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An Optimized Single-Molecule Pull-Down Assay for Quantification of Protein Phosphorylation

Elizabeth M. Bailey
Department of Pathology, University of New Mexico School of Medicine

Emanuel Salazar-Cavazos
Department of Pathology, University of New Mexico School of Medicine

Rachel M. Grattan
Department of Pathology, University of New Mexico School of Medicine

Michael J. Wester
Department of Physics and Astronomy, University of New Mexico

David J. Schodt
Department of Physics and Astronomy, University of New Mexico

See next page for additional authors

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Quantification of protein phosphorylation using single molecule pull-down

Elizabeth M. Bailey[1], **Emanuel Salazar-Cavazos**[1], **Rachel M. Grattan**[1], **Michael J. Wester**[2,3], **David J. Schodt**[2], **Julian A. Rojo**[1], **Keith A. Lidke**[2,3], **Diane S. Lidke**[1,3,*]

[1]Department of Pathology, University of New Mexico School of Medicine, Albuquerque, New Mexico, 87131, USA

[2]Department of Physics and Astronomy, University of New Mexico, Albuquerque, NM, USA

[3]Comprehensive Cancer Center, University of New Mexico Health Sciences Center, Albuquerque, NM 87131, USA

Abstract

Phosphorylation is a critical post-translational modification that regulates protein function and directs cell signaling outcomes. Current methods to measure protein phosphorylation lack the ability to preserve the heterogeneity in phosphorylation across individual proteins. The single molecule pull-down (SiMPull) assay was developed to investigate the composition of macromolecular complexes via immunoprecipitation of proteins on a glass coverslip followed by single molecule imaging. Herein is an adaptation of SiMPull that provides robust quantification of the phosphorylation state of full-length membrane receptors at the single molecule level. Imaging of thousands of individual receptors in this way allows for quantification of protein phosphorylation patterns. The following protocol includes details on the optimized SiMPull procedure, from sample preparation to imaging and data analysis. While this work focuses on phosphorylation of the epidermal growth factor receptor (EGFR), the protocol is generalizable to other membrane receptors as well as cytosolic signaling molecules.

SUMMARY:

A protocol detailing SiMPull sample preparation and data analysis for quantification of protein phosphorylation.

INTRODUCTION:

Membrane associated signaling is tuned by a combination of ligand-induced membrane receptor activation and recruitment of downstream accessory proteins that propagate

*Correspondence: dlidke@salud.unm.edu.
ESC Current address:
Immunodynamics Group, Laboratory of Integrative Cancer Immunology, Center for Cancer Research, National Cancer Institute, Bethesda MD USA

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Nothing to disclose.

the signal. Phosphorylation of key tyrosines in receptor cytoplasmic tails is critical to initiating formation of signaling complexes, or signalosomes. Therefore, an important question in biology is how phosphorylation patterns are created and maintained in order to recruit signaling partners and dictate cellular outcomes. This includes understanding the heterogeneity of receptor phosphorylation, both in abundance and in the specific phosphotyrosine patterns that can provide a means of manipulating signaling outputs by dictating the composition of the signalosome[1-5]. However, there are limitations in current methods to interrogate protein phosphorylation. Western blot analysis is excellent for describing trends of protein phosphorylation but does not provide information on the heterogeneity of the system because thousand to millions of receptors are averaged together. While western blots do allow for probing a sample using phospho-specific antibodies to distinct tyrosines, they cannot provide information on multisite phosphorylation patterns within the same protein. Quantitative phosphoproteomics report on phosphotyrosine abundance, but there are limitations to detection of multisite phosphorylation, as the residues of interest need to be located within the same peptide (typically 7-35 amino acids) that is generated by enzymatic digestion[6-8].

To overcome these limitations, the single molecule pull-down (SiMPull) assay has been adapted to quantify the phosphorylation states of intact receptors at the single molecule level. SiMPull was first demonstrated as a powerful tool for interrogating macromolecular complexes by *Jain et al*[9,10]. In SiMPull, macromolecular complexes were immunoprecipitated (IP) on antibody-functionalized glass coverslips and then analyzed through single molecule microscopy for protein subunit number and co-IP with complex components[9]. A modification by *Kim et al.*, termed SiMBlot, was the first to use a variation of SiMPull to analyze phosphorylation of denatured proteins[11]. The SiMBlot protocol relies on capturing biotinylated cell surface proteins using NeutrAvidin-coated coverslips, which are then probed for phosphorylation with phospho-specific antibody labeling[11]. Despite these advances, improvements were needed to make the quantification of post-translational modification more robust and applicable to a broader range of proteins.

Here, we present an optimized SiMPull approach to quantify phosphorylation patterns of intact epidermal growth factor receptor (EGFR) in response to a range of ligand conditions and oncogenic mutations[12]. While this work focuses on EGFR, this approach can be applied to any membrane receptor, as well as cytosolic proteins of interest (POI), for which quality antibodies are available. The protocol includes steps to reduce sample autofluorescence, a sample array design that requires minimal sample volume with simultaneous preparation of up to 20 samples, and optimization of antibody labeling and fixation conditions. Additionally, data analysis algorithms have been developed for single molecule detection and quantification of phosphorylated proteins.

PROTOCOL:

1. Coverslip Preparation.

CAUTION: Required personal protective equipment (PPE): double layer of nitrile gloves; safety glasses or face shield; lab coat.

1.1. Piranha etch the glass to remove organic debris.—CAUTION: Piranha solution is a strong oxidizing agent that is corrosive and highly reactive when in contact with organic materials. Reaction with organic debris is exothermic and potentially explosive. The procedure must be done in a chemical fume hood with the sash lowered. Pyrex glassware is required to handle piranha solution.

1.1.1. Prepare the workspace in a chemical fume hood. Arrange coverslips without overlap in the bottom of a 4 L glass "reaction" beaker, and place the reaction beaker on a hotplate with gentle heat. Allow the glassware to warm for 10 min. Place a "waste" 1 L glass beaker with 500 mL ddH_2O proximal to the reaction beaker.

1.1.2. Add 49 mL of 12 N sulfuric acid (H_2SO_4) slowly to the reaction beaker with a glass serological pipette. Rinse the pipette in the waste beaker before disposal.

1.1.3. Add 21 mL of 30% hydrogen peroxide (H_2O_2) dropwise with a glass serological pipette to the reaction beaker. Distribute the H_2O_2 droplets evenly across the bottom of the reaction flask at a slow rate to prevent localized quenching of the piranha etching reaction. Rinse the pipette in the waste beaker before disposal.

CAUTION: Always add H_2O_2 into the H_2SO_4, and never vice-versa.

1.1.4. Piranha etch the coverslips for 30 min. Gently agitate the contents of the reaction beaker every 5 min.

1.1.5. Quench the piranha solution by pouring the contents of the waste beaker into the reaction beaker. Transfer the liquid slowly down the wall of the reaction beaker to minimize splashing. Remove the reaction beaker from the hotplate.

1.1.6. When the reaction is quenched and cooled, pour the piranha solution back into the waste beaker for neutralization without removing the etched coverslips from the reaction beaker.

1.1.7. Neutralize the piranha solution with the gradual addition of a weak base. For example, use an excessive mass of 20 g sodium bicarbonate ($NaHCO_3$)/ 100 mL piranha solution.

CAUTION: Do not store piranha solution in sealed waste containers. The solution must always be neutralized before disposal. The neutralization reaction produces vigorous bubbles and may be explosive if not controlled by the gradual addition of the weak base.

1.1.8. Stir the neutralized solution with a glass stir rod and let it react for 2 h. Raise the pH to >4 and dispose of the solution.

1.1.9. Transfer the etched coverslips from the reaction beaker into a Buchner funnel with a glass stir rod and rinse for 5 min in running ddH_2O.

CHOICE: Proceed immediately to the next step or store the piranha etched coverslips for up to 2 weeks in ddH_2O in a sealed glass jar or petri dish (wrap with sealing film).

1.2. Bath-sonicate the coverslips in organic solvents.—1.2.1. Place the coverslips in a glass Coplin jar and cover with methanol (CH_3OH). Seal the lid to the jar with sealing film and bath-sonicate for 10 min. Carefully pour the CH_3OH out of the Coplin jar into a glass storage bottle.

1.2.2. Fill the Coplin jar with acetone (C_3H_6O), seal the lid, and bath sonicate for 10 min. Carefully pour the C_3H_6O out of the Coplin jar into a glass storage bottle.

CAUTION: Methanol is flammable and acutely toxic. Use in a chemical fume hood. Acetone is flammable and an irritant. Handle and store with glass. Use in a chemical fume hood. Dispose as hazardous waste according to local and state regulation.

NOTE: CH_3OH can be reused up to 5 times. C_3H_6O can be reused up to 5 times.

1.3. Activate the coverslip surface for silane functionalization.—1.3.1. Bath sonicate with 1 M potassium hydroxide (KOH) for 20 min. Carefully pour the KOH out of the Coplin Jar into a 50 mL conical tube for reuse.

CAUTION: KOH is corrosive and an irritant. Use in a chemical fume hood. Do not store in glass. Store in polypropylene tubes. Dispose as hazardous waste according to local and state regulation.

NOTE: KOH can be reused up to 5 times.

1.3.2. Rinse twice with ddH_2O. Drain ddH_2O from the coverslips, and then heat each coverslip by waving through the flame of a Bunsen burner to drive off all surface moisture. Place the coverslips in a dry Coplin jar.

1.4. Coverslip Aminosilanization—CAUTION: Acetic acid (CH_3COOH) is flammable and corrosive. Handle and store with glass. Use in a chemical fume hood. Dispose as hazardous waste according to local and state regulation.

CAUTION: N-(2-aminoethyl)-3-aminopropyltrimethoxysilane (aminosilane) is an acutely toxic inhalation hazard, a sensitizer, and an irritant. It is harmful to aquatic life. Use in a chemical fume hood. Dispose as hazardous waste according to local and state regulation.

NOTE: Aminosilane is photosensitive and hydrolyses rapidly in water. All steps with this reagent should be performed under minimal light conditions to retain activity. Purge the bottle with nitrogen gas and apply sealing film prior to storage in a dark desiccator. Replace every 6-9 months.

1.4.1. Prepare the aminosilane solution. Mix 69.4 mL of CH_3OH with 3.6 mL of CH_3COOH in a conical flask. Add 720 μL of aminosilane and mix well.

1.4.2. Immediately add the aminosilane solution to the Coplin jar. Cover and apply sealing film, continuing to protect from light.

1.4.3. Incubate the coverslips in the aminosilane solution for 10 min in the dark at room temperature (RT). Bath sonicate for 1 min, and then incubate for another 10 min. Carefully pour the aminosilane solution off into a waste container designated for CH_3OH with trace aminosilane and CH_3COOH.

1.4.4. Rinse the coverslips with CH_3OH and pour off into a waste container designated for CH_3OH.

1.4.5. Rinse the coverslips 3 times for 2 min each with ddH_2O. Drain the coverslips, dab away excess moisture and air dry completely for 10 min.

1.5. Array Preparation/Biotin-PEG Functionalization of Coverslips—1.5.1. Make 1 M $NaHCO_3$ pH 8.5 working stock: dissolve 84.5 mg of $NaHCO_3$ into 1 mL ddH20. For final concentration of 10 mM $NaHCO_3$, dilute 1 M $NaHCO_3$ 1:100 in ddH20.

1.5.2. Draw a grid array on the dry aminosilanized coverslips with a hydrophobic barrier pen and wait for the ink to dry. Write an identifier on the coverslip to mark the proper orientation. The array should consist of 16-20 squares approximately 4 x 4 mm in size. Place the coverslips in a humidified chamber.

1.5.3. To make the biotin-PEG succinimidyl valerate (biotin-PEG)/mPEG succinimidyl valerate (mPEG) solution, first remove mPEG and biotin-PEG from freezer storage and equilibrate to RT. Add 153 mg mPEG and 3.9 mg Biotin-PEG (~ 1:39 biotin-PEG:mPEG) to a 1.5 mL microcentrifuge tube, and resuspend in 609 μL of 10 mM $NaHCO_3$ by gentle pipetting. Centrifuge at 10,000 x g for 1 min at RT to remove bubbles.

CRITICAL STEP: The hydrolysis half-life of succinimidyl valerate in pH 8.5 buffer is ~30 min. After adding the buffer to mPEG, proceed with the following steps as rapidly as possible.

1.5.4. Apply the biotin-PEG/mPEG solution to completely cover each square in the coverslip arrays, typically 10-15 μL per square. Do not allow liquid to overflow the defined space. Store the coverslips in a humidity chamber in the dark for 3-4 h at RT.

1.5.5. Wash the coverslips with copious amounts of water by sequentially dipping them into 3 x 250 mL glass beakers filled with ddH_2O for 10 s each.

1.5.6. Drive off all moisture from the coverslips with nitrogen gas. Store the coverslips back-to-back in a nitrogen-filled 50 mL conical tube wrapped with sealing film at −20 °C.

CHOICE: Proceed immediately to Step 2 or store coverslips at −20 °C for up to one week before use.

2. SiMPull Lysate Preparation

NOTE: This section is described for adherent CHO cells expressing EGFR-GFP that have been plated in a 60 mm tissue culture (TC60) dish overnight[12,13]. CHO cells are cultured in DMEM supplemented with 10% fetal bovine serum, 1% L-glutamine, 1% Penicillin-

Streptomycin, and 500 ng/mL geneticin. Other adherent cell lines or suspension cells can be used with appropriate adjustments to the protocol.

CAUTION: Required PPE for the remainder of the protocol: nitrile gloves; safety glasses; lab coat.

2.1. Cell Plating.—2.1.1. Wash culture dish of cells with 1 mL PBS. Incubate for 5 min at 37 °C in 1 mL Trypsin. Gently pipette to detach cells from dish and transfer to a 1.5 mL centrifuge tube.

2.1.2. Take 10 μL of trypan blue and mix with 10 μL of cell suspension in a separate centrifuge tube. Count cells using 10 μL of cell mixture in an automatic cell counter, according to manufacturer's instructions.

2.1.3. Plate $8x10^5$ cells overnight in a TC60 petri dish. Plate one dish per condition.

2.2. Prepare solutions—2.2.1. Ice cold PBS pH 7.4.

2.2.2. Lysis Buffer: 1% IGEPAL CA-630 in 50 mM Tris pH 7.2/150 mM NaCl + Protease/Phosphatase Inhibitor (PPI) (1:100 from stock). Keep on ice.

2.2.3. Tyrode's Buffer: 135 mM NaCl, 10 mM KCl, 0.4 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES pH 7.2, 20mM Glucose, 0.1% Bovine Serum Albumin (BSA). Warm to 37 °C.

2.3. Optional: Positive Control for phosphorylation – 1 mM Pervanadate Treatment—NOTE: Pervandate is the peroxidized form of vanadate—an inhibitor of protein tyrosine phosphatases. Preventing protein dephosphorylation by inhibition of phosphatase activity results in a highly phosphorylated sample.

2.3.1. Make a 200 mM stock of activated sodium orthovanadate (Na_3VO_4). To prepare 100 mL, add 3.89 g Na_3VO_4 to 90 mL ddH_2O and dissolve while stirring. Adjust the pH to 10 by adding HCl or NaOH dropwise. Adding HCl will turn the solution yellow. Bring volume to 100 mL with ddH_20. Boil the solution by heating in the microwave. After boiling, the solution will be colorless. Cool the solution to RT and readjust pH to 10. Repeat the boiling, cooling, and pH adjustment 2-4 more times, until the pH stabilizes at 10. Aliquot and store at −20 °C.

2.3.2. Make a stock of 30 mM pervanadate (PV): mix 20.4 μL of 3% H_2O_2 with 100 μL of 200 mM Na_3VO_4, and 546.8 μL ddH_2O (equimolar concentrations of H_2O_2 and activated Na_3VO_4). Incubate in the dark at RT for 15 min.

2.3.3. Make 1 mM PV in Tyrode's buffer. For 10 mL, add 0.33 mL 30 mM PV stock to 9.67 mL of 37 °C Tyrode's buffer. Treat cells immediately.

2.3.4. Wash cells once with 3 mL Tyrode's buffer. Add 3 mL of 1 mM PV in Tyrode's buffer to the cells and incubate for 15 min at 37 °C.

2.4. Ligand stimulation—Stimulate cells with ligand of interest using appropriate concentration, time, and temperature. For maximal EGFR stimulation, incubate with 1 mL of 50 nM Epidermal Growth Factor (EGF) + 1 mM PV in Tyrode's for 5 min at 37 °C to achieve maximal phosphorylation.

2.5. Cell Lysis—2.5.1. After desired cell treatment, place the dish on ice and wash with ice cold PBS. Completely remove the full volume of PBS.

2.5.2. Add 180 μL of Lysis Buffer to the plate. Use a cell scraper to pull buffer around the plate to fully cover the cells. Apply firm, consistent pressure with the cell scraper across the entire cultured surface to fully lyse the cells.

NOTE: Volume of Lysis Buffer should be kept at a minimum to ensure a high protein concentration.

2.5.3. Pipette the lysed cells and transfer to a 1.5 mL tube. Keep the tube on ice for 30 min. Vortex lysates every 5 min.

NOTE: If the POI consists of multiple subunits or is sensitive to dissociation, do not vortex lysates.

2.5.4. Centrifuge the lysed cells at 16,000 x g for 20 min at 4 °C. Transfer the supernatant to a new 1.5 mL tube. This contains the total protein lysate.

2.5.5. Reserve 10 μL of the lysate and dilute into 90 μL of lysis buffer for Bicinchoninic Colorimetric Assay (BCA) analysis[14]. Store the remaining total protein lysate at −80 °C.

2.5.6. Determine lysate total protein concentration using BCA analysis.

CHOICE: Total protein lysates can be prepared on the day of the experiment and used fresh or stored as single use aliquots at −80 °C for up to 12 weeks. Do not freeze/thaw.

3. Functionalize the array with biotinylated antibody

3.1. Prepare Solutions—3.1.1. T50 Buffer: 10 mM Tris pH 8.0, 50 mM NaCl. Stable for 1 month at RT.

3.1.2. T50-BSA: T50 supplemented with 0.1 mg/mL BSA. Keep on ice.

3.1.3. 10 mg/mL sodium borohydride ($NaBH_4$) in PBS - Prepare immediately before use.

3.1.4. 0.2 mg/mL NeutrAvidin in T50

CAUTION: $NaBH_4$ is a reducing agent and is flammable. Always purge the container with nitrogen gas after use and store in a desiccator. Naturally degas $NaBH_4$ solutions for 1 h before disposal by leaving tube open in the chemical fume hood.

3.2. Functionalize the array with the biotinylated antibody—3.2.1. Remove the PEG-biotin functionalized arrays from the freezer and equilibrate the conical tube to RT

before opening. Place the coverslip with the array oriented up onto a sealing film lined 100 mm tissue culture (TC100) TC100 dish.

NOTE: Minimize overhead lighting. All solutions should "bead up" on the squares defined by the hydrophobic array. Add an appropriate volume of solution to completely cover each square (typically 10-15 μL) and do not allow liquid to overflow the defined space. For rapid removal of liquids, use an in-house vacuum line attached to a vacuum flask to capture waste. Allow $NaBH_4$ to degas for 1 h before disposal.

CRITICAL STEP: $NaBH_4$ treatment is necessary to reduce background autofluorescence, thereby reducing false positive single molecule detections.

3.2.2. Treat each square of the array with 10 mg/mL $NaBH_4$ in PBS for 4 min at RT. Wash 3 times with PBS.

3.2.3. Incubate each square for 5 min with 0.2 mg/mL NeutrAvidin in T50. NeutrAvidin binds to PEG-biotin and provides binding site for biotinylated antibody. Wash 3 times with T50-BSA.

3.2.4. Incubate each square for 10 min with 2 μg/mL biotinylated POI-specific antibody in T50-BSA; wash 3 times with T50-BSA. This protocol uses biotinylated anti-EGFR IgG to capture EGFR-GFP.

4. SiMPull of POI from Whole Cell Lysates

CRITICAL STEP: Place the TC100 dish of functionalized SiMPull arrays on ice for the remainder of the SiMPull preparation. This step is the pulldown of a POI from total protein lysate. It is critical that the lysate is not reused after thawing.

4.1. Prepare Solutions—4.1.1. 4% paraformaldehyde (PFA)/0.1% glutaraldehyde (GA) in PBS.

CAUTION: PFA and GA are toxic chemical fixatives and potential carcinogens. Wear PPE. Dispose as hazardous waste according to local and state regulation.

4.1.2. 10 mM Tris-HCl pH 7.4.

4.2. Thaw and mix the lysate by gently pipetting up and down. Keep on ice.

4.3. Dilute the lysate into ice cold T50-BSA/PPI.—NOTE: If needed, determine the appropriate dilution factor of the total protein lysate by applying a range of dilutions to the array. The optimal density of SiMPull receptors per array area is 0.04-0.08/μm^2. Lysate dilutions can be assessed in Step 6: Data Analysis.

4.4. Incubate the lysate on the array for 10 min; then wash 4 times with ice cold T50-BSA/PPI.

4.5. Dilute AF647-conjugated anti-phosphotyrosine antibody in ice cold T50-BSA/PPI and incubate on the array for 1 h. Here a pan anti-pTyr (PY99)–AF647 IgG is used to identify the phosphorylated population of EGFR-GFP.—NOTE:

The use of directly labeled antibodies removes the need for secondary antibodies, increasing the labeling options and improving the consistency of the results. Fluorescently-labeled antibodies can often be purchased. If not commercially available, antibodies can be custom labeled using standard bioconjugation techniques, and commercial bioconjugation kits are available. Each batch of fluorescently labeled antibodies should be tested for optimal labeling conditions by performing SiMPull to measure a dose curve and find the saturation point.

4.6. Wash 6 times with ice cold T50-BSA for a total of 6-8 min.

4.7. Wash twice with ice cold PBS.

4.8. Incubate the array with 4% PFA/0.1% GA solution for 10 min to prevent antibody dissociation.

4.9. Wash twice for 5 min each with 10 mM Tris pH 7.4/PBS to inactivate the fixatives.—NOTE: For experiments using more than one antibody, e.g., detection of multiple phosphotyrosine sites, repeat steps 4.5-4.9. See section 6.2.9 for information on determining steric hindrance between two antibodies.

5. Image Acquisition

NOTE: Single molecule image acquisition here is performed using a 150x TIRF objective and an image splitter that captures each spectral channel in a specific quadrant of the emCCD camera. Calibration images are first acquired to allow for channel registration and camera gain calibration with a nanopatterned channel alignment grid (nanogrid) that contains a 20 × 20 array of 200 ± 50 nm holes at an intra-hole distance of 3 ± 1 μm (total size ~60 × 60 μm). Images with the nanogrid are acquired using transmitted light which passes through the nanogrid and is detected in all spectral channels. Alternatively, one can use multi-fluorescent beads that emit fluorescence detected in each channel. Image acquisition will need to be optimized according to each microscope set up.

5.1. Channel Registration.—CRITICAL STEP: Accurate channel registration is needed to properly calculate colocalization of emitters.

5.1.1. Clean the oil objective and deposit a drop of oil on the objective. Place nanogrid on the stage for imaging. Using transmitted white light, focus on the grid pattern.

5.1.2. Acquire a series of 20 images of the grid. Ensure that pixels are not saturated. Save the image series as “Fiducial.”

5.1.3. Defocus the nanogrid to create airy rings. Acquire a series of 20 images for gain calibrations. Save the image as “Gain.”

5.1.4. Acquire a series of 20 images for camera offset by blocking all light from going to the camera. Save the image as “Background.”

5.2. Acquire SiMPull images.—NOTE: Before proceeding to imaging of the coverslip array, exchange the Tris solution for T50-BSA and equilibrate the array to RT.

5.2.1. Clean the oil objective and deposit additional oil on the objective. Secure the coverslip array on the microscope stage.

5.2.2. Optimize the excitation power, TIRF angle, and camera integration time for each fluorophore. The goal is to achieve the highest signal-to-noise while minimizing photobleaching of the sample. Record the laser power for consistency in future measurements. For the setup described, use 300 ms exposure time for the far-red channel and 1 s for the green channel. The 642 nm laser was used at approximately 500 μW laser power while the 488 nm laser was used at 860 μW, measured before the tube lens.

5.2.3. Acquire images for each sample. To reduce photobleaching, image the far-red channel first, followed by each lower wavelength fluorophore. Due to the low volume used for each sample, the buffer level should be checked every 30-45 min and replenished as needed.

6. Data Analysis

6.1. Demo Code—NOTE: The provided demo code and example data sets demonstrate the full data analysis work flow (Supplemental File 1-4). System requirements listed in SiMPullMain.m, found in Supplemental File 1.

6.1.1. Supplemental Files 1-4. **Unzip** and **save** into a personal Documents/MATLAB (MacOS/Linux) or Documents\MATLAB (Windows) directory. This generates four new folders: SiMPull_class, smite-master, Sample Data, Sample Analysis Outputs.

6.1.2.**Open** ReadMe_Setup.txt file found in the SiMPull_class folder.

6.1.3.**Install** MATLAB (The MathWorks, Inc.) and MATLAB Toolboxes: Curve Fitting Toolbox, Parallel Computing Toolbox, and Statistics and Machine Learning toolbox.

6.1.4. **Install** DipImage[15] according to download instructions.

6.1.5. **Install** *smite* single molecule analysis package as described in ReadMe_setup.txt.

6.1.6. **Open** SiMPullMain.m, found in SiMPull_class folder, by dragging the file into the MATLAB window.

6.1.7. Change directory to …\MATLAB\Sample Data\ by **clicking** “Browse for folder” icon and **selecting** the folder.

6.2. Overview of Data Processing Steps—NOTE: Run SiMPullMain.m - following instructions for each section. Execute each section individually by placing the cursor in that section and **clicking the “Run Section” icon**. The general steps for data analysis are described in this section. Detailed instructions are found in the accompanying SiMPullMain.m code.

6.2.1. **Run** “Initialization” Section to set the path, define spectral channels and image size.

6.2.2. **Run** “Find Camera Gain and Offset” Section to convert camera gain to photons using the Gain and Background datasets.

6.2.3. **Run** “Channel Registration” Section to calculate the local weighted mean transform for image registration.

6.2.4. Format and curate the data. **Run** “Join Sequential Channels into a Quad Image” Section. **Run** “Remove Bad Frames.”

6.2.5. **Run** “Fit Single Molecules and Find Overlapping Molecules” Section. This section executes multiple functions to localize single molecules in each channel and determine colocalization events between spectral channels.

6.2.6. To determine the minimum photon count per true GFP fit, as described in Figure 3, **run** “Optimize Minimum Photon Threshold” Section. This is an iterative process. First, set smf.Thresholding_MinPhotons = [0, 0, 0] and **run** the section. Select “Blank Data” files when prompted. Repeat with the “CHO-EGFR-GFP” files. Select an appropriate minimum threshold value by comparing the two histograms as described in Figure 3D. **Set** smf.Thresholding_MinPhotons = [475, 0, 0] and **run** the section again.

6.2.7. **Run** “Calculate Percentage of GFP fits Positive for FR Signal” Section to correct for background localizations and calculate final values.

6.2.8. Option: Correction for the number of receptors available at the plasma membrane for ligand binding can be applied[12]. First, label surface receptors with saturating levels of fluorescent NHS Ester dye (NHS-AF647). Then perform a SiMPull experiment to determine the percentage of GFP localizations that colocalize with AF647. This provides an estimate of the fraction of receptors available for NHS labeling, and therefore the ratio of receptors on the surface (SR). Apply the SR correction in the final calculation: NGFP = (NLOC - NBG)*SR, where NBG is the number of background localization and NLOC is the number of total localization. In the provided example, this correction is not applied because the action of phosphatase inhibition is not limited to surface receptors.

6.2.9. Option: For multisite phosphorylation measurements, the potential for steric hindrance when two antibodies are used needs to be considered. Steric hindrance will cause a reduction in the observed percentage of phosphorylated receptors for Antibody 1 when it is in the presence of Antibody 2 (P12) as compared to when Antibody 1 was alone (P1). Use SiMPull to determine P1 and P12 and calculate the correction factor for steric hindrance, as described in ref[12].

REPRESENTATIVE RESULTS:

A cartoon depicting the SiMPull process is shown in Figure 1A. Coverslips are functionalized using NeutrAvidin as an anchor for biotinylated anti-EGFR antibody to capture EGFR-GFP from total protein lysates. After washing away unbound protein, the phosphorylated receptors are labeled with an anti-phosphotyrosine (anti-PY) antibody[12]. Figure 1B shows an image of the hydrophobic array, where multiple samples can be prepared and imaged on the same coverslip. One advantage of this sample holder is that minimal sample volumes of ~10 μL are required. The coverslip can be imaged by placing it directly on the microscope stage. However, it is helpful to stabilize the coverslip by using

a coverslip holder. The coverslip holder shown in Figure 1B was created using a 3D-printer and the blueprint is provided in Supplemental File 5. Note that the autofluorescence of the hydrophobic ink is a useful guide to find the focal plane of the sample (Fig. 1C). An example of a multi-channel raw image is shown in Figure 1D. An overlay of the raw green and far-red channels is shown in Figure 1E.

Figure 2 outlines the analysis workflow and provides representative data. Data acquisition first starts with acquiring fiducials for channel registration, which is used to overlay the individual spectral channels data (Figure 2A). Bright field images are taken using a nanogrid pattern that passes white transmission light and is detected in each spectral channel of the image splitter (not shown). The green channel acts as the reference channel and the far-red channel is the shifted channel. The local weighted mean transform is calculated using the *fitgeotrans* function in MATLAB that is used to shift far-red coordinates into the coordinate frame of the green channel. This transform uses a second order polynomial model at every control point. Multi-channel data of the SiMPull array is then acquired. This work-flow consisted of a semi-automated acquisition, where a starting ROI was selected for the specific sample square and three regions around this area were imaged. Each dataset then contains full quad-view images (as seen in Figure 1D) from three independent ROIs. In each spectral channel, the emitter candidate locations are found by applying a difference of Gaussians filter to images and identifying local maxima. Subregions (boxes, Figure 2B) are drawn around local maxima and emitter photon counts are estimated by assuming each subregion contains only one emitter. Subregions containing emitter candidates with photon counts above a minimum value are retained for fitting. A Gaussian point spread function (PSF) is fit to each emitter candidate within small subregions roughly centered around each emitter. The resulting localizations are thresholded based on their photon count, background, Cramér-Rao lower bound of the fit coordinates, PSF variance (i.e., PSF width), and a p-value describing the goodness of fit of the PSF model. A Gaussian image is created for each spectral channel, with uniform intensity Gaussian blobs placed at the coordinates for each good fit (Figure 2C). Colocalization is visualized by overlaying the Gaussian images from each spectral channel using the transform calculated from the fiducial sample (Figure 2D). It is important to fluorescently label the receptor for identification since there is still non-specific binding of the anti-phosphotyrosine antibodies to the surface when cell lysate is present. The EGFR-GFP (green channel) is used to generate a mask of the receptor locations and only the AF647-anti-PY signal (far-red channel) within that mask is counted (Figure 2D). Pairs within 1 pixel (106.7 nm pixel size) are assumed to be colocalized and saved to a list containing the coordinates of the reference channel and the percentage of AF647 colocalized with GFP is calculated to determine the fraction of phosphorylated receptors (Figure 2E).

There are several critical steps to ensure proper data quality. One such step is to incubate the coverslip array with $NaBH_4$ as described in the protocol, to quench autofluorescence in the green channel. This autofluorescence refers to nonspecific signal due to possible impurities on the glass, containing single or conjugated π bonds[16]. Such impurities are potentially from the aminosilane and PEG reagents used in the functionalization process, or dust from the air, and tend to fluoresce in the green spectral channel. These molecules may also be generated through oxidation that occurs in storage, despite efforts to keep glass stored under nitrogen. $NaBH_4$ has also been used to reduce fluorescence from impurities on slides and

microarrays, including those with silane coating[16]. Figure 3A shows the reduction in the amount of background detections that occur when the piranha etched glass is treated with $NaBH_4$. While $NaBH_4$ dramatically reduces the background fluorescence, some emitters are still detected in the green channel. One can correct for this by acquiring background images from lysate free samples (Figure 3D) and subtracting the average number of background localizations from the GFP-containing samples. Fluorescence from impurities was not detected in the far-red channel. Note that at high receptor density multiple GFP emitters can be found in a diffraction limited spot (data not shown). Using step-photobleaching to identify the number of GFP per spot, we found that a receptor density between 0.04-0.08 proteins/μm^2 provided sufficient spacing between single emitters to remove the potential of finding multiple emitters per spot (see reference[12]). The receptor density can be optimized by varying the amount of IP antibody bound to the glass surface or the amount of lysate added. It is critical to ensure that the antibody targeting the POI is used at saturating levels. It is recommended to acquire an antibody concentration curve on phosphorylated samples to determine the appropriate labeling conditions (Figure 3B). In addition, the phospho-specificity of an antibody should be validated with resting samples and/or treatment with protein-specific kinase inhibitors (Figure 3B). Antibodies will dissociate from the receptor during the imaging time window. Treating the sample with a combination of PFA and GA prevented the loss of signal (Figure 3C).

Finally, it is important to optimize the single molecule fitting parameters. The first “box finding” step that identifies potential emitter candidates (Figure 2B) should be generous to allow many candidates to undergo the Gaussian Fitting. Thus, the minimum photon threshold for box finding can be relatively low to capture all real emitters as well as some background spots. It is also important to not set the box size and overlap allowance too small. Keeping the box size of 5-7 pixels, and allowing two pixel overlap is ideal for emitters at the recommended density. After box finding, the minimum photons threshold in the fitting step needs to be optimized. The minimum photons parameter contributes to determining which of the Gaussian fitted emitters passes as a true fit. To determine the proper minimum photon threshold for true GFP fits, the code includes a histogram plotting function to examine the photons/localization in both background (no cell lysate) and GFP-containing (plus cell lysate) samples (Figure 3D). This step is important because, while $NaBH_4$ reduces the amount of fluorescence from impurities, it does not remove all background localizations. Figure 3D demonstrates the need to set a minimum photon threshold to reduce the amount of detections from impurities. To determine this threshold, a histogram of background emitter intensities is calculated from imaging a sample that is not exposed to cell lysate (Fig. 3D, top left). The majority of the background emitters were found to have values less than 475 photons. In comparison, the sample containing true GFP emitters showed a significant fraction of the distribution above 475 (Fig. 3D, top right). The threshold is chosen by inspection to remove as many background counts as possible while minimizing the amount of signal loss from the lysate sample (Fig. 3D, bottom row). Note that remaining background count density at this threshold is accounted for in the quantitative analysis, as described above.

DISCUSSION:

The protocol described here was optimized to enable quantitative measurements of receptor phosphorylation at the single protein level. We developed several straight-forward but important modifications to the SiMPull protocol that improved the reliability of the measurements for phospho-tyrosine detection, including reduction of autofluorescence with NaBH4 treatment and post-fixing of the sample to prevent antibody dissociation. The use of the green channel mask to identify receptor locations for calculation of colocalization with the anti-PY antibody also improves the accuracy of the measurement by removing potential artifacts from nonspecific binding of the antibody to cell lysate. Detailed here, two-color imaging was utilized to detect the fraction of receptors phosphorylated. In this scenario, the receptor was genetically tagged with GFP and the antibody directly-labeled with a far-red dye. The SiMPull approach is applicable to other protein targets for which specific antibodies are available, including intracellular proteins. In addition, because denaturing conditions are not required, multi-subunit receptors/complexes can also be captured. However, denaturation may be incorporated if the PTMs of interest are located in structured regions of the protein. Ultimately, SiMPull can be readily expanded to include simultaneous labeling of distinct phospho-tyrosines on individual receptors for quantification of multisite phosphorylation patterns (see section 6.2.9). The interrogation of full-length, intact receptors in such a way cannot be achieved by other standard methods, including western blotting and phospho-mass spectrometry.

Along with the advantages of SiMPull, there are limitations that need to be considered. As with any antibody-based technique, the affinity and specificity of antibodies used is critical to the success of the measurement. It is, therefore, important to optimize antibody labeling conditions and ideally to avoid secondary antibodies by using directly-labeled primary antibodies. Furthermore, the surface-bound antibodies will precipitate proteins that were localized to the plasma membrane as well as within cytosolic compartments. This can lead to an underestimate of phosphorylation since cytosol-localized proteins are not accessible to the exogenously added ligand. Extra steps must be taken to correct for the receptor surface levels (see section 6.2.8). Note that the anti-phosphotyrosine antibodies exhibited some non-specific binding once lysate was present. To avoid this artifact, the EGFR was genetically-tagged with GFP to identify the location of receptors, which allowed us to exclude anti-PY signal off the receptor. If endogenous proteins are to be interrogated, then counterstain with a total protein antibody can provide the mask image, with appropriate correction for any non-specific binding. Finally, while SiMPull provides information on heterogeneity at the protein level, the lysate generated in this protocol is from thousands of cells and cell-to-cell variability is lost. However, advances towards single cell SiMPull have been made by the Ha Lab. Using a flow chamber consisting of a coverslip and a microscope side with a 10 μm gap, bacteria were sparsely plated on the coverslip while the slide is functionalized with antibody to capture the desired proteins. Upon lysis of the bacteria, the proteins from each cell were capture in a confined area on the antibody-coated slide[17]. Similar single cell SiMPull analysis of mammalian cells and protein phosphorylation may be possible in the future.

In addition to these modifications to optimize the method, there are a number of critical steps in the protocol that ensure success. For example, the protocol includes an elaborate preparation of the coverslip glass. Piranha etching coverslips both thoroughly cleans the glass and increases hydroxide groups and hydrophilicity, which are needed to provide optimal functionalization of the coverslip surface. Following several washes with organic solvents, KOH treatment provides additional hydroxide groups for aminosilanization, which coats the glass with amine groups for PEG and biotin-PEG binding. Improper cleaning or functionalization at any of these steps will interfere with protein pull-down. Control of the molar ratio of PEG:biotin-PEG, along with lysate concentration, are key factors in obtaining appropriate protein IP density on the SiMPull substrate. As with any biological assay, there is variability between cell lysate preparations and small differences between phosphorylation percentages may be seen between sample replicates. To account for this, is it important to measure the phosphorylation of different tyrosine sites within the same sample. Having a system to collect a large number of data points, like the sample chamber described here, allows for averaging over multiple SiMPull experiments.

On the image acquisition side, it is important to properly obtain the fiducial sample to ensure accurate channel overlay, otherwise colocalization will not be accurate. It is also important to optimize laser power and camera setting to maximize signal-to-noise, while at the same time minimizing photobleaching. Lastly, while the sample array requires a small amount of sample and reagents to be use, the low volumes are susceptible to evaporation during the imaging session. It is important to periodically check the sample array (~30-45 minutes) and add buffer as needed to prevent samples from drying.

This protocol demonstrated the use of SiMPull to quantify membrane receptor phosphorylation states. While focused on EGFR, the approach can be applied to other cell surface receptors as well as intracellular proteins and protein complexes, as long as appropriate antibodies are available. Another potential use for SiMPull is to interrogate the contents and phosphorylation status of phase-separated condensates. In addition, SiMPull can be used to measure other PTMs, such as ubiquitination. Therefore, SiMPull provides a unique tool for cell biologists to interrogate PTMs on intact proteins and correlate PTM patterns with cellular outcome.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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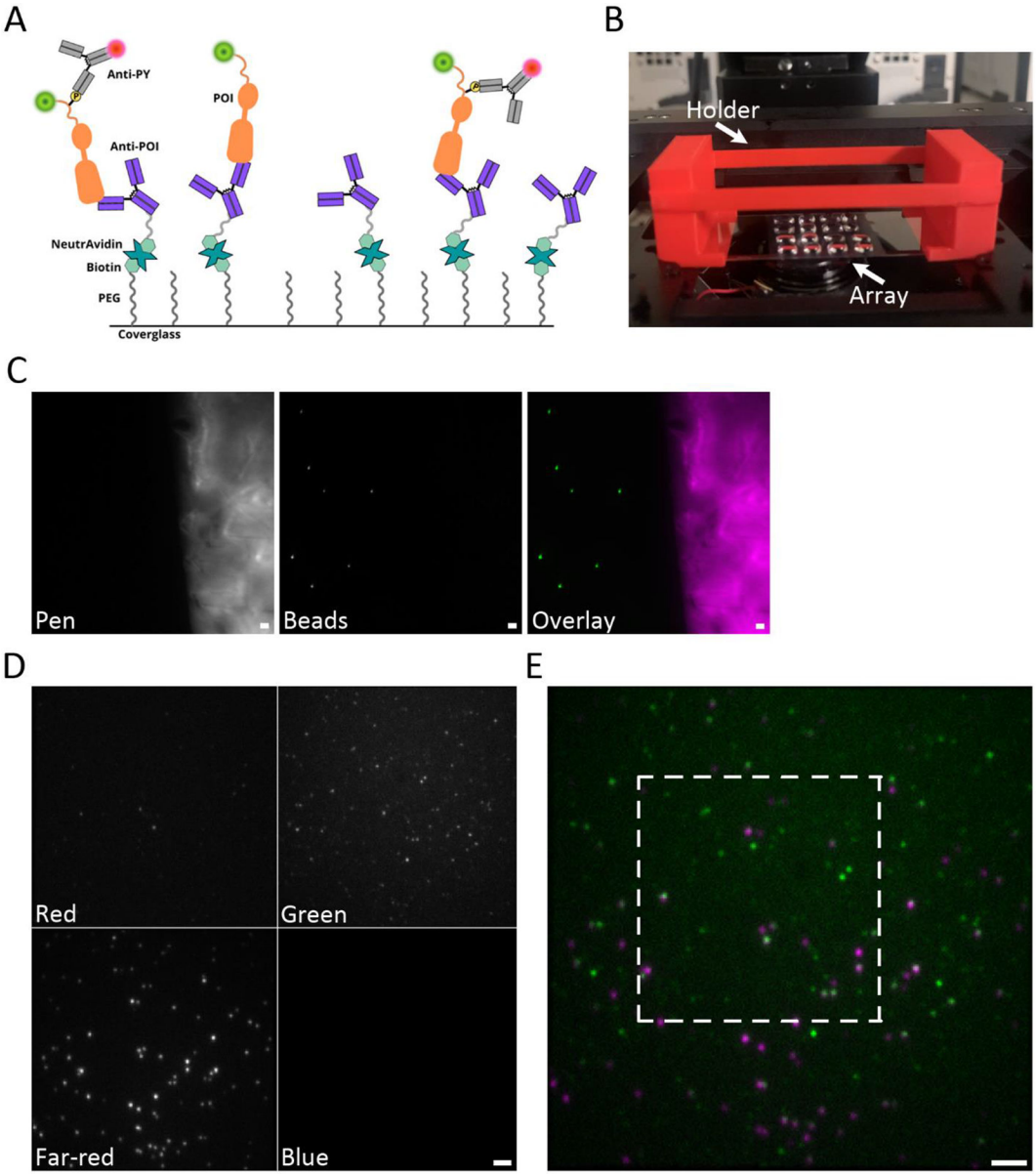

Figure 1: Overview of sample preparation.

(A) Cartoon depicting the SiMPull approach. Coverslips are functionalized with an antibody that recognizes the POI to capture that POI from whole cell lysates. The glass is first coated with PEG and biotin-PEG. NeutrAvidin is then bound to the biotin-PEG and acts as an anchor for the biotinylated anti-POI antibody. Phosphorylated proteins are then detected with a fluorescently labeled anti-PY antibody. (B) Picture of the coverslip holder (red) with coverslip array in place and mounted on the microscope stage. The multi-sample arrays are generated by using hydrophobic ink to create up to 20 individual sample squares on a single glass coverslip. The coverslip is 60 mm x 24 mm. (C) Example images of the hydrophobic ink autofluorescence (magenta) with respect to fluorescent beads (green). The autofluorescence of the hydrophobic ink is a useful guide to find the focal plane at the coverslip surface. (D) Example of a raw data image with spectral channels separated on

the camera chip by the Quad-view image splitter. The Quad-view filter set includes the following emission filters: blue (445/45 nm), green (525/45 nm), red (600/37 nm), far-red (685/40 nm). (E) Raw overlay of green and far-red channels. The white box indicates the region further examined in Figure 2B-D. Scale bars, 2 μm.

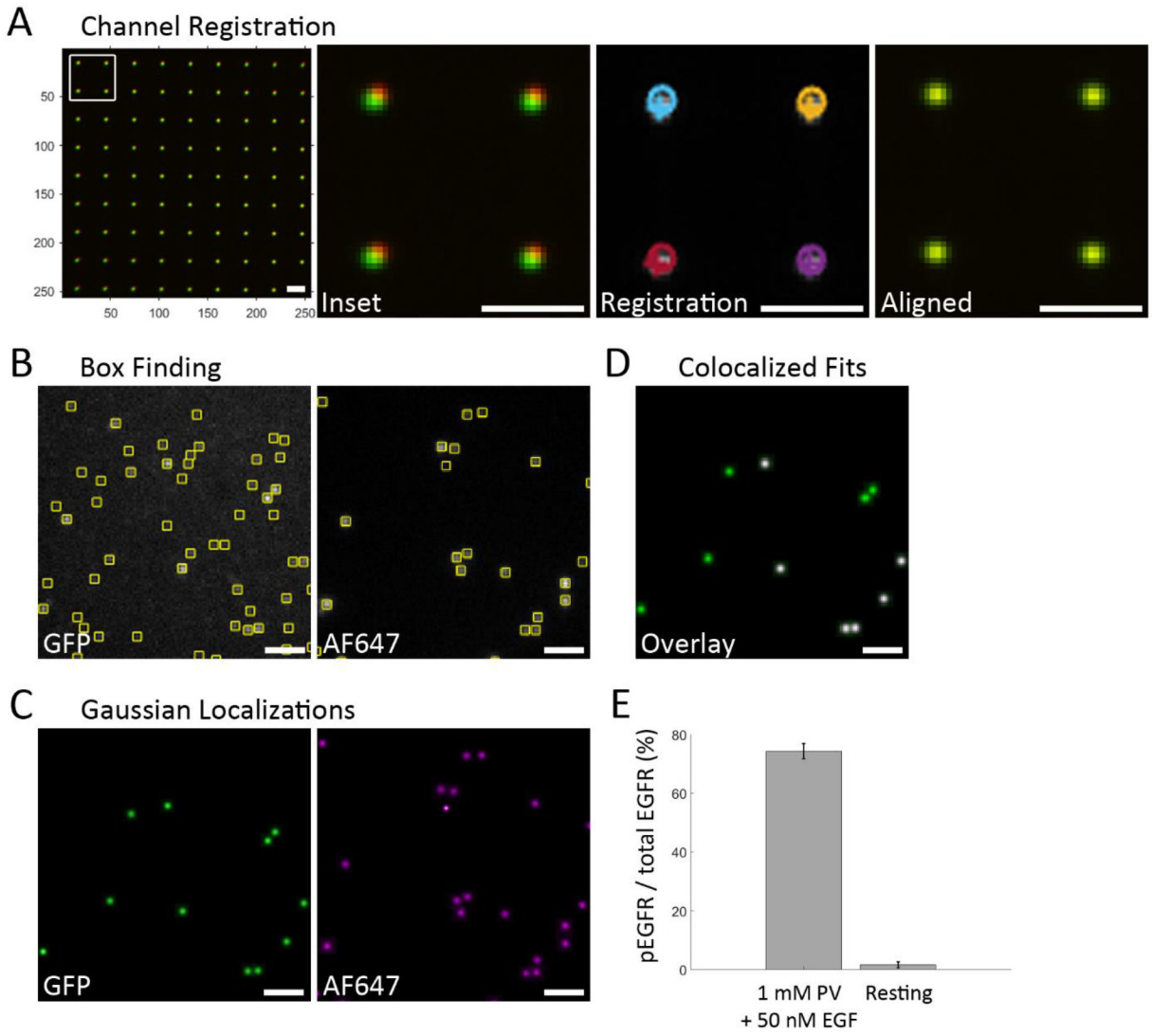

Figure 2: Data Analysis workflow.

(A) Channel registration is first performed on images acquired from the nanogrid. After cropping the two spectral channels of interest (here, green and far-red), the fiducial images for each channel are overlaid (left). Enlargement of the box in the left image (Inset) shows that the images are not yet truly registered. The emitters in each channel are then fit to a Gaussian model and localized (Registration). Localization of emitters is shown as circles for the far-red channel and crosses for the green channel. The final step is to apply a local weighted mean transform to shift the far-red channel localization coordinates into the green channel reference frame (Aligned). The calculated local weighted mean transform is then used to register the subsequent SiMPull data. (B) Representative images of the green/EGFR-GFP channel and the far-red/AF647-anti-PY channel. Single emitters above the background photon count are identified and marked with boxes. (C) The emission profile within each selected box is fit to a Gaussian model and the emitters that fit the model of a single fluorophore PSF are kept. (D) A mask is created from the GFP emitters to identify location of EGFR-GFP (green). Colocalization of EGFR-GFP and AF647-anti-PY identifies

phosphorylated receptors (white). (E) The fraction of phosphorylated receptors is calculated from the colocalized EGFR-GFP and AF647-anti-PY fits. Bar graph compares PV+EGF treatment to resting cells, averaged for multiple measurements. Error bars represent standard error calculated assuming a binomial distribution. Scale bars, 2 μm.

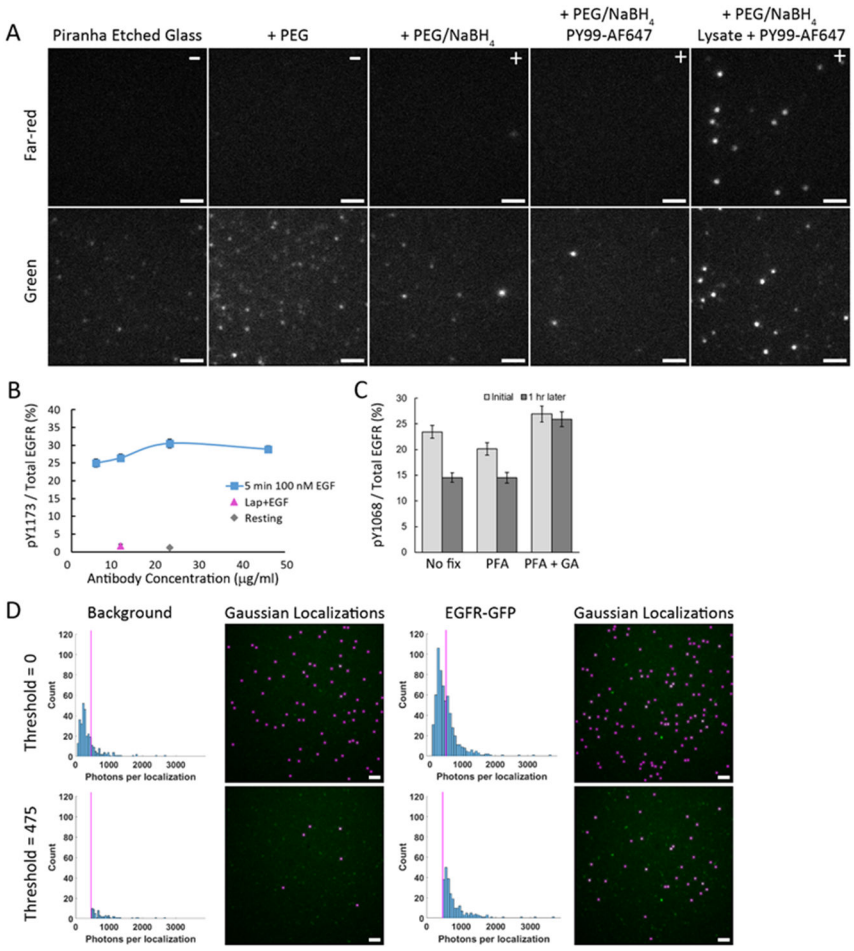

Figure 3: Critical steps to ensure data quality.

(A) From left to right, the first three panels are representative images of the autofluorescence on glass under the respective conditions: after piranha etching, with PEG, and PEG plus $NaBH_4$ treatment (indicated with +). Additionally, surface functionalization is retained after $NaBH_4$ treatment as demonstrated by minimal non-specific PY99-AF647 binding, while retaining robust binding of EGFR-GFP from the lysate. (B) To ensure optimal antibody labeling, a saturation curve should be acquired for each batch of antibody used. This figure shows the concentration curve for labeling EGFR with the site specific phosphotyrosine antibody, anti-EGFR-pY1173. Minimal phosphorylation is detected in untreated cells (Resting, gray diamond). As a control for non-specific binding, cells were also treated with the EGFR kinase inhibitor Lapatinib before addition of 100 nM EGF (magenta triangle), which shows the expected prevention of EGFR phosphorylation. Error bars represent standard error assuming a binomial distribution. (C) Fixation of the sample with a combination of PFA and GA prevents antibody dissociation over time. Error bars represent standard error assuming a binomial distribution. (D) False positives are excluded by selecting the appropriate threshold for Gaussian fitting. Comparing the histogram of fit intensities at a low threshold (Threshold = 0; top) between background (no lysate) and real data (plus cell lysate) allows for selection of appropriate value (Threshold = 475; bottom) to remove fits from autofluorescent spots in the green channel. Vertical magenta line indicates

475 photon threshold. Histograms are calculated from the same number of ROIs for each sample type (n=3). Scale bars, 2 μm.

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Table of Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1.5 mL microcentrifuge tubes	MTC Bio	C2000	
10 mM Tris-HCl pH 8.0/ 50 mM NaCl			T50 Buffer
10 mM Tris-HCl pH 7.4			
100 mm Tissue Culture dish	CELLTREAT	229620	Storage of piranha etched glass/arrays
15 mL conical tube			
16% Paraformaldehyde Aqueous Solution	Electron Microscopy Sciences	15710	Hazardous
50 mL conical tube			Functionalized Glass storage/ KOH reuse
50 mM Tris-HCl pH 7.2/150 mM NaCl			Lysis Buffer Component
60 mm Tissue Culture dish	Corning	430166	
8% Glutaraldehyde Aqueous Solution	Electron Microscopy Sciences	16020	Hazardous
Acetone (C_3H_6O)	Millipore Sigma	270725	Hazardous
Alexa Fluor® 647 NHS Ester	Thermo Fisher Scientific	A-20006	
Animal-Free Recombinant Human EGF	Peprotech	AF-100-15	
Anti-Human EGFR (External Domain) – Biotin	Leinco Technologies, Inc	E101	
Anti-p-Tyr Antibody (PY99) Alexa Fluor® 647	Santa Cruz Biotechnology	sc-7020 AF647	
Bath-sonicator	Branson	1200	
BCA Protein Assay Kit	Pierce	23227	
Biotin-PEG	Laysan Bio	Biotin-PEG-SVA, MW 5,000	
Bovine serum albumin	Gold Biotechnology	A-420-1	Tyrode's Buffer Component
Buchner funnel			
Bunsen burner			
Calcium Chloride ($CaCl_2$)	Millipore Sigma	C4901	Tyrode's Buffer Component
Cell Scraper	Bioworld	30900017-1	
Conical Filtering Flask	Fisher Scientific	S15464	
Coplin Jar	WHEATON	900470	
Countess II Automated Cell Counter	Thermo Fisher Scientific	AMQAX1000	

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Coverslips 24 x 60 #1.5	Electron Microscopy Sciences	63793	
DMEM	Caisson Labs	DML19-500	
emCCD camera	Andor iXon		
Fetal Bovine Serum, Optima	Bio-Techne	S12450H	Heat Inactivated
Geneticin G418 Disulfate	Caisson Labs	G030-5GM	
Glacial Acetic Acid (CH_3COOH)	JT Baker	JTB-9526-01	Hazardous
Glass serological pipettes			
Glass Stir Rod			
Glucose (D-(+)-Glucose)	Millipore Sigma	D9434	Tyrode's Buffer Component
Halt Phosphotase and Protease Inhibitor Cocktail (100X)	Thermo Fisher Scientific	78446	Lysis Buffer Component
HEPES	Millipore Sigma	H3375	Tyrode's Buffer Component
Hydrochloric Acid (HCl)	VWR	BDH7204-1	Hazardous
Hydrogen Peroxide (H_2O_2) (3%)		HX0645	
Hydrogen Peroxide (H_2O_2) (30%)	EMD Millipore	HX0635-2	
Ice			
IGEPAL® CA-630 (NP-40)	Sigma Aldrich	I8896	Lysis Buffer Component
ImmEdge Hydrophobic Barrier Pen	Vector Laboratories	H-4000	
Immersol 518F immersion oil	Zeiss	444960-0000-000	
in-house vacuum line			
L-glutamine	Thermo Fisher Scientific	25030-164	
Magnessium Chloride Hexahydrate ($MgCl_2$-$6H_2O$)	MPBio	2191421	Tyrode's Buffer Component
Methanol (CH_3OH)	IBIS Scientific	MX0486-1	Hazardous
Milli-Q water			
Mix-n-Stain™ CF® Dye Antibody Labeling Kits	Biotium	92245	Suggested conjugation kit
mPEG	Laysan Bio	mPEG-succinimidyl valerate, MW 5,000	
N-(2-aminoethyl)-3-aminopropyltrimethoxysilane	UCT United Chemical	A0700	Hazardous
Nanogrid	Miraloma Tech		
NeutrAvidin Biotin Binding Protein	Thermo Fisher Scientific	31000	
Nitrogen (compressed gas)			

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Olympus iX71 Microscope	Olympus		
Parafilm M Sealing Film	The Lab Depot	HS234526C	
PBS pH 7.4	Caisson Labs	PBL06	
PC-200 Analog Hot Plate	Corning	6795-200	
Penicillin-Streptomycin (10,000 U/mL)	Thermo Fisher Scientific	15140-163	
Phospho-EGF Receptor (Tyr1068) (1H12) Mouse mAb	Cell Signaling Technology	2236BF	
Potassium Chloride (KCl)	Millipore Sigma	529552	Tyrode's Buffer Component
Potassium Hydroxide (KOH)	Millipore Sigma	1050330500	Hazardous
Pyrex 1 L beaker			
Pyrex 250 mL beakers			
Pyrex 4 L beaker			
PYREX® 100 mL storage bottles	Corning	1395-100	CH_3OH/C_3H_6O reuse
Quad-view Image Splitter	Photometrics	Model QV2	
Refrigerated centrifuge	Eppendorf	EP-5415R	
RevCount Cell Counters, EVE Cell Counting Slides	VWR	10027-446	
Semrock emission filters: blue (445/45 nm), green (525/45 nm), red (600/37 nm), far-red (685/40 nm).	Semrock	LF405/488/561/635-4X4M-B-000	
Serological pipette controller			
Serological Pipettes			
Sodium Bicarbonate ($NaHCO_3$)	Sigma Aldrich	S6014	Hazardous
Sodium Borohydride ($NaBH_4$)	Millipore Sigma	452874	Tyrode's Buffer Component
Sodium Chloride (NaCl)	Millipore Sigma	S9625	Activate by successive heat and pH cycling
Sodium Hydroxide	VWR	BDH3247-1	
Sodium Orthovanadate (Na_3VO_4)	Millipore Sigma	S6508	Hazardous
Sulfuric Acid (H_2SO_4)	Millipore Sigma	258105	Hazardous
TetraSpeck Microspheres	Thermo Fisher Scientific	T7279	multi-fluorescent beads
Tris (Trizma) base	Millipore Sigma	T1503	
Trypan blue stain, 0.4%	Thermo Fisher Scientific	15250061	
Trypsin-EDTA 0.05%	Thermo Fisher Scientific	25300120	

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
3D Printer:			
Pro2 3D printer	Raise 3D		
Fusion 360 software	Autodesk		
Premium PLA Filament, 1.75 mm diameter	Raise 3D	PMS:2035U/RAL:3028	Printing temperature range: 205-235 °C
Data Analysis:			
Matlab	Mathworks		Curve Fitting Toolbox, Parallel Computing Toolbox, and Statistics and Machine Learning toolbox
DipImage			https://diplib.org
smite single molecule analysis package			https://github.com/LidkeLab/smite.git
NVIDIA GPU with CUDA			

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