down to $\sim$ 20 μ L. Refill with 500 μ L 1x PBS, thus achieving a 25x dilution of the QDot-SpA mixture. Repeat centrifugation 5 more times. Following the last centrifugation round, dilute concentrated QDot-SpA solution up to 100 μ L with 1x PBS and transfer this purified QDot-SpA stock to a new 1.7-mL microcentrifuge tube. **CRITICAL STEP** Typically, a 100KDa MWCO centrifugal filter concentrates QDots in the top chamber, while letting free SpA pass through the membrane; confirm the absence of QDots in the wash-through fraction using a handheld UV lamp.

TROUBLESHOOTING

- 20 Measurement of the QDot-SpA concentration with spectrophotometer. Baseline spectrophotometer with 1x PBS. Transfer 100 μ L QDot-SpA stock solution to a new low-volume spectrophotometry cuvette, place it into the spectrophotometer and measure QDot absorbance on the 500–700 nm spectral range in reference to 1x PBS. QDot concentration can be determined in two ways, depending on availability of appropriate extinction coefficients:
 - A. If extinction coefficients are known (provided by the manufacturer)
 - i. Measure absorbance at a wavelength for which the extinction coefficient is known.
 - ii. Calculate QDot concentration using formula: $C = A / (e \cdot l)$, where C is concentration (mole/L, or M), A is absorbance, e is extinction coefficient (L/mole-cm), l is path length (cm, typically 1 cm).
 - B. If extinction coefficients are not known
 - i. Measure absorbance at the lowest-energy absorbance peak.
 - ii. Obtain reference absorbance from color-matched original QDot stock. Prepare 100 μ L 1 μ M QDot solution in PBS

from stock (concentration should be provided by the manufacturer). Transfer QDot solution to a new low-volume spectrophotometry cuvette, place into spectrophotometer, and record absorbance on the 500–700 nm spectral range in reference to 1x PBS. Measure absorbance at the same wavelength as in previous step.

- iii. Calculate QDot concentration in QDot-SpA sample using formula: $C = A_{\text{sample}}/A_{\text{reference}}$, where C is concentration of QDot-SpA ($\mu\text{mole/L}$, or μM), A_{sample} is absorbance of QDot-SpA sample, and $A_{\text{reference}}$ is absorbance of reference 1 μM QDot solution.

TROUBLESHOOTING

- 21 Transfer QDot-SpA solution from the spectrophotometry cuvette into a new 1.7-mL microcentrifuge tube, label with QDot type, concentration, and preparation date, and place into a 4°C refrigerator for storage.

PAUSE POINT QDot-SpA bioconjugates can be stored at 4°C for several weeks.

Building reference QDot spectral library with HSI

TIMING 30 min

CRITICAL Steps 22–26 should be performed for each new QDot-SpA preparation and each microscope setup used for analysis of stained specimens. The procedure is optimized for the Exemplar microscope setup (see Equipment Setup); modifications to the procedure might be necessary when used with other setups.

- 22 Preparation of a microscope slide with samples of each QDot-SpA color. Combine 4.5 μL 1x PBS and 0.5 μL 1 μM QDot-SpA solution in a 0.65-mL microcentrifuge tube to obtain 5 μL 100 nM aliquots. Spot 5 μL aliquots onto a no. 1.5 cover glass. Place droplets at a distance of at least 5–10 mm from each other (Supplementary Fig. 4a).
- 23 Turn on the excitation light source (e.g., mercury lamp burner), start Nuance Image Analysis software, and wait for HSI camera to initialize. On the microscope, use the Wide-UV filter cube and 20x dry objective (see Equipment setup). Transfer cover glass with QDot samples onto the microscope stage.
- 24 Collecting images of QDot droplets with HSI camera. Completely lower the 20x objective and place it directly underneath the center of a QDot droplet. Open the shutter of the excitation light source to illuminate the droplet (you should see bright QDot fluorescence, Supplementary Fig. 4b). Open HSI camera optical port. In the Nuance Image Analysis software, under “Acquire” tab select the desirable spectral range for scanning. Click “Autoexpose Cube” to automatically determine optimal exposure time for all wavelengths, then click “Acquire Cube” to collect a hyperspectral image of the droplet. Close the shutter. Save the cube (it is helpful to include QDot emission peak wavelength in the file name).

Repeat this step for all QDot droplets on the cover slide. **CRITICAL STEP** Use the same spectral range for all QDots and imaging of stained cells. **CRITICAL STEP** Image collection should be performed under reduced ambient illumination (preferably in a dark room) to minimize contribution of non-QDot light.

- 25 Extract QDot spectra from image cubes collected in the previous step. Load an image cube in the Nuance Image Analysis software. Under the “Spectra” tab, click “Draw” icon, and draw a line through the center of the image. A QDot spectrum should be recorded (Supplementary Fig. 4c). Name this spectrum with the QDot emission peak wavelength. Repeat for all image cubes to build up a spectral library (Supplementary Fig. 4d). Once all spectra are recorded, go to *File -> Save Spectral Library* and save this library at a suitable location on PC (it is helpful to include QDot-SpA preparation date or lot ID in the file name). **CRITICAL STEP** Do not exit the software throughout this procedure.

TROUBLESHOOTING

- 26 Optional: record cell autofluorescence spectrum if contribution of autofluorescence is substantial. Place a 24-well glass-bottom plate containing fixed cells onto the microscope stage. Focus on cells using 20x dry objective. Collect a hyperspectral image of cells. In the Nuance Image Analysis software, go to the “Spectra” tab, click “Draw” icon, and draw a line through an area of autofluorescence. Save spectrum at a suitable location on PC. **CRITICAL STEP** For samples containing several sources of autofluorescence with distinct spectra (e.g., fixed tissue sections), each spectrum must be recorded separately in a spectral library; do not create an average spectral profile for all autofluorescence sources.

PAUSE POINT Reference QDot spectral library can be created at any time before or after staining experiments, as it is used only for post-staining data analysis.

Measuring differential QDot brightness with HSI

TIMING 1.5 h

CRITICAL Steps 27–30 should be performed for each new QDot-SpA preparation and each microscope setup used for analysis of stained specimens. The procedure is optimized for the Exemplar microscope setup (see Equipment Setup); modifications to the procedure might be necessary when used with other setups.

CRITICAL If QDot concentration cannot be accurately determined, differential brightness should be directly assessed via cell staining (see Box 2).

- 27 For each QDot color combine 285 μL 1x PBS and 15 μL 1 μM QDot-SpA in a 0.65-mL microcentrifuge tube to obtain 300 μL 50 nM QDot samples. Transfer 100 μL /well of QDot samples onto a clear-bottom 96-well plate, preparing triplicates of each sample.

- 28 Prepare imaging setup and collect hyperspectral images of each well as instructed in Steps 23 and 24.
- 29 Determine QDot fluorescence intensity from the image cubes collected through previous step. In the Nuance Image Analysis software, go to the “Spectra” tab, click “Import Spectra” and import the spectral library created at Steps 22–25. Then load an image cube and click “Unmix”. The software should extract individual QDot channels in accordance to similarity to reference spectra. Click on the image corresponding to measured QDot, go to “Measure” tab, set “Threshold” to zero, and record *Total Scaled Signal*. Repeat for all image cubes. **CRITICAL STEP** *Total Signal* can be used instead of *Total Scaled Signal*, but it must be normalized to the exposure time used for each QDot.
- 30 Calculating differential QDot brightness. In a spreadsheet, record triplicate measurements of *Total Scaled Signal* intensity for each QDot color and calculate an average intensity for each color. Normalize average intensities by a suitable QDot color to create a list of correction factors. Typically, QDot fluorescence intensity increases from green to red emission color.

PAUSE POINT Differential brightness of QDots can be measured at any time before or after staining experiments, as it is used only for post-staining data analysis.

Cell staining with QDot-SpA-IgG probes

TIMING 3.5–4 h for one cycle

CRITICAL Steps 31–38 must be performed on the same day and at room temperature.

CRITICAL All buffers should be freshly prepared immediately before performing cell staining.

CRITICAL Specimen regeneration is used as part of multicycle staining to remove QDot signal after each cycle. Preservation of antigenicity during this step varies for different molecular targets. It is recommended to confirm adequate staining of targets of interest using conventional immunofluorescence procedure with secondary QDot probes (see Box 1). Targets incompatible with regeneration conditions can be stained during the first cycle, skipping the initial regeneration part of Step 32.

- 31 Equilibrate a 24-well plate containing fixed cells to room temperature. Prepare Regeneration buffer, Blocking buffer, Staining buffer, Washing buffer, and 1x TBS (see Reagent Setup).

CRITICAL Steps 32 and 33 should be performed in parallel.

- 32 Regeneration of cells. Aspirate storage solution from the well and rinse cells with 1 mL/well fresh 1x TBS. Aspirate TBS and wash cells for 5 min 3 times with 500 μ L/well Regeneration buffer. Aspirate Regeneration buffer, rinse twice with 1 mL/well 1x TBS, and wash for 5 min 2 times with 500 μ L/well Blocking buffer. Replace with fresh 500 μ L/well Blocking buffer and incubate for 30 min at room temperature. **CRITICAL STEP** We recommend performing cell

regeneration even on unstained cells to ensure consistent conditions between different staining cycles.

- 33** Preparation of QDot-IgG probes. To stain cells in 1 well, for each QDot-IgG probe combine 2.5 μ L 1x PBS, 1.5 μ L 0.2 mg/mL primary antibody, and 6 μ L 1 μ M QDot-SpA in a separate 0.65-mL microcentrifuge tube. Mix by pipetting. Incubate at room temperature for 1 h. **CRITICAL STEP** Keep individual QDot-antibody pairs in separate tubes; do not mix different QDot colors. **CRITICAL STEP** Only whole IgG antibodies that exhibit strong binding between Fc region and SpA can be used; avoid using IgG antibodies that also exhibit binding between Fab region and SpA, as it might lead to QDot cross-linking and aggregation; avoid using antibody stocks containing non-targeting IgG.
- 34** Labeling cells with multicolor QDot-IgG probes. Mix all pre-assembled QDot-IgG probes from Step 33 in the same 1.7-mL microcentrifuge tube and dilute up to 300 μ L with Staining buffer. Vortex briefly. Optionally, centrifugate tubes at 1,000g for 1 min to collect the solution from tube walls and cap. Aspirate Blocking buffer from the well and replace it with 300 μ L/well QDot-IgG cocktail in Staining buffer. Incubate at room temperature for 2 h. **CRITICAL STEP** Do not add detergents (e.g., Tween-20, SDS, Triton X-100) to Staining buffer, as it might lead to increased non-specific staining by QDots.
- 35** Aspirate the QDot staining cocktail, briefly rinse cells with 1 mL/well Washing buffer, and wash for 10 min once with 1 mL/well Washing buffer. Then wash for 5 min 3 times with 1 mL/well 1x TBS. Keep cells immersed in 1x TBS for imaging.

CRITICAL STEP It is recommended to evaluate non-specific staining by QDot-SpA bioconjugates for each new preparation of cells and each new batch of QDot-SpA bioconjugates. Follow general staining guidelines outlined in Steps 31–35, but do not add target-specific primary antibodies to QDot-SpA bioconjugates.

CRITICAL STEP Proceed with imaging immediately after staining and washing.

Imaging of QDot-labeled cells

TIMING Typically 15–30 min, but varies depending on type of analysis performed.

CRITICAL The following procedure is optimized for Exemplar microscope setup (see Equipment Setup); modifications to the procedure might be necessary when used with other setups.

CRITICAL Imaging can be done directly on stained cells immersed in 1x TBS. Optionally, TBS can be replaced by 500 μ L/well 80% (vol/vol) Glycerol/TBS to reduce specimen damage by extended UV illumination. It is advisable to confirm the accuracy of QDot analytical calibration (see Steps 22–30 and Box 2) with 80% Glycerol/TBS used as an imaging buffer, as QDot fluorescence might be affected by glycerol.

- 36** Turn on the excitation light source (e.g., mercury lamp burner), start the Nuance Image Analysis software, and wait for HSI camera to initialize. On the microscope, use Wide-UV filter cube (see Equipment setup). Optional: place a reference mark (e.g., cross) on the bottom glass surface of the well containing stained cells using permanent fine-point marker. Transfer a 24-well plate containing stained cells onto the microscope stage.
- 37** Collecting images of stained cells with HSI camera. Open the shutter of the excitation light source to illuminate the cells. Focus on cells using suitable objective. Optional: find the location of the reference mark using brightfield illumination first; then step a set distance away from the mark for cell imaging. Record this position for imaging during subsequent staining cycles. Open HSI camera optical port. In the Nuance Image Analysis software, under “Acquire” tab select the desirable spectral range for scanning. Click “Autoexpose Cube” to automatically determine optimal exposure time for all wavelengths, then click “Acquire Cube” to collect a hyperspectral image of the cells. Close the shutter. Save the cube (it is helpful to specify Antibody-QDot pairs used for staining in the file name). **CRITICAL STEP** Selected spectral range should match QDot spectral library created in Steps 22–25. **CRITICAL STEP** Image collection should be performed under reduced ambient illumination (preferably in a dark room) to minimize contribution of non-QDot light.

TROUBLESHOOTING

- 38** Resetting setup for further use. If glycerol was used for imaging, wash cells for 5 min 3 times with 1 mL/well 1x TBS. If oil-immersion objective was used, gently wipe the oil from the objective and the glass bottom of the 24-well plate.

CRITICAL STEP If further staining cycles are necessary, repeat steps 32–38 for a new sub-set of molecular targets. Use reference mark and unique cell morphology features to find the same sub-set of cells through several staining cycles. You might choose to re-stain the same molecular target through several cycles to aid in precise alignment of images.

PAUSE POINT Regenerated cells can be stored in TBS at 4°C for several days before performing subsequent staining cycles. Should such a break be necessary, de-stain cells following the regeneration procedure outlined in Step 32, then wash with 1x TBS instead of Blocking buffer. Cover the plate, seal with parafilm, and transfer to 4°C.

Analysis of hyperspectral image cubes

TIMING Typically 10–15 min per image, but varies depending on type of analysis performed.

CRITICAL The Nuance HSI camera does not have to be connected to the PC in order to perform data analysis.

- 39** In the Nuance Image Analysis software, go to the “Spectra” tab, click “Import Spectra”, and import the QDot spectral library created through Steps 22–25. If

an autofluorescence spectral library was created in Step 26, import it and merge with the QDot spectral library.

- 40 Unmixing multicolor images into their individual components. Load an image cube with sample of interest and click “Unmix” under the “Spectra” tab. The software should extract individual QDot channels in accordance to similarity to reference spectra and create a false-color composite image. **CRITICAL STEP** Typically, brightness of each channel is adjusted automatically for best visual presentation; such images cannot be used for direct evaluation of relative staining intensity.

TROUBLESHOOTING

- 41 Customizing false-color composite images. Click on the *Composite* image window to make it active, then click “Display Control” icon. A *Display Control* dialog box should appear. Adjust brightness and contrast of each channel, select a color scheme for each channel, and choose channels to be “visible” in the composite image. Save false-color composite images by selecting *File -> Save Image -> Save (As displayed)*, while keeping the *Composite* image window active.
- 42 Recording intensity-normalized images for each QDot channel. Selecting *Tools -> Compare Images* should bring up the *Compare Images* dialog box. Drag and drop images for individual QDot channels from the *Thumbnails* box into empty cells of the *Compare Images* dialog box. All images should be automatically normalized to the brightest signal. Normalized images can be used for direct comparison of staining intensity; however, this procedure does not take into account differential QDot brightness. Apply appropriate scaling factors to compensate for differential QDot brightness (measured in Steps 27–30). Save normalized images: right-click on each image and select *Save Image As Displayed* (it is helpful to specify scaling factor in the file name).
- 43 Measuring average staining intensity over the cell population within the field of view. Click on a window corresponding to one QDot channel to make it active. Go to “Measure” tab and adjust “Threshold Level” to include stained cells and to exclude unstained areas in between. Save measurements as a text file: select *File -> Save Measurements*. Calculate an average intensity per pixel using a spreadsheet software: $I = \text{SUM}[\text{Total Signal (scaled counts/s)}] / \text{SUM}[\text{Area(pixels)}]$. Repeat for all QDot channels. **CRITICAL STEP** We recommend using *Total Scaled Signal* rather than *Total Signal*, as it accounts for exposure time used for acquisition of each image cube.
- 44 Measuring total staining intensity for individual cells. Click on a window corresponding to one QDot channel to make it active. Go to “Measure” tab and click “Draw” button under “Manual Draw Regions” box. Draw a region of interest around a single cell or intracellular compartment. Record *Total Scaled Signal* value in a spreadsheet or save measurements as a text file: select *File -> Save Measurements*. Repeat for all cells of interest. Optional: as manual

selection of individual cells is time-consuming, third-party automated algorithms might be employed for high-throughput single-cell analysis.

TROUBLESHOOTING

Troubleshooting advice can be found in Table 3.

TIMING

Steps 1–6, Growing cell culture in a glass-bottom 24-well plate: 30 min plus 48–72 h for cell growth

Steps 7–14, Cell fixation and permeabilization: 1.5 h

Steps 15–21, QDot-SpA bioconjugation: 2.5 h plus 4 h (or overnight) incubation

Steps 22–26, Building reference QDot spectral library with HSI: 30 min

Steps 27–30, Measuring differential QDot brightness with HSI: 1.5 h

Steps 31–35, Cell staining with QDot-SpA-IgG probes: 3.5–4 h for one cycle

Steps 36–38, Imaging of QDot-labeled cells: Typically 15–30 min, but varies depending on type of analysis performed.

Steps 39–44, Analysis of hyperspectral image cubes: Typically 10–15 min per image, but varies depending on type of analysis performed.

Box 1, Conventional immunofluorescence with secondary QDot probes: 3.5–4.5 h

Box 2, Determination of QDot differential brightness from cell staining: 3.5 h plus image acquisition and analysis

ANTICIPATED RESULTS

The M3P profiling procedure utilizing self-assembled QDot-SpA-IgG probes should enable robust quantitative molecular imaging at sub-cellular resolution and offer a set of data necessary for measurement of protein expression at a single-cell level (Fig. 4). Further application-specific analyses of, for example, target colocalization, translocation, and cell morphology, should be straightforward. At least 5 molecular targets can be stained and visualized simultaneously using a standard epifluorescence microscope equipped with a single UV excitation light source and hyperspectral imaging camera. Narrow symmetrical emission profiles of QDot probes help with accurate unmixing of multicolor images using reference spectral library. Direct comparison of staining intensity between different QDot colors should be readily achievable with the use of proper correction factors determined from bulk fluorescence measurements (Steps 27–30) or cell staining (Box 2). It is important, however, to build a reference spectral library and determine correction factors for each QDot-SpA preparation and each microscope setup, as minor deviations often result in unmixing errors and inaccurate quantitative analysis.

Versatile design of the QDot-SpA bioconjugates should offer an easy, one-step, purification-free preparation of target-specific QDot-SpA-IgG probes via a self-assembly procedure. All types of whole IgG compatible with SpA (e.g., rabbit IgG, mouse IgG2a) are expected to form stable probes that exhibit lack of cross-linking and cross-talk. It is important, however, to have QDot-SpA in excess to IgG to avoid QDot surface saturation with antibodies, which might result in probe disassembly and antibody exchange between different QDot colors.

Multicycle staining methodology is expected to enable analysis of at least 25 molecular targets (5 targets in 5 cycles) without damaging specimen antigenicity and morphology³⁶. At the same time, properly processed specimens (e.g. using the methodology described in Steps 1–14) should be amenable to re-probing for at least 10 staining cycles (Fig. 5a), thus enabling evaluation of 100-target single-cell molecular profiles when combined with 10-color QDot libraries and 10-channel HSI. Optimization of the specimen processing procedure might be required to achieve staining of certain targets (e.g., easily damaged targets, or tightly packed targets) depending on the application requirements; however, compatibility with specimen regeneration conditions must be ensured, as poorly processed specimens typically exhibit substantial target degradation in just a few cycles (Fig. 5b).

Overall, the M3P procedure consists of straightforward steps that do not require specialized technical skills or expertise in nanoparticle engineering. As a result, it can be readily employed by a wide range of biomedical laboratories for multiplexed staining and single-cell analysis of adherent cell cultures. Further optimization should make this procedure suitable for examination of other biological specimens (e.g., clinical tissue sections).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

1. de Souza N. Single-cell methods. *Nat. Meth.* 2012; 9:35–35.
2. Chaurand P, Sanders ME, Jensen RA, Caprioli RM. Proteomics in diagnostic pathology: profiling and imaging proteins directly in tissue sections. *Am J Pathol.* 2004; 165:1057–1068. [PubMed: 15466373]
3. Liotta L, Petricoin E. Molecular profiling of human cancer. *Nat Rev Genet.* 2000; 1:48–56. [PubMed: 11262874]
4. Gorg A, Weiss W, Dunn MJ. Current two-dimensional electrophoresis technology for proteomics. *Proteomics.* 2004; 4:3665–3685. [PubMed: 15543535]
5. Aebersold R, Mann M. Mass spectrometry-based proteomics. *Nature.* 2003; 422:198–207. [PubMed: 12634793]

6. Schwamborn K, Caprioli RM. Molecular imaging by mass spectrometry--looking beyond classical histology. *Nat. Rev. Cancer*. 2010; 10:639–646. [PubMed: 20720571]
7. Wollscheid B, et al. Mass-spectrometric identification and relative quantification of N-linked cell surface glycoproteins. *Nat. Biotechnol*. 2009; 27:378–386. [PubMed: 19349973]
8. Caldwell RL, Caprioli RM. Tissue profiling by mass spectrometry - A review of methodology and applications. *Molecular & Cellular Proteomics*. 2005; 4:394–401. [PubMed: 15677390]
9. Chattopadhyay PK, et al. Quantum dot semiconductor nanocrystals for immunophenotyping by polychromatic flow cytometry. *Nat. Med*. 2006; 12:972–977. [PubMed: 16862156]
10. Bendall SC, et al. Single-cell mass cytometry of differential immune and drug responses across a human hematopoietic continuum. *Science*. 2011; 332:687–696. [PubMed: 21551058]
11. Ma C, et al. A clinical microchip for evaluation of single immune cells reveals high functional heterogeneity in phenotypically similar T cells. *Nat. Med*. 2011; 17:738–743. [PubMed: 21602800]
12. Salehi-Reyhani A, et al. A first step towards practical single cell proteomics: a microfluidic antibody capture chip with TIRF detection. *Lab. Chip*. 2011; 11:1256–1261. [PubMed: 21347466]
13. Umemura S, Osamura RY. Utility of immunohistochemistry in breast cancer practice. *Breast Cancer*. 2004; 11:334–338. [PubMed: 15604987]
14. True LD. Quantitative immunohistochemistry: a new tool for surgical pathology? *Am J Clin Pathol*. 1988; 90:324–325. [PubMed: 3046322]
15. Micheva KD, Busse B, Weiler NC, O'Rourke N, Smith SJ. Single-synapse analysis of a diverse synapse population: proteomic imaging methods and markers. *Neuron*. 2010; 68:639–653. [PubMed: 21092855]
16. Schubert W, et al. Analyzing proteome topology and function by automated multidimensional fluorescence microscopy. *Nat. Biotechnol*. 2006; 24:1270–1278. [PubMed: 17013374]
17. Medintz IL, Mattoussi H. Quantum dot-based resonance energy transfer and its growing application in biology. *Phys. Chem. Chem. Phys*. 2009; 11:17–45. [PubMed: 19081907]
18. Medintz IL, Mattoussi H, Clapp AR. Potential clinical applications of quantum dots. *Int. J. Nanomedicine*. 2008; 3:151–167. [PubMed: 18686776]
19. Misra RD. Quantum dots for tumor-targeted drug delivery and cell imaging. *Nanomed*. 2008; 3:271–274.
20. Resch-Genger U, Grabolle M, Cavaliere-Jaricot S, Nitschke R, Nann T. Quantum dots versus organic dyes as fluorescent labels. *Nat. Methods*. 2008; 5:763–775. [PubMed: 18756197]
21. Smith AM, Duan H, Mohs AM, Nie S. Bioconjugated quantum dots for in vivo molecular and cellular imaging. *Adv Drug Deliv Rev*. 2008; 60:1226–1240. [PubMed: 18495291]
22. Tholouli E, et al. Quantum dots light up pathology. *J. Pathol*. 2008; 216:275–285. [PubMed: 18814189]
23. Xing Y, Rao J. Quantum dot bioconjugates for in vitro diagnostics & in vivo imaging. *Cancer Biomark*. 2008; 4:307–319. [PubMed: 19126959]
24. Zrazhevskiy P, Gao X. Multifunctional quantum dots for personalized medicine. *Nano Today*. 2009; 4:414–428. [PubMed: 20161004]
25. Zrazhevskiy P, Sena M, Gao XH. Designing multifunctional quantum dots for bioimaging, detection, and drug delivery. *Chem. Soc. Rev*. 2010; 39:4326–4354. [PubMed: 20697629]
26. Yezhelyev MV, et al. In situ molecular profiling of breast cancer biomarkers with multicolor quantum dots. *Adv. Mater*. 2007; 19:3146–3151.
27. Matsuno A, Itoh J, Takekoshi S, Nagashima T, Osamura RY. Three-dimensional imaging of the intracellular localization of growth hormone and prolactin and their mRNA using nanocrystal (Quantum dot) and confocal laser scanning microscopy techniques. *J Histochem Cytochem*. 2005; 53:833–838. [PubMed: 15995141]
28. Shi C, et al. Quantum dots-based multiplexed immunohistochemistry of protein expression in human prostate cancer cells. *Eur. J. Histochem*. 2008; 52:127–134. [PubMed: 18591159]
29. Liu J, Lau SK, Varma VA, Kairdolf BA, Nie S. Multiplexed detection and characterization of rare tumor cells in Hodgkin's lymphoma with multicolor quantum dots. *Anal. Chem*. 2010; 82:6237–6243. [PubMed: 20565106]

30. Liu J, et al. Molecular mapping of tumor heterogeneity on clinical tissue specimens with multiplexed quantum dots. *ACS Nano*. 2010; 4:2755–2765. [PubMed: 20377268]
31. Chen C, et al. Quantum-dot-based immunofluorescent imaging of HER2 and ER provides new insights into breast cancer heterogeneity. *Nanotechnology*. 2010; 21:095101. [PubMed: 20110584]
32. Sweeney E, et al. Quantitative multiplexed quantum dot immunohistochemistry. *Biochem. Biophys. Res. Commun.* 2008; 374:181–186. [PubMed: 18621021]
33. Fountaine TJ, Wincovitch SM, Geho DH, Garfield SH, Pittaluga S. Multispectral imaging of clinically relevant cellular targets in tonsil and lymphoid tissue using semiconductor quantum dots. *Mod. Pathol.* 2006; 19:1181–1191. [PubMed: 16778828]
34. Huang DH, et al. Comparison and Optimization of Multiplexed Quantum Dot-Based Immunohistofluorescence. *Nano Research*. 2010; 3:61–68.
35. Xing Y, et al. Bioconjugated quantum dots for multiplexed and quantitative immunohistochemistry. *Nat. Protoc.* 2007; 2:1152–1165. [PubMed: 17546006]
36. Zrazhevskiy P, Gao X. Quantum dot imaging platform for single-cell molecular profiling. *Nat Commun.* 2013; 4:1619. [PubMed: 23511483]
37. Denysenko T, et al. Glioblastoma cancer stem cells: heterogeneity, microenvironment and related therapeutic strategies. *Cell Biochem. Funct.* 2010; 28:343–351. [PubMed: 20535838]
38. Sachs K, Perez O, Pe'er D, Lauffenburger DA, Nolan GP. Causal protein-signaling networks derived from multiparameter single-cell data. *Science*. 2005; 308:523–529.
39. Resch-Genger U, Grabolle M, Cavaliere-Jaricot S, Nitschke R, Nann T. Quantum dots versus organic dyes as fluorescent labels. *Nat. Meth.* 2008; 5:763–775.
40. Ciftlik AT, Lehr H-A, Gijs MAM. Microfluidic processor allows rapid HER2 immunohistochemistry of breast carcinomas and significantly reduces ambiguous (2+) read-outs. *Proc. Natl. Acad. Sci. U. S. A.* 2013; 110:5363–5368. [PubMed: 23479638]
41. Chang KH, et al. Novel 16-minute technique for evaluating melanoma resection margins during Mohs surgery. *J. Am. Acad. Dermatol.* 2011; 64:107–112. [PubMed: 21167405]
42. Goldman ER, et al. Avidin: a natural bridge for quantum dot-antibody conjugates. *J. Am. Chem. Soc.* 2002; 124:6378–6382. [PubMed: 12033868]
43. Jaiswal JK, Mattoussi H, Mauro JM, Simon SM. Long-term multiple color imaging of live cells using quantum dot bioconjugates. *Nat. Biotechnol.* 2003; 21:47–51. [PubMed: 12459736]
44. Jin T, et al. Antibody-protein A conjugated quantum dots for multiplexed imaging of surface receptors in living cells. *Mol. Biosyst.* 2010; 6:2325–2331. [PubMed: 20835432]
45. Mattoussi H, et al. Self-assembly of CdSe-ZnS quantum dot bioconjugates using an engineered recombinant protein. *J. Am. Chem. Soc.* 2000; 122:12142–12150.
46. Levenson RM. Spectral Imaging and Pathology: Seeing More. *Lab Medicine*. 2004; 35:244–251.
47. True LD, Gao X. Quantum dots for molecular pathology: their time has arrived. *J. Mol. Diagn.* 2007; 9:7–11. [PubMed: 17251330]
48. Byers RJ, et al. Semiautomated multiplexed quantum dot-based in situ hybridization and spectral deconvolution. *J. Mol. Diagnostics*. 2007; 9:20–29.
49. Ghazani AA, et al. High throughput quantification of protein expression of cancer antigens in tissue microarray using quantum dot nanocrystals. *Nano Lett.* 2006; 6:2881–2886. [PubMed: 17163724]
50. Toth ZE, Mezey E. Simultaneous visualization of multiple antigens with tyramide signal amplification using antibodies from the same species. *J. Histochem. Cytochem.* 2007; 55:545–554. [PubMed: 17242468]
51. Glass G, Papin JA, Mandell JW. SIMPLE: a sequential immunoperoxidase labeling and erasing method. *J. Histochem. Cytochem.* 2009; 57:899–905. [PubMed: 19365090]
52. Pirici D, et al. Antibody elution method for multiple immunohistochemistry on primary antibodies raised in the same species and of the same subtype. *J. Histochem. Cytochem.* 2009; 57:567–575. [PubMed: 19223296]
53. Wahlby C, Erlandsson F, Bengtsson E, Zetterberg A. Sequential immunofluorescence staining and image analysis for detection of large numbers of antigens in individual cell nuclei. *Cytometry*. 2002; 47:32–41. [PubMed: 11774347]

Box 1: Conventional immunofluorescence with secondary QDot probes**TIMING** 3.5–4.5 h**Additional materials**

- Biotinylated secondary antibodies matching the species origin of primary antibodies, 0.5 mg/mL stock concentration
- Qdot 565 goat F(ab')₂ anti-rabbit IgG conjugate (H+L), 200 µL, 1 µM (Invitrogen, cat. no. Q11431MP)
- Qdot 565 goat F(ab')₂ anti-mouse IgG conjugate (H+L), 200 µL, 1 µM (Invitrogen, cat. no. Q11031MP)
- Qdot 565 streptavidin conjugate, 200 µL, 1 µM (Invitrogen, cat. no. Q10131MP)
- Endogenous biotin blocking kit (Invitrogen, cat. no. E-21390)

Perform 1-color 2-step (primary antibody → QDot-secondary antibody) or 3-step (primary antibody → biotinylated secondary antibody → QDot-Streptavidin) staining in separate wells for each target tested. As a control for secondary QDot probes, skip incubation with primary antibodies.

- 1 Equilibrate a 24-well plate containing fixed cells to room temperature. To stain 1 well prepare: 500 µL Blocking buffer, 600 µL (for a 2-step staining) or 900 µL (for a 3-step staining) Staining buffer, 2 mL Washing buffer, and 1x TBS (see Reagent Setup). Specimen regeneration requires 1.5 mL Regeneration buffer and additional 1 mL Blocking buffer.
- 2 Block cells with Blocking buffer. This step can be done using option A or option B depending on which effect of specimen regeneration needs to be tested:
 - A. Effect of cell processing without regeneration is tested
 - i. Aspirate storage solution from the well and rinse cells with 1 mL/well fresh 1x TBS. Replace TBS with 500 µL/well Blocking buffer and incubate for 30 min at room temperature. **CRITICAL STEP** If the specimen exhibits high levels of endogenous biotin, it must be blocked (e.g., by Endogenous biotin blocking kit) prior to incubation with Blocking buffer to avoid off-target staining by QDot-Streptavidin probes.
 - B. Combined effect of cell processing and regeneration is tested
 - i. Aspirate storage solution from the well and rinse cells with 1 mL/well fresh 1x TBS. Aspirate TBS and wash cells for 5 min 3 times with 500 µL/well Regeneration buffer. Aspirate Regeneration buffer, rinse twice with

1 mL/well 1x TBS and wash twice for 5 min with 500 μ L/well Blocking buffer.

- ii. Replace with fresh 500 μ L/well Blocking buffer and incubate for 30 min at room temperature. **CRITICAL STEP** If the specimen exhibits high levels of endogenous biotin, it must be blocked (e.g., by Endogenous biotin blocking kit) prior to incubation with Blocking buffer to avoid off-target staining by QDot-Streptavidin probes.

- 3 Incubating cells with target-specific primary antibodies. Combine 300 μ L Staining buffer and 1.5 μ L 0.2 mg/mL primary IgG in a 1.7-mL microcentrifuge tube. Vortex briefly. Aspirate Blocking buffer from the well and replace it with 300 μ L/well IgG in Staining buffer. Incubate at room temperature for 1 h. Wash for 5 min 3 times with 1 mL/well 1x TBS.
- 4 Labeling primary antibodies with secondary QDot probes. This step can be done using option A or option B depending on availability of secondary QDot probes:
 - A. If species-matched QDot-secondary antibody bioconjugates are available (2-step staining):
 - i. In a 1.7-mL microcentrifuge tube, combine 300 μ L Staining buffer and 1 μ L 1 μ M secondary QDot probe matching the species of the primary IgG used in Step 3 (e.g., use Qdot 565 goat F(ab')₂ anti-rabbit IgG conjugate (H+L) to detect primary IgG raised in rabbit). Vortex briefly. Aspirate TBS from the well and replace it with 300 μ L/well QDot in Staining buffer. Incubate at room temperature for 1 h.
 - B. If species-matched QDot-secondary antibody bioconjugates are not available (3-step staining):
 - i. In a 1.7-mL microcentrifuge tube, combine 300 μ L Staining buffer and 1 μ L 0.5 mg/mL biotinylated secondary antibodies matching the species of the primary IgG used in Step 3. Vortex briefly. Aspirate TBS from the well and replace it with 300 μ L/well secondary antibody in Staining buffer. Incubate at room temperature for 1 h.
 - ii. Wash cells for 5 min 3 times with 1 mL/well 1x TBS.
 - iii. In a 1.7-mL microcentrifuge tube, combine 300 μ L Staining buffer and 1 μ L 1 μ M Qdot streptavidin probes. Vortex briefly. Aspirate TBS from the well

and replace it with 300 μ L/well QDots in Staining buffer. Incubate at room temperature for 1 h.

CRITICAL STEP Do not add detergents (e.g., Tween-20, SDS, Triton X-100) to Staining buffer, as it might lead to increased non-specific staining by QDots.

- 5** Wash cells with Washing buffer and TBS. Aspirate QDot solution, briefly rinse cells with 1 mL/well Washing buffer, and wash with 1 mL/well Washing buffer for 10 min. Then wash for 5 min 3 times with 1 mL/well 1x TBS. Keep cells immersed in 1x TBS for imaging.
- 6** Proceed with regular fluorescence microscopy to evaluate staining intensity and specificity (see Equipment Setup for exemplar microscope setup).

Box 2: Determination of QDot differential brightness from cell staining**TIMING** 3.5 h plus image acquisition and analysis

Accuracy of differential QDot brightness measurement from bulk solutions depends on preparation of samples with precisely known QDot concentrations. When such information is not available, differential brightness can be determined directly from cell staining with QDot-SpA probes.

CRITICAL Perform 1-color 2-step staining in separate wells for each QDot-SpA color. Choose a molecular target that is uniformly and abundantly expressed throughout all cells in cell culture for this procedure (e.g., heat shock protein 90, HSP90, in HeLa cells). Include control for each QDot-SpA color by skipping incubation with primary antibodies.

CRITICAL Only whole IgG antibodies that exhibit strong binding between Fc region and SpA can be used.

1. Equilibrate 24-well plate containing fixed cells to room temperature. For each QDot-SpA color prepare: 1 mL Blocking buffer, 1.2 mL Staining buffer, 4 mL Washing buffer, and 1x TBS (see Reagent Setup). **CRITICAL STEP** Use 2 wells for each QDot-SpA color: one for “Positive” staining and one as control.
2. Block cells with Blocking buffer. Aspirate storage solution from the well and rinse cells with 1 mL/well fresh 1x TBS. Then replace TBS with 500 µL/well Blocking buffer and incubate for 30 min at room temperature.
3. Incubation with target-specific primary antibodies. For each “Positive” well, combine 300 µL Staining buffer and 1.5 µL 0.2 mg/mL primary IgG in a 1.7-mL microcentrifuge tube. Vortex briefly. Aspirate Blocking buffer from the well and replace it with 300 µL/well IgG in Staining buffer. For control wells, replace Blocking buffer with 300 µL/well Staining buffer without IgG. Incubate at room temperature for 1 h. Wash for 5 min 3 times with 1 mL/well 1x TBS.
4. Incubation with QDot-SpA probes. For each QDot-SpA color, combine 600 µL Staining buffer and 12 µL ~1 µM QDot-SpA in a separate 1.7-mL microcentrifuge tube. Approximate QDot concentrations can be successfully used with this procedure. Aspirate TBS from the wells and transfer 300 µL QDot-SpA in Staining buffer to a “Positive” well and another 300 µL QDot-SpA to a control well. Incubate at room temperature for 1 h. **CRITICAL STEP** Do not add detergents (e.g., Tween-20, SDS, Triton X-100) to Staining buffer, as it might lead to increased non-specific staining by QDots.
5. Wash cells with Washing buffer and TBS. Aspirate the QDot-SpA solution, briefly rinse cells with 1 mL/well Washing buffer, and wash with 1 mL/well Washing buffer for 10 min. Then wash for 5 min 3 times with 1 mL/well 1x TBS. Keep cells immersed in 1x TBS for imaging.
6. Recording of hyperspectral images of each well. Follow general guidelines outlined in Steps 36–38 of the main PROCEDURE. Use low-magnification 20x

dry objective for image collection. Record images from at least 3 different areas on each well.

7. Perform quantitative analysis of staining intensity. Follow general guidelines outlined in Steps 39, 40, and 43 of the PROCEDURE. Use only one QDot reference spectrum that matches QDot-SpA color tested for image unmixing (include autofluorescence reference spectrum, if applicable). Repeat this analysis for all images recorded in Step 6, thus obtaining at least 6 measurements (3 from “Positive” sample and 3 from controls) for each QDot-SpA color.
8. Calculate the differential QDot brightness. For each QDot-SpA color calculate an average of 3 “Positive” measurements and an average of 3 control measurements, then subtract control from “Positive”, obtaining an average specific staining intensity. Normalize average specific staining intensities by a suitable QDot color to create a list of correction factors. Typically, QDot fluorescence intensity increases from green to red emission color.

Figure 1.

Workflow of the multicolor multicycle molecular profiling (M3P) technology. The procedure consists of (i) preparation of specimens for analysis, (ii) preparation of universal QDots, and (iii) multicolor multicycle staining, imaging, and image analysis.

Figure 2.

Multiplexed 1-step staining of clinical tissue specimens with self-assembled QDot-SpA-Antibody probes. Incubation of formalin-fixed paraffin-embedded prostate tissue sections with a cocktail of 3 QDot probes targeting Histone H3, β -Tubulin, and Ki-67 produces characteristic staining patterns in individual QDot channels (**a**) and yields a 3-color image (**d**, with autofluorescence false-colored in cyan). In a control experiment, same probes lacking primary antibodies produce nearly no non-specific staining (**b**). Notably, staining patterns observed with a 1-step multiplexed staining methodology are completely consistent with those obtained with a conventional single-color 2-step procedure performed on separate sequential tissue sections with commercial QDot-secondary antibody bioconjugates (**c**, shown in grayscale). Scale bar, 250 μ m.

Figure 3.

Purification of activated QDots with NAP-5 column. Column is equilibrated with 1x PBS at room temperature. After PBS completely enters the column (a), activated QDots are carefully loaded onto the top frit (b and c). Handheld UV lamp is used to trace QDot position inside the column (d). About 500 μ L QDot solution is collected as it elutes from the column (e), then transferred to centrifugal concentrator (f) and concentrated down to $\sim$ 20 μ L (g). Concentrated QDots are immediately mixed with excess SpA for bioconjugation.

Figure 4.

Single-cell molecular profiling with the M3P technology. Simultaneous QDot labeling of at least 5 molecular targets can be achieved in a straightforward, 1-step procedure (model targets Ki-67, HSP90, Lamin A, Cox-4, and β-Tubulin are shown here). HSI and spectral unmixing are performed to isolate individual QDot channels and perform quantitative analysis of staining intensity. The total abundance of each molecular target is measured for 2 exemplar cells, showing heterogeneity in molecular composition (note, fluorescence intensities have been adjusted according to correction factors determined through Steps 27–30 of the PROCEDURE). High-magnification imaging shows intracellular localization of each molecular target with great detail. Images are false-colored for clarity. Scale bar, 50μm.

a *Properly processed cells*

b *Poorly fixed cells*

Figure 5.

Antigen preservation via proper pre-staining specimen processing. Prostate cancer (LNCaP) cells were treated for 1–10 (**C1–C10**) regeneration cycles, after which androgen receptor was stained. Properly processed cells (**a**) yield uniform staining intensity and specificity irrespective of the number of regeneration cycles performed, thus being amenable to an extensive re-probing via multicycle staining. In contrast, substantial target degradation (as indicated by a drop in staining intensity) is observed with poorly fixed cells (**b**), indicating the need for a more thorough fixation. Images are normalized by intensity and false-colored with a heat-map for clarity. Scale bar, 500 μ m.

Table 1

Comparison of analytical capacities of single-cell molecular profiling technologies

Method	Parallel multiplexing	Quantitative analysis	Sub-cellular resolution	Specimen morphology
Microfluidic assay	up to 12	Moderate	No	No
FL Flowcytometry	up to 17	High	No	No
Mass-cytometry	up to 34	High	No	No
IF / IHC microscopy	1–4	Low	Yes	Yes
QDot technology	up to 10	High	Yes	Yes

Table 2

Comparison of QDot-based staining methodologies

	Target detection	Multiplexing capacity	Method complexity/cost	1' Antibody modification	Methodology flexibility
QDot-2'Ab	via 1'/2' Ab bond	Low	Low / \$	None	Moderate
Covalent QDot-1'Ab	direct	High	High / \$\$\$	Moderate	Low
Self-assembled QDot-1'Ab	direct	High	Low / \$	None	High

Table 3

Troubleshooting table

Step	Problem	Possible reason	Solution
5	Cells do not attach and/or grow in a glass-bottom 24-well plate	Poor cell adherence to glass surface	Treat glass surface with cell-compatible coating (e.g., poly-L-lysine). Avoid using coatings with high autofluorescence (e.g., collagen)
18 & 19	QDots appear in the wash-through fraction after concentration	Damaged, “leaky” concentrator used	Use a new centrifugal concentrator. Avoid mechanical damage to the membrane by the pipet tip. Only use compatible solvents (check manufacturer’s instructions)
		Excessively high centrifugal force used	Use recommended centrifugal force and duration. Check manufacturer’s instructions for maximum centrifugal force permitted
	QDots aggregate onto the filter membrane	QDot scaffolds have poor (or degraded) PEG coverage	Use new stock of PEG-coated QDots for preparation of QDot-SpA bioconjugates
		Poor QDot stability under reaction conditions	Use recommended buffers, reagents, and reaction conditions. Avoid vortexing and heating during reaction
20	Low QDot yield (final QDot concentration below 1 μM)	Degraded/aggregated SpA is used	Use fresh stock of SpA in PBS. Use recommended SpA storage and reaction conditions.
		QDot loss during NAP-5 column purification	Load no more than 100 μL QDot solution. Use handheld UV lamp to accurately collect eluted QDots
		QDot loss during purification with centrifugal filter	Ensure proper retention of QDots in the top part of the centrifugal filter. Do not damage the filter. Use suggested centrifugation parameters
25	QDot spectra have more than 1 peak	QDot solutions are cross-contaminated	Prepare new reference solutions each composed of a single, pure QDot color
	QDot spectra have high tails	More than one reference spot is imaged at once	Place reference spots farther apart. Ensure that only 1 spot is illuminated during imaging
		Low QDot signal is recorded (high exposure time used)	Use recommended QDot concentration (100–500 nM). Increase excitation light intensity, if possible. Place objective closer to the cover slide
37	No staining is observed	No SpA on QDot probes	Repeat QDot-SpA bioconjugation. Use at least 1 μM [QDot] during reaction. Avoid buffers containing primary amines and contaminating proteins
		No target binding by QDot probes (target is degraded or not accessible)	Check for proper target staining with secondary QDot probes (see Box 1). Adjust cell processing conditions to achieve proper staining (see Experimental Design section for cell processing strategies)
	High non-specific staining is observed	Old blocking buffer is used	Prepare fresh buffers before each staining procedure
		High QDot concentration is used	Reduce final QDot concentration to below 20 nM in Staining buffer
40	Cross-talk between QDots is observed	QDot scaffolds have poor (or degraded) PEG coverage	Use new stock of PEG-coated QDots for preparation of QDot-SpA bioconjugates
		Reference QDot spectral library is inaccurate	Record reference spectral library for each new QDot-SpA stock. Avoid over-exposure of HSI camera to collect

Step	Problem	Possible reason	Solution
			accurate spectra
	Some QDot channels are very noisy	Excess IgG is used during QDot-IgG probe assembly	Use at least 3x molar excess of QDot-SpA over IgG during probe assembly
		Combination of very high and very low signals is analyzed	Avoid parallel staining of molecular targets with >10x difference in abundance. Match high-abundance targets with low-intensity QDots
		Lack of staining with particular QDot probe	Noise might appear in channels with negative staining due to unmixing artifacts. Set proper threshold level during data analysis to subtract noise