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Imaging Molecular Adhesion in Cell Rolling by Adhesion Footprint Assay

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TITLE:

Imaging Molecular Adhesion in Cell Rolling by Adhesion Footprint Assay

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rolling adhesion, leukocyte, selectin, P-selectin glycoprotein ligand-1 (PSGL-1), molecular force sensor, adhesion footprint assay, tension gauge tether (TGT), DNA-based point accumulation for imaging in nanoscale topography (DNA-PAINT)

SUMMARY:

This protocol presents the experimental procedures to perform the adhesion footprint assay to image the adhesion events during fast cell rolling adhesion.

ABSTRACT:

Rolling adhesion, facilitated by selectin-mediated interactions, is a highly dynamic, passive motility in recruiting leukocytes to the site of inflammation. This phenomenon occurs in postcapillary venules, where blood flow pushes leukocytes in a rolling motion on the endothelial cells. Stable rolling requires a delicate balance between adhesion bond formation and their mechanically-driven dissociation, allowing the cell to remain attached to the surface while rolling in the direction of flow. Unlike other adhesion processes occurring in relatively static environments, rolling adhesion is highly dynamic as the rolling cells travel over thousands of microns at tens of microns per second. Consequently, conventional mechanobiology methods such as traction force microscopy are unsuitable for measuring the individual adhesion events

and the associated molecular forces due to the short timescale and high sensitivity required. Here, we describe our latest implementation of the adhesion footprint assay to image the P-selectin: PSGL-1 interactions in rolling adhesion at the molecular level. This method utilizes irreversible DNA-based tension gauge tethers to produce a permanent history of molecular adhesion events in the form of fluorescence tracks. These tracks can be imaged in two ways: (1) stitching together thousands of diffraction-limited images to produce a large field of view, enabling the extraction of adhesion footprint of each rolling cell over thousands of microns in length, (2) performing DNA-PAINT to reconstruct super-resolution images of the fluorescence tracks within a small field of view. In this study, the adhesion footprint assay was used to study HL-60 cells rolling at different shear stresses. In doing so, we were able to image the spatial distribution of the P-selectin: PSGL-1 interaction and gain insight into their molecular forces through fluorescence intensity. Thus, this method provides the groundwork for the quantitative investigation of the various cell-surface interactions involved in rolling adhesion at the molecular level.

INTRODUCTION:

The rolling adhesion cascade describes how circulating cells tether to and roll along the blood vessel wall[1]. Passive rolling is primarily mediated by selectins, a major class of cellular adhesion molecules (CAMs)[1]. Under the shear flow of blood, leukocytes expressing P-selectin glycoprotein ligand-1 (PSGL-1) form highly transient bonds with P-selectin, which may be expressed on the surface of inflamed endothelial cells. This process is critical for leukocytes to migrate to a site of inflammation[2]. In addition, PSGL-1 is also a mechanosensitive receptor capable of triggering the subsequent firm adhesion stage of the rolling adhesion cascade upon its engagement with P-selectin[3].

Genetic mutations affecting CAM function can severely affect the immune system, such as in the rare disease of leukocyte adhesion deficiency (LAD), where malfunction of adhesion molecules mediating rolling leads to severely immunocompromised individuals[4–6]. In addition, circulating tumor cells have been shown to migrate following a similar rolling process, leading to metastasis[7,8]. However, because cell rolling is fast and dynamic, conventional experimental mechanobiology methods are unsuitable for studying molecular interactions during cell rolling. While single-cell and single-molecule manipulation methods like atomic force microscopy and optical tweezer were able to study molecular interactions such as P-selectin's force-dependent interaction with PSGL-1 at the single-molecule level[9], they are unsuitable for investigating live adhesion events during cell rolling. Additionally, the interaction characterized *in vitro* cannot directly answer the question about molecular adhesion *in vivo*. For instance, what molecular tension range is biologically relevant when cells are functioning in their native environment? Computational methods such as adhesive dynamics simulation[10] or simple steady-state model[11] have captured certain molecular details and how they influence the rolling behavior but are highly dependent on the accuracy of the modeling parameters and assumptions. Other techniques such as traction force microscopy can detect forces during cell migration but do not provide sufficient spatial resolution or quantitative information on molecular tension. None of these techniques can provide direct experimental observations of the temporal dynamics, spatial distribution, and magnitude heterogeneity of molecular forces, which directly relate to cell

function and behavior in their native environment.

Therefore, implementing a molecular force sensor capable of accurately measuring selectin-mediated interactions is crucial to improving our understanding of rolling adhesion. Here, we describe the protocol for the adhesion footprint assay[12] where PSGL-1 coated beads are rolled on a surface presenting p-selectin functionalized tension gauge tethers (TGTs)[13]. These TGTs are irreversible DNA-based force sensors that result in a permanent history of rupture events in the form of fluorescence readout. This is achieved through the rupturing of the TGT (dsDNA) and then subsequent labeling of the ruptured TGT (ssDNA) with a fluorescently labeled complementary strand. One major advantage of this system is its compatibility with both diffraction-limited and super-resolution imaging. The fluorescently labeled complementary strand can either be permanently bound (>12 bp) for diffraction-limited imaging or transiently bound (7–9 bp) for super-resolution imaging through DNA PAINT. This is an ideal system to study rolling adhesion as the TGTs are ruptured during active rolling, but the fluorescence readout is analyzed post-rolling. The two imaging methods also provide the user with more freedom to investigate rolling adhesion. Typically, diffraction-limited imaging is useful for extracting molecular rupture force through fluorescence intensity[13], whereas super-resolution imaging allows for quantitative analysis of receptor density. With the ability to investigate these properties of rolling adhesion, this approach provides a promising platform for understanding the force-regulation mechanism on the molecular adhesion of rolling cells under shear flow.

PROTOCOL:

1. Oligonucleotide labeling and hybridization

1.1 Reduction of Protein G disulfide bonds

1.1.1. Dissolve 10 mg of Protein G (ProtG) in 1 mL of ultrapure water.

NOTE: The Protein G here is modified with a single cysteine residue at the C-terminus and an N-terminus poly-histidine tag.

1.1.2. Buffer exchange ≥20 µL of ProtG (10 mg/mL) into 1x PBS (pH 7.2) with a P6 column.

1.1.3. Measure the protein concentration following buffer exchange.

NOTE: Typical concentration of 7–8 mg/mL.

1.1.4. Prepare 120 mM Tris(2-carboxyethyl)phosphine (TCEP) by dissolving 3 mg of TCEP into 90 µL of 1x PBS (pH 7.2) followed by 10 µL of 0.5 M EDTA.

NOTE: TCEP should be freshly prepared.

1.1.5. Add 4 µL of 120 mM TCEP (480 nmol) to 20 µL of ProtG (4–5 nmol).

NOTE: Aim for a molar ratio of ~100:1 TCEP to protein.

1.1.6. Allow the reaction to proceed for 30 min at room temperature (RT).

1.1.7. Remove excess TCEP from the reduced ProtG with a P6 column (buffer exchanged in 1x PBS, pH 7.2).

1.1.8. Measure the concentration of the reduced ProtG with a UV/Vis spectrophotometer and save the spectra.

NOTE: Typical concentration of 3.5–4.5 mg/mL.

1.2 Amine-labeled ssDNA reaction with Sulfo-SMCC

1.2.1. Dissolve amine-labeled ssDNA (amine-ssDNA) with nuclease-free water to a concentration of 1 mM. Verify the strand concentration with a UV/Vis spectrophotometer.

1.2.2. Prepare 11.5 mM sulfo-SMCC (a hetero-bifunctional crosslinker with sulfo-NHS ester and maleimide) solution fresh by dissolving 2 mg of sulfo-SMCC in 400 µL of ultrapure water and vortex to mix.

1.2.3. Add 6 µL of the 1 mM amine-ssDNA (6 nmol) to 5.2 µL of 11.5 mM sulfo-SMCC (60 nmol) and 88.8 µL of 1x PBS (pH 7.2). Vortex for 5 s followed by centrifugation at 8600 x *g* for 3 min.

1.2.4. Allow the reaction to proceed for 30 min at RT.

1.2.5. Remove excess sulfo-SMCC from the SMCC conjugated amine-ssDNA (mal-ssDNA) with a P6 column (buffer exchanged in 1x PBS, pH 7.2).

1.2.6. Measure the concentration of the mal-ssDNA with a UV/Vis spectrophotometer and save the spectra.

NOTE: Typical concentration of 35–45 µM.

1.3 ProtG-ssDNA conjugation

1.3.1. Add 21 µL of 4.5 mg/mL reduced ProtG (3 nmol) to the mal-ssDNA (~4–5 nmol).

NOTE: Volumes and concentrations here are typical values. Adjust according to individual experimental measurement. Always ensure an excess amount of mal-ssDNA over ProtG at a ratio of ~1.5:1.

1.3.2. Vortex for 5 s and allow the reaction to proceed for 3 h at RT.

1.4 ProtG-ssDNA purification and characterization

1.4.1. Purify the conjugated ProtG-ssDNA through his-tag isolation with magnetic nickel-nitrilotriacetic acid (Ni-NTA) beads.

1.4.2. Remove excess imidazole (Ni-NTA elution buffer) from the product with a P6 column (buffer exchanged in 1x PBS, pH 7.2).

NOTE: This step is essential for quantifying the conjugation, as imidazole has significant absorption at 280 nm.

1.4.3. Use a UV/Vis spectrophotometer to record the spectra of the product ProtG-ssDNA as well as the Ni-NTA elution buffer (1x).

NOTE: Typical ProtG-ssDNA absorbance at 260 nm and 280 nm is 0.8 and 0.6, respectively.

1.4.4. To determine the conjugation efficiency and ratio of ProtG to ssDNA, use the custom-written MATLAB script (**Supplemental Coding File 1)** to decompose the final product spectrum based on the three spectra collected previously (ProtG, SMCC-strand, Ni-NTA bead elution buffer).

NOTE: Briefly, the code works as described in steps 1.4.5–1.4.8. The typical concentration is 4 µM of ProtG-ssDNA with ProtG and ssDNA at a ~1:1 molar ratio (**Figure 2A**).

1.4.5. Input ProtG, SMCC-strand, Ni-NTA bead elution buffer, and the ProtG-ssDNA UV/Vis spectra into the MATLAB script

1.4.6. Perform a multidimensional unconstrained nonlinear minimization to reconstruct the ProtG-ssDNA spectra from the source spectra (ProtG, SMCC-strand, and Ni-NTA bead elution buffer spectra)

NOTE: The minimization function outputs three transformation factors, one for each source spectra.

1.4.7. Reconstruct the ProtG-ssDNA spectra by multiplying the spectra by their corresponding factor and combining the transformed source spectra.

1.4.8. Multiply the initial concentration of the ProtG and SMCC-strand by the corresponding transformation factors to determine the concentrations of SMCC-strand and ProtG in the ProtG-ssDNA product.

1.4.9. (OPTIONAL) Run native PAGE according to **Figure 2B** to help ensure each component and step works as expected.

1.5 TGT hybridization

1.5.1. Hybridize ProtG-ssDNA (top strand) with biotinylated bottom strand at a molar ratio of 1.2:1 with concentrations of 240 nM and 200 nM respectively in T50M5 buffer (10 mM Tris, 50 mM NaCl, 5 mM $MgCl_2$) to hybridize the full TGT construct. Let hybridize at RT ≥1 h.

2. Surface PEGylation

2.1 Surface cleaning

2.1.1. Clean one Erlenmeyer flask and two staining jars for every 8 coverslips. Fill each container with 1 M KOH solution and sonicate for 1 h at RT. Thoroughly wash each container with ultrapure water and dry with N_2 or in an oven.

NOTE: Fill the KOH to the top to touch the lid, so they are also cleaned.

2.1.2. Thoroughly rinse each coverslip with ultrapure water and place them into one of the cleaned staining jars.

NOTE: Ensure that they are not stuck to each other.

2.1.3. In a fume hood, freshly prepare a piranha solution by adding 30 mL of 30% hydrogen peroxide to 90 mL of concentrated (95%–98%) sulfuric acid in a 250 mL beaker.

CAUTION: Concentrated sulfuric acid is highly corrosive. Add the hydrogen peroxide very slowly to the sulfuric acid and carefully swirl to mix.

2.1.4. Fully submerge the coverslips in the staining jar with the piranha solution. Leave coverslips in piranha for 30 min in the fume hood.

CAUTION: Cool down the piranha solution to no more than 80 °C before pouring to prevent cracking the staining jar.

2.1.5. Discard the piranha solution into a 1000 mL beaker and neutralize with the 1 M KOH from glass cleaning.

2.1.6. (OPTIONAL) Repeat piranha cleaning (steps 2.1.3–2.1.5) with a fresh piranha solution.

2.1.7. Rinse the coverslips with copious amounts of ultrapure water to remove all residual piranha solution. Gently shake the staining jar during each rise to facilitate removal (10 rinses are recommended).

2.1.8. Rinse the coverslips with methanol 3 times to remove water from the coverslip surface

and keep the coverslips submerged in methanol.

2.2 Surface silanization

2.2.1. Prepare a 1% aminosilane solution by thoroughly mixing 94 mL of methanol, 1 mL of aminosilane, and 5 mL of glacial acetic acid in the cleaned and dried Erlenmeyer flask. Pour into the second cleaned and dried staining jar[14].

2.2.2. Transfer the coverslips submerged under the methanol solution to the staining jar containing 1% aminosilane solution and keep the jar covered.

NOTE: Do not allow the coverslips to dry while transferring to aminosilane to limit glass surface exposure to air.

2.2.3. Incubate the staining jar containing coverslips in aminosilane for 1 h at 70 °C in an oven[15].

2.2.4. Carefully discard the aminosilane solution in a separate waste container and rinse the coverslips in the staining jar with methanol 5 times to remove the aminosilane solution.

2.2.5. Rinse coverslips in the staining jar with ultrapure water 5 times and dry them with N_2.

2.2.6. Bake the dried coverslips in the staining jar in an oven at 110 °C for 20 min. Allow the coverslips to cool to RT, then place them on the PEGylation rack.

NOTE: Cover the staining jar with a lid during the bake to minimize particular and chemical deposition on the surface[15].

2.3 PEG solution preparation

2.3.1. Thaw PEG (Polyethylene glycol) and PEG-biotin to RT for about 30 min.

NOTE: This step minimizes moisture condensation that can degrade the NHS ester on PEG.

2.3.2. Make PEG buffer by adding 84 mg of sodium bicarbonate to 10 mL of ultrapure water. This formulation should provide a buffer at pH 8.4.

2.3.3. For 8 coverslips, each with one PEGylated side with 20:1 PEG:PEG-biotin: measure 100 mg of PEG and 5 mg of PEG-biotin to add to 400 µL of PEG buffer. Vortex the solution for 30 s and centrifuge for 1 min at the max speed (≥18000 x *g*).

NOTE: This step is time-sensitive, as the SVA NHS-ester hydrolysis starts immediately and has a half-life of 34 min at pH 8.0, and shorter at pH 8.4.

2.4 PEG incubation and coverslip storage

2.4.1. Set up the humidity chamber and place the coverslips inside.

2.4.2. Add 90 µL of PEG solution to a coverslip in the humidity chamber and place a second coverslip on top of the PEG solution using coverslip holding tweezers to evenly spread the PEG solution.

2.4.3. Ensure there are no bubbles in the solution dropped onto the coverslip. Lower one end of the second coverslip on the first coverslip and slowly drop the other end so that there are no bubbles sandwiched between the coverslips.

NOTE: Bubbles will cause certain areas to be poorly PEGylated.

2.4.4. Repeat until all coverslips have PEG (i.e., 8 coverslips = 4 PEG sandwiches). Incubate the PEG solutions overnight (~12 h) at RT in a humidity chamber in the dark[16].

2.4.5. Separate the coverslip pairs and place them into a staining jar. Note the PEGylated sides.

2.4.6. Rinse the coverslips thoroughly with ultrapure water and dry with N_2.

NOTE: Hold the coverslips with tweezers and blow N_2 across the surface towards the tweezer to prevent contaminants from drying onto the surface.

2.4.7. Mark the non-PEGylated side with a dot at a corner using a permanent marker or a diamond pen.

2.4.8. Place 2 PEGylated coverslips in mailer tubes with the PEGylated sides facing each other to help identify the PEGylated side before use.

2.4.9. Vacuum the tube for 5 min and backfill with N_2. Seal the tube with parafilm.

2.4.10. Store the PEGylated coverslips at -20 °C in the sealed mailer tubes for up to 6 months.

NOTE: Warm the storage tubes to RT before chip assembly. Condensation on the coverslips during sealing will cause leaking.

3. Flow chamber preparation

3.1 Chip assembly

3.1.1. Thinly spread a small amount of epoxy on both sides of double-sided tape with a razor blade.

NOTE: Too much epoxy may spread into the channel during assembly.

3.1.2. Laser-cut the epoxy coated tape to create 4 channels. Create the flow chip by sandwiching the epoxy tape between a 4-hole slide and PEG coverslip (**Figure 1A**).

3.1.3. Using a pipette tip, apply gentle pressure along the length of the channels to create a good seal. Cure the epoxy for ≥1 h.

NOTE: Do not shear the glass to avoid sliding against epoxy.

3.2 Chamber assembly

3.2.1. Align the chip so that the opening of each channel is positioned at the centers of the adapter (**Figure 1A**). Place two transparent acrylic spacers on top of the chip, apply firm pressure in the middle of the block, and screw in two 4-40 screws at the ends of each spacer.

NOTE: Do not force the screw or press too hard on the spacers, or the chip may crack.

3.2.2. On the other side of the bracket, screw the inlets into the threaded holes. Monitor the sealing condition through the transparent acrylic block.

3.2.3. As the tubing makes contact with the chip's opening, seal the connection by gently twisting the tubing clockwise.

NOTE: Overtighten inlets may cause flow blockage, while loose contacts will cause leakage.

4. Surface preparation

NOTE: Refer to **Figure 1B** for the overall workflow.

4.1 Blocking agents to prevent nonspecific binding

4.1.1. Use a pipette to flow 200 µL of wash buffer (10 mM Tris, 50 mM NaCl, 5 mM $MgCl_2$ and 2 mM $CaCl_2$, 0.05% Tween 20) into the chamber to check for leakage. If bubbles form in the channel, aggressively push an additional 200 µL to remove the bubbles.

NOTE: Large air bubbles not removed at this step can dislodge and destroy surface functionalization later.

4.1.2. Add 40 µL of BSA (1% w/v) to the flow chamber to prevent nonspecific binding and incubate for 10 min.

4.1.3. Ensure to add enough volume during each incubation period to fill the flow chambers and

form droplets at the inlets and outlets. Adjust incubation volumes accordingly and perform all incubations in a humidity chamber.

4.1.4. Add 40 µL of Tween 20 (5% v/v) to the flow chamber. Incubate for 10 min to further reduce nonspecific binding.

4.1.5. (OPTIONAL) Check the surface for adequate passivation by flowing polystyrene beads through the channel. Add 40 µL of ProtG coated polystyrene beads (0.01% w/v) and image using a darkfield microscope with 10x objective. If beads do not adhere to the surface, proceed to the next step.

4.1.6. Wash the channel with 200 µL of wash buffer to remove all passivation agents.

4.2 Chamber surface functionalization

4.2.1 Add 40 µL of streptavidin (100 µg/mL) to the flow chamber and incubate for 20 min. Then, wash with 200 µL of wash buffer.

4.2.2 Add 40 µL of hybridized ProtG-TGT (100 nM) to the flow chamber and incubate for 20 min. Then, wash with 200 µL wash buffer.

4.2.3 Add 40 µL of ProtG-TGT top strand (100 nM) for 20 min to complete any unhybridized TGT bottom strand on the surface. Wash with 200 µL of wash buffer.

4.2.4 Add 40 µL of P-selectin-Fc (10 µg/mL) to the flow chamber and incubate for 60 min. Then, wash with 200 µL of wash buffer.

NOTE: Incubation duration is critical.

5. Experiment and imaging

5.1 Flow system setup

5.1.1. Fill a 5 mL glass syringe with the rolling buffer (HBSS with 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES, 0.1% BSA). Ensure there are no air bubbles in the syringe by tapping the sides of the syringe to dislodge the bubbles and pushing them out as they float towards the tip.

5.1.2. Insert a sterile needle (26 G, 5/8 Inch Length) into a ~200 mm of polyethylene tubing (I.D: 0.38 mm; O.D: 1.09 mm) and connect the needle to the glass syringe.

NOTE: Ensure no air is trapped anywhere in the needle connector.

5.1.3. Fix the syringe onto the syringe pump and tilt the syringe pump such that the plunger side is elevated to prevent air bubbles from entering the channel. Insert the end of the tube into the

flow chamber inlet.

NOTE: Ensure liquid to liquid contact when making the connection by depositing droplets onto the inlets and having droplets at the end of the syringe tubing. Ensure no air bubbles enter the channel by allowing a small drop to form on the end of the syringe tubing before inserting it into the inlet.

5.1.4. Insert one end of another 200 mm of the polyethylene tubing into the outlet, and the other end submerged in a waste beaker.

5.2 Setting up for cell rolling

5.2.1. Grow HL-60 cells in 25 cm^2 ventilated culture flasks in IMDM media supplemented with 20% fetal bovine serum and 1% antibiotics at 37 °C with 5% CO_2. Maintain cell densities between 1 x 10^5–2 × 10^6 cells/mL.

5.2.2. (OPTIONAL) Differentiate the HL-60 cells in a complete IMDM medium containing 1.25% DMSO at an initial density of 2 x 10^5 cells/mL. Incubate cells to be most active for 5–6 days.

5.2.3. Take a sample (1–2 mL) from the cell suspension and centrifuge (200 x *g*, 3 min) to pellet the cells.

5.2.4. Remove the medium and gently resuspend the cells in 500 µL of the rolling buffer. Repeat the wash step twice to remove cellular debris.

5.2.5. Measure the cell density with a hemocytometer. Resuspend the cell pellets with rolling buffer to a density of 2 x 10^5 cells/mL.

5.2.6. Carefully disconnect the tubing from the inlets/outlet and pipette 40 µL of the cell suspension into the flow chamber. Reconnect the tubing as described previously, ensure no bubbles are introduced into the flow channel.

5.2.7. Begin cell rolling experiment by starting the syringe pump at desired flow rates.

NOTE: The intensity of fluorescent tracks depends on the shear stress, and lower cell velocity shear stress/cell velocity will generally produce dimmer tracks (**Figure 4A**). Avoid high cell density on the surface or excessive rolling duration to ensure separable single-cell tracks (**Figure 4B**,**C**).

5.2.8. Use a darkfield microscope with a 10x objective to ensure cell rolling is observed.

5.2.9. Once the experiment is completed, remove the cells from the channel by infusing the rolling buffer at 100 mL/h until the surface is cell-free.

5.3 Imaging local tracks by "DNA-based Point Accumulation for Imaging in Nanoscale Topography" (DNA-PAINT)

5.3.1. Add 40 µL of DNA-PAINT imager strand (500 pM) in DNA-PAINT buffer (0.05% Tween-20, 5 mM Tris, 75 mM $MgCl_2$, 1 mM EDTA) to the channel.

5.3.2. Perform Total internal reflection fluorescence (TIRF) microscopy using a 100x oil-immersion TIRF objective lens. Acquire 40000+ frames with 25 ms exposure time per frame at an electron-multiplying (EM) gain of 300 using an Electron-multiplying charge-coupled device (EMCCD) camera.

5.3.3. Use Picasso software package[17] to localize and render the super-resolution images (**Figure 4D**) following steps 5.3.4–5.3.5.

5.3.4. Load the DNA-PAINT movie into Localize program to determine the localization of each fluorophore in every frame.

NOTE: Optimize box side length and Min. Net Gradient parameters until only fluorophores are accurately tracked. Min. Net Gradient parameter can often go above 100000 to achieve optimal tracking. Fit setting: MLE, integrated Gaussian method produces the best result. Lastly, if the movie is too long, split it into stacks of 10000 frames in order for the preview tracking in Localize to work properly before recombining them into a final hdf5 file.

5.3.5. Then, load the resulting hdf5 file into the Render program to perform drift correction and rendering.

NOTE: Perform multiple drift correction via **Undrift by RCC** to improve the final result.

5.4 Imaging long tracks by permanent labeling

5.4.1. Add the permanent imager strand and incubate for 120 s in T50M5 buffer. Wash the channel by infusing 200 µL of wash buffer.

5.4.2. Record an image with the excitation laser off to obtain background camera noise. Image a large area in a grid pattern by TIRF microscopy.

NOTE: Frame-over-frame overlap of $\geq$10% is recommended for subsequent stitching.

5.4.3. Program the microscope to scan over the area of 400 x 50 images (20000 images in total). Using FIJI program, split raw data into individual tiff files, each containing a maximum of 10000 images.

5.4.4. Flatten all images using the illumination profile (**Figure 3A–C**) following steps 5.4.5–5.4.7.

5.4.5. Subtract the background camera noise from every frame. Obtain the mean stack projection (illumination profile) of every background-subtracted frame.

5.4.6. Normalize the illumination profile by its max value. Divide every background-subtracted frame by the normalized illumination profile.

5.4.7. Rescale the corrected frames to the appropriate range for the corresponding bit depth.

5.4.8. Use MIST[18] to stitch the images (**Figure 3D**,**E**).

REPRESENTATIVE RESULTS:

The protocol above describes the experimental procedure of the adhesion footprint assay. The general experiment workflow is illustrated in **Figure 1**, from the flow chamber assembly (**Figure 1A**) to the surface functionalization (**Figure 1B**) and experiment and imaging steps (**Figure 1C**).

Figure 2 is a representative result for the ProtG-ssDNA bioconjugation characterization. The UV/Vis spectra of three components in the final product, namely, ProtG, mal-ssDNA, and imidazole elution buffer, were collected prior to the final conjugation (**Figure 2A**), each corresponding to a known concentration. These spectra form the orthogonal basis for fitting to the bioconjugation product spectrum, where the three unknown parameters are their concentrations. A custom function in MATLAB was used to determine the concentrations. The results show a nearly 1:1 ratio of ProtG to ssDNA (**Figure 2A**). This is as expected because the ssDNA has only one amine modification, and the ProtG has a single cysteine engineered at its C-terminus. This approach is more advantageous to the previously reported approach[19] using a single thiol modified DNA to target the multitude of primary amines on ProtG, where the conjugation ratio cannot be easily maintained.

Additionally, native PAGE was used to confirm the bioconjugation (**Figure 2B**). DNA is stained by GelGreen and proteins by Coomassie blue, respectively. As GelGreen stains dsDNA more strongly than ssDNA, it is expected that any ssDNA bands to be dimmer than the equal molar concentration of dsDNA bands (lanes 3, 4). Because the stock ProtG contains a C-terminal cysteine residue, a fraction of the proteins form dimers through a disulfide bond, as seen in lane 5 (**Figure 2B**). The reduced ProtG, on the other hand, shows a single band (lane 6). When using the stock ProtG in the DNA conjugation directly, the disulfide dimerized ProtG does not react to the DNA and shows as a band without any GelGreen staining (lanes 7, 8). The ProtG dimer band disappears in the conjugation product using reduced ProtG (lanes 9, 10). Because an excess of mal-ssDNA to ProtG (1.5:1) is used during the conjugation, TGT only bands are visible in the final conjugation product (lanes 8, 10). The bright GelGreen bands coinciding with the monomeric ProtG band indicate successful conjugation and good yield.

Figure 3 illustrates representative raw microscopy images and the workflow to correct them for subsequent image-stitching and analysis. The TIRF illumination profile introduced from a single-mode fiber is generally brighter in the middle of the field of view and dimmer around the edges (**Figure 3A**,**B**). To compensate for the uneven illumination and flatten the images for quantitative

analysis, the illumination profile was determined by averaging thousands of individual frames (**Figure 3B**). Flattened images were produced by subtracting the camera noise from both raw and illumination profiles and then normalizing by the illumination profile (**Figure 3C**). The effect of the image flattening is clearly illustrated when the images are stitched to form a large image. Image intensity in the background regions without any cell tracks shows clear periodic patterns corresponding to the uncorrected images (**Figure 3D**). The same field of view stitched from flattened images produces a flat background (**Figure 3E**). Having a flat background is critical for interpreting the intensities fluctuations along a cell track. As a first experiment, a ramp-up flow profile similar to the one illustrated in **Figure 3F** is used to determine the range of shear stress suitable for the experiment ensuring both stable cell rolling and clear fluorescent cell tracks. A typical single-cell adhesion footprint under this flow profile is shown in **Figure 3G**, where the intensity increases as the shear stress increases until the cell can no longer sustain rolling at high shear stress and detach from the surface, marking the end of a single track.

Figure 4 illustrates potential outcomes from suboptimal to optimal representative experimental results. **Figure 4A** illustrates a suboptimal outcome where the fluorescent tracks have a low signal-to-noise ratio. This is likely caused by either a low surface density of the fully conjugated probes or a low flow rate. **Figure 4B** illustrates another suboptimal outcome where the fluorescent tracks are too densely packed to resolve and isolate individual tracks for subsequent analysis. **Figure 4C** is an example of a good outcome where individual tracks are resolvable over a long distance and clear against the background. **Figure 4D** is an example of the diffraction-limited TIRF image (left half) in comparison to the same track imaged by DNA-PAINT (right half). The DNA-PAINT in this setup produces a NeNA (nearest-neighbor-based analysis) precision of 28.8 nm.

FIGURE AND TABLE LEGENDS:

Figure 1: Experiment workflow of the adhesion footprint assay. (A) Assembly of the flow cell and flow chamber. **(B)** Surface passivation and functionalization. Each incubation step is marked by the duration, followed by a wash step. **(C)** Cell rolling experiment and imaging. Cells rolling on the surface will unzip the DNA where adhesion interactions form, leaving ssDNA on the surface that marks the location of each adhesion event. The surface is labeled with the permanent imager ssDNA for extensive area imaging, requiring 2 min staining before washing off. For super-resolution DNA-PAINT imaging, the imager strand is kept in the buffer.

Figure 2: Bioconjugation characterization. (A) UV-VIS absorbance of the Ni-NTA purified bioconjugation product (blue) and the curve fit (red) to determine the conjugation ratio. The absorption spectra of ProtG (magenta), mal-ssDNA (green), and imidazole (gray) were used as components to create the best fit (red) to the product spectrum (blue). The residue of the fit is shown as the black dashed line. This allows us to determine the concentration of ProtG and ssDNA in the purified bioconjugation product and their molar ratio. **(B)** Native PAGE of components in the bioconjugation procedure. The first lane shows a low molecular weight DNA ladder (25, 50, 75, 100, 150, 200, 250, 300, 350, 500, 766 bp). The gel image is false-colored, with DNA-staining GelGreen in green and Coomassie blue protein stain (inversed) in magenta.

Figure 3: Image processing and representative results from extensive area imaging. (A) Thousands of raw TIRF images tiling an extensive area. **(B)** Illumination profile derived from the raw images. **(C)** Corrected images by flattening the illumination profile. **(D)** Stitched image from raw image tiles. The uneven illumination profile can be seen as periodic patterns in the image. The blue and red boxes indicate the image sections where the mean intensity profiles are projected (blue and red traces). The mean intensity values (arbitrary unit) represent those of 8-bit images (0-255). **(E)** Same area as (D) but stitched using flattened images. The projections do not show any large-scale periodic patterns. **(F)** The shear stress profile to use in the experiments to determine the range of shear stresses that result in cell rolling and yield fluorescence tracks. **(G)** A sample fluorescent track from a single cell under the flow profile illustrated in (**F**). The cell travels from left to right, the fluorescence intensity increases as the shear stress ramps up until the cell can no longer sustain rolling and detaches from the surface.

Figure 4: Representative suboptimal and optimal results. (A) An example of fluorescent tracks with insufficient contrast. **(B)** An example of excessive fluorescent track density. **(C)** An example of optimal track density and contrast. All three images were acquired under the same condition, flattened and stitched. The red boxes in each image represent the area where the intensity projections (right) were taken. **(D)** A fluorescent track shown in diffraction-limited (left half) and DNA-PAINT (right half) imaging.

Table 1: Troubleshooting. The table lists the possible reasons and solutions for problems occurring when performing this assay

Supplemental Coding File 1: The custom-written MATLAB script to decompose the final product spectrum based on the three spectra collected previously (ProtG, SMCC-strand, Ni-NTA bead elution buffer) to determine the conjugation efficiency and ratio of ProtG to ssDNA.

DISCUSSION:

The adhesion footprint assay enables visualization of the molecular adhesion events between PSGL-1 and P-selectin during cell rolling adhesion. This process is initiated by P-selectin-mediated capturing followed by rolling under fluidic shear stress. Potential issues during the experiment usually involve poor cell rolling or missing fluorescent tracks even when cells roll well. These problems are often resulting from quality controls at the critical steps in the protocol, as listed in the troubleshooting table (**Table 1**).

Biomolecules and buffers are required to be filtered and stored at 4 °C to prevent contamination because surface preparation involves multiple steps. High-quality surface passivation is a requirement to achieve appropriate surface functionalization density and reduce the nonspecific binding of biomolecules. Nonspecific binding of biomolecules to the surface can create a high fluorescence background, interfering with the single-molecule fluorescence imaging and statistical data analysis. Multiple factors can affect surface passivation. Hydrolysis of aminosilane and PEG-NHS yield much lower efficiency of PEGylation. Sufficient KOH washing and piranha cleaning enhance the hydrophilicity by generating free hydroxyl groups on the glass surface, increasing the density of the chemically reactive group. Cells would be stuck on poorly passivated

surfaces. The quality of surface passivation is checked by measuring background fluorescence intensity before and after PEGylation using fluorescent biomolecules.

The surface density of ligands is a critical factor for cell rolling, which is controlled by the PEG: PEG-biotin ratio, TGT hybridization, and P-selectin binding. In this system, a PEG: PEG-biotin ratio of 20:1 is sufficient for surface functionalization with sufficient P-selectin for cell rolling. Efficient TGT hybridization also improves the surface density and the signal-to-noise ratio of the fluorescent tracks. This protocol includes a replenishment step of top-strand-TGT-ProtG to ensure any unhybridized TGT bottom strand is complemented before experiments. Conjugation of DNA to ProtG also affects the surface density. Sulfo-SMCC linker was added to DNA at 10-fold molar excess so that all DNA reacted with the linker. ProtG with a single cysteine residue (ProtG-Cys) at the C-terminus was used to achieve a 1:1 DNA: ProtG conjugation ratio. Because the ProtG-Cys can form dimers through disulfide bonds, treatment of TCEP is needed to reduce the disulfide bond before sulfhydryl-reactive cross-linking reactions. Protein-conjugated DNA and TGT hybridization can be validated with native PAGE analysis, in which conjugate DNA and DNA duplex will show the retarded mobility due to the increase of molecular weight. Assembly efficiency can also be estimated by gel densitometry (**Figure 2B**). The careful PEGylation and bioconjugation processes are crucial for producing a consistent surface. Occasionally, cell state may affect the cell rolling and track formation. Although PSGL-1 expression over cell density has not been reported, cell density is a potent regulator of the cell cycle and protein expression during the growth phase.

Given that PSGL-1 also functions as a signal transduction receptor and regulates cell proliferation[20,21], culture conditions such as cell density are maintained for consistent expression level and binding ability of PSGL-1. Attachment of P-selectin-Fc mediated by ProtG onto the surface is crucial to the adhesion and rolling of cells. The binding kinetics of P-selectin to ProtG is dependent on the concentration. Lower concentrations of P-selectin lead to an increase of time to reach equilibrium. At least 30 min is required for saturation binding for concentrations lower than 100 nM[22]. 10 nM of P-selectin was used to reduce the nonspecific binding to the surface and increased incubation time for sufficient interaction with ProtG. A 1 h incubation is enough time to induce cell adhesion and rolling in this system.

The TGT and its corresponding force-dependent lifetime is an important factor in the results of this assay. During rolling adhesion, the force on the tether is transmitted through both the TGT and the P-selectin: PSGL-1 interaction. Each of these individual components has a unique force-dependent lifetime, and depending on the applied force, the rupture probability will favor one over the other. For example, it has been shown that when using the TGT described in this article, at forces below 13.6 pN, P-selectin: PSGL-1 primarily dissociates, whereas above 13.6 pN, the TGT primarily dissociates[13]. This is important to understand when performing this assay because if the shear stress is too low or the beads are rolling too slow, the rupture events will primarily be the P-selectin: PSGL-1 interaction, and there will be minimal or no measurable fluorescence signal from the TGTs. The tension threshold of the TGT will also influence the results. If the TGT ruptures at too high of a force, the rupture events will primarily be P-selectin: PSGL-1, and there will be minimal fluorescence signal.

The method described here allows for the analysis of the molecular rupture forces, as well as the locations of molecular adhesion events involved in rolling adhesion. Instead of real-time detection of adhesion, the most significant advantage of this method is that it allows for post-experiment imaging and analysis. Once the adhesion footprint has been left on the surface in the form of ssDNA, the tracks can still be imaged after 12 h if the flow channels are maintained in a 4 °C fridge in a dark humidity chamber with both inlets and outlets blocked to prevent drying. The interpretation of the fluorescence readout for this assay is dependent on the chosen imaging method. Through super-resolution imaging, this assay achieves high spatial resolution (<50 nm) that allows for quantitative analysis of the density of ruptured TGTs[13]. The analysis of receptor density or ruptured TGT density would be useful in investigating rolling adhesion behavior under different conditions. Contrarily, diffraction-limited imaging does not provide a high spatial resolution; however, it allows a large surface area to be imaged to analyze the fluorescence tracks of multiple beads over hundreds of fields of view. This is advantageous as the fluorescence intensity of a track can be analyzed for a single bead over a large distance providing information on changes in rolling behavior over time. Such an example is changing the shear stress over time and observing the corresponding changes in fluorescence intensity. Recently, it has been shown that through a simple model, the fluorescence intensity of the tracks can be used to estimate the molecular force distribution[13]. There is also potential application of ratiometric methods to achieve force quantification with this assay[23].

Because cell rolling happens rapidly (10s of µm/s) and over an extended distance (1000s of µm), studying their molecular tension has been challenging with traditional real-time molecular tension sensors. The adhesion footprint assay breaks this demanding constraint to allow for post-event imaging. Although the TGT rupture event does not directly report the magnitude of tension experienced prior to rupture, promising developments have been made in the analysis of the fluorescence tracks to allow for the quantitative investigation of the molecular forces involved in rolling adhesion[13,23,24].

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors declare no conflict of interest.

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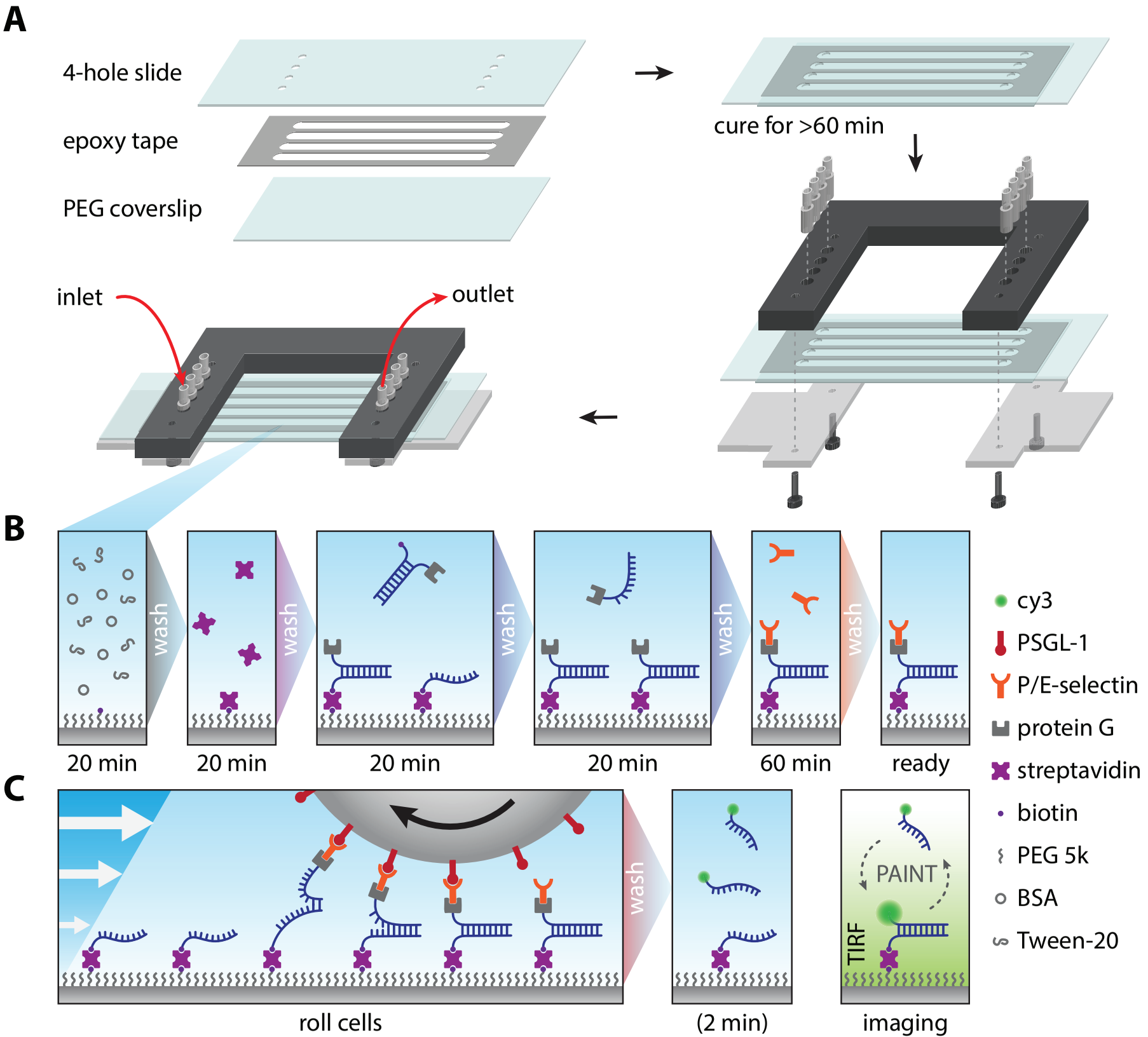

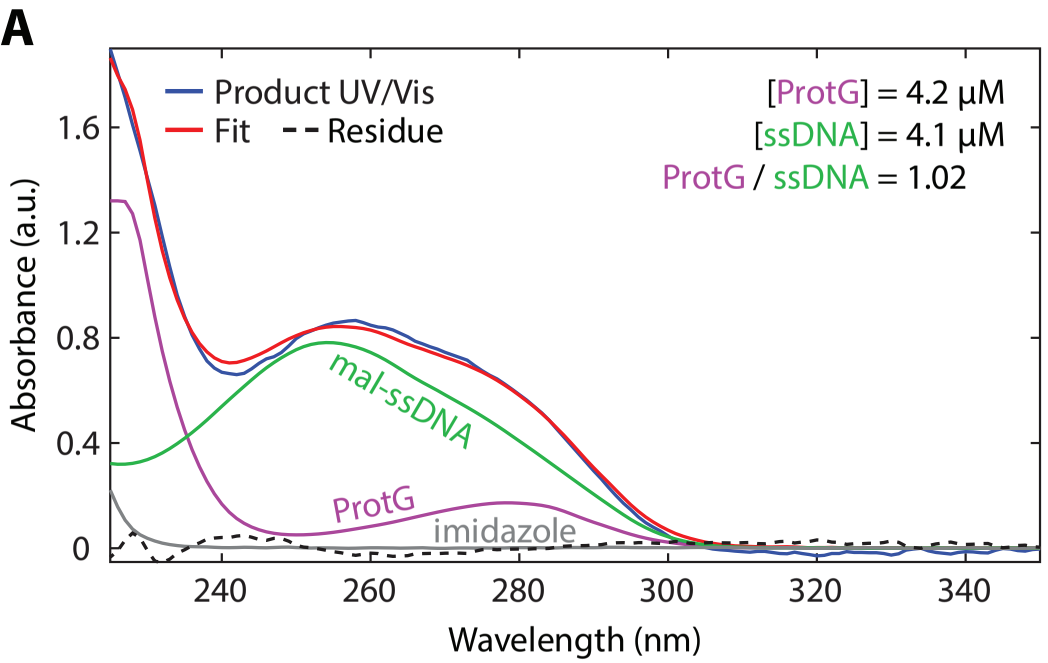

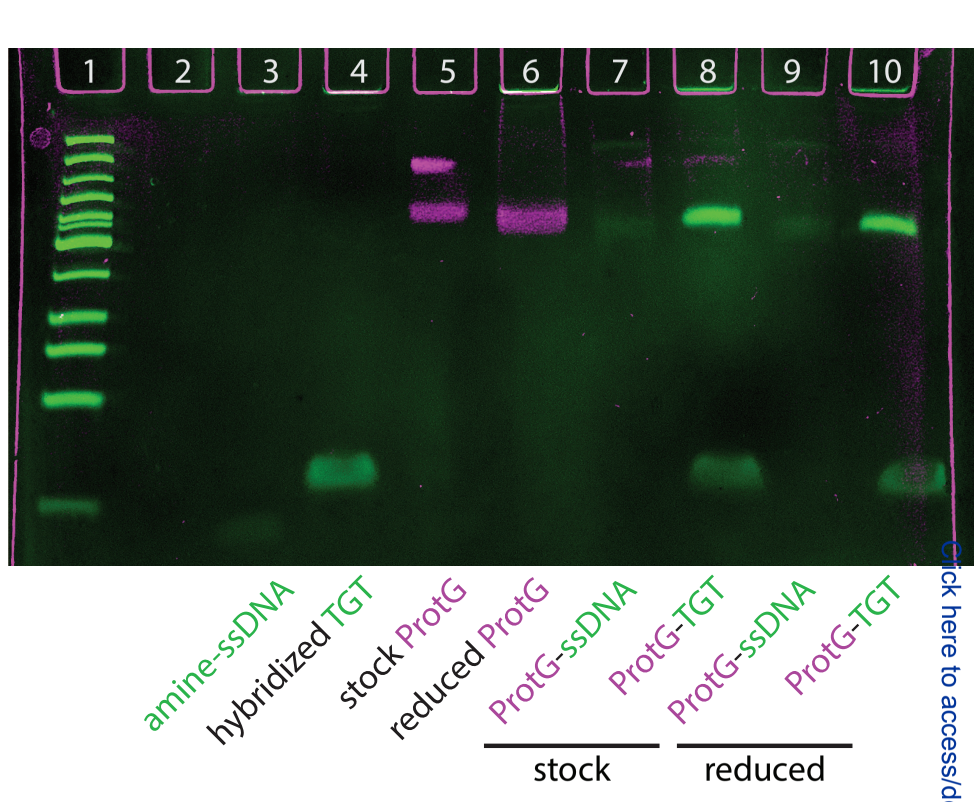

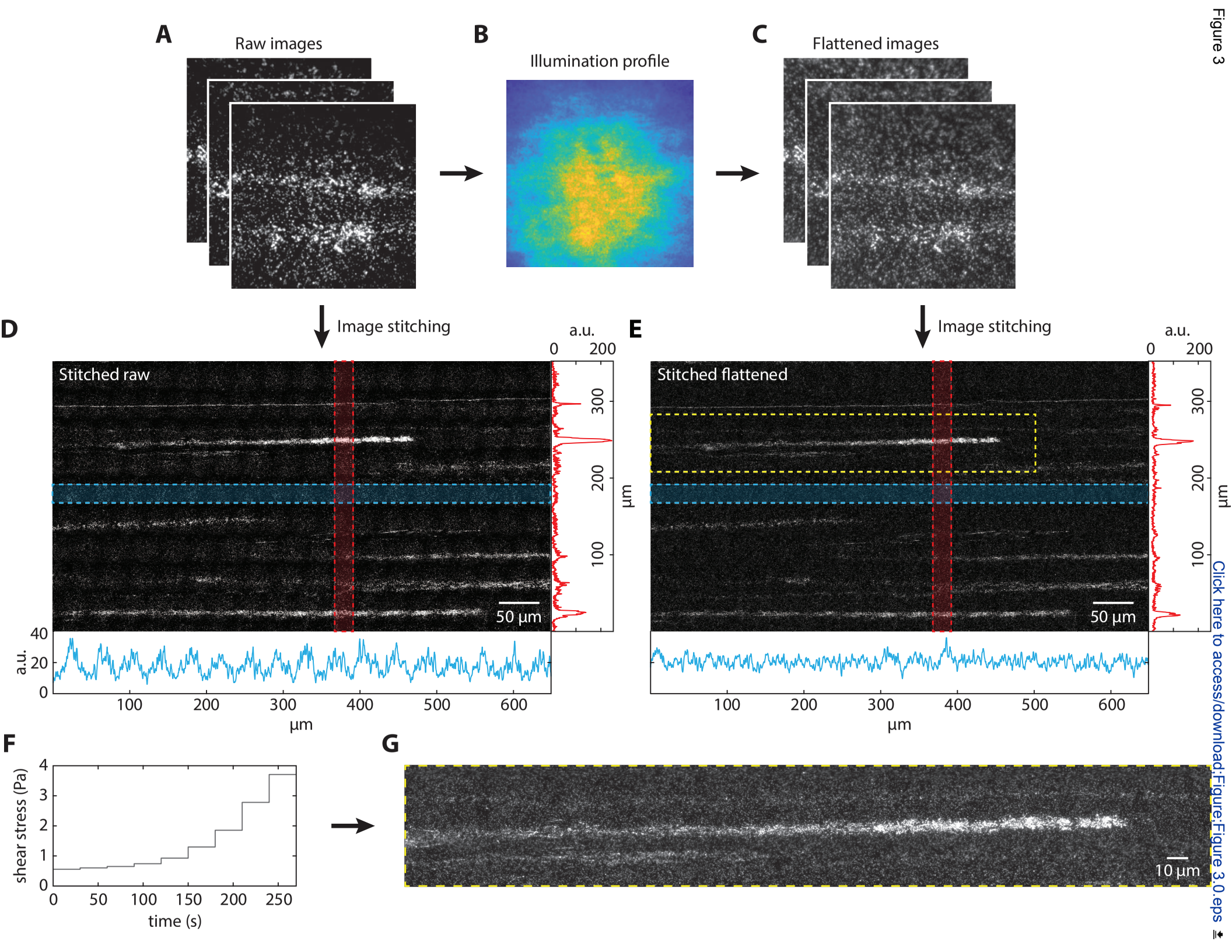

Figure 4

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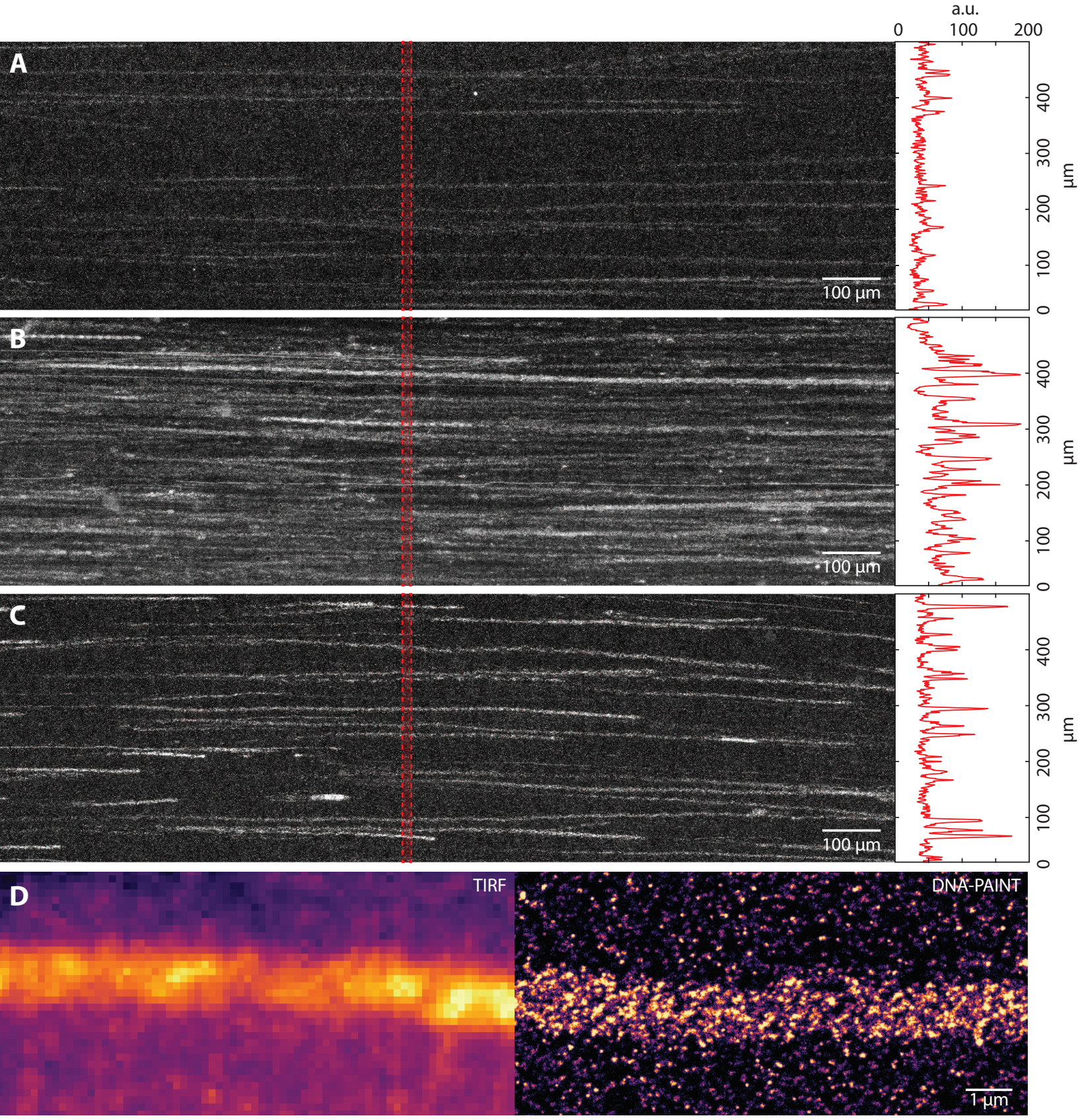

Problem	Possible Reason
Epoxy tape not cut properly	Epoxy layer too thick Laser engraver power and speed not optimized
Bubbles stuck on sides of flow chamber	Improper initial liquid introduction
Bubbles pass through flow chamber	Improper liquid introduction through inlet and outlet Bubbles from the syringe got into the flow line
Liquid cannot enter channels	Inlets screwed on too tight
Channel leakage	Epoxy hasn't cured completely Inlets not sealing properly
Cells stuck	Poor PEGylation leading to non-specific binding Surface passivation destroyed by large air bubbles passing through the channel Problem with the cells
Cells do not or have sparse interactions with the surface	P-selectin density too low, as a result of poor surface functionalization and/or poor bioconjugation
Cells roll but do not produce fluorescent tracks	Adhesion interactions too weak to rupture TGT Bad quality surface leads to high background fluorescence and insufficient contrast to see tracks

Solution
Thin the epoxy layer as much as possible with the razer blade Optimize laser engraver power and speed
Push buffer solutions through channel at high flow rate to wash the bubbles out
Ensure a liquid bubble form at the inlet tubing when introducing liquid through tubing or pipette Tilt the syringe pump to ensure bubbles are trapped at the plunger end
Adjust to optimize seal. The inlet tubing should just touch the channel opening when screwed in properly.
Re-make channels, ensure using 5 min fast curing epoxy and let cure for at least 1 h Adjust to optimize seal
Ideally, remake PEG. Additionally, can try to incubate additional blocking agents (BSA & Tween-20) and add blocking agents to wash buffer. Ensure no bubbles go through channel Use HL-60 2 weeks after restarting cell culture from frozen. Confirm cell rolling on control surface with only P-selectin.
First, use ProtG-biotin instead of ProtG-TGT as a control to determine whether the problem is due to bioconjugation or surface biotin density.After ensuring bioconjugation quality and surface biotin density, increase TGT-ProtG and P-selectin-Fc concentration and incubation time.
Increase flow rate during rolling experiment to increase the interaction force. We recommend an initial ramp up flow profile (**Figure 3F**) to find the optimal flow rate that produces both stable rolling and fluorescence tracks (**Figure 3G**) Check surface passivation

Response to Review

We thank the editors and reviewers for the comments that helped improve this article.

All comments were responded in blue text. Changes to the main text were marked red in this response letter.

All changes were marked blue in the revised manuscript to track changes.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

Response: We have thoroughly proofread the manuscript and addressed the issues listed below from the editorial office and reviewers.

2. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript (this includes the figures and the legends) and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. Avoid advocating the use of any particular product, e.g., line 321. But if you have optimized your protocol with certain commercial products (reagents such as dyes, software (MATLAB, DNA-PAINT) etc), mention them once and then switch to a more generic term that you can mention in the Table of Materials in the comments column next to that material.

Response: We have removed all commercial language and mentioned optimized our protocol with commercial products in the Table of Materials.

3. Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Response: We have revised the manuscript and removed all personal pronouns.

4. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

Response: We have revised the protocol and included all safety procedures.

5. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

Response: We have edited the sections to include sufficient details for video production.

6. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step,

substep and note in the protocol section. Please use Calibri 12 points and one-inch margins on all the side. Please include a ONE LINE SPACE between each protocol step and then HIGHLIGHT up to 3 pages of protocol text for inclusion in the protocol section of the video.

Response: We have formatted the manuscript as requested by the editorial office.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
It is critical to facilitate the transfer of mechanobiology protocols to the hands of biologists, and this is an important contribution. I anticipate the video will be extremely helpful in aspects of this protocol, especially setting up the flow chamber and in the surface preparation. This work will be greatly strengthened by more background and introduction to the assay, and description of its potential impact.

Major Concerns:
The introduction is not clear or as developed as it should be. A deeper introduction to the approach would greatly help establish expectations for this protocol. Some things that should be included are - A description of TGTs and how they measure forces, a description of the set up including the details of functionalized surface presenting ligand which cells will be flowed over and then imaged, and how surfaces be imaged using two different types of imager strands and why you would pick one method or the other. For a reader who has never made a tension probe surface before, the protocol itself will not provide enough context as it currently stands.

Response: We thank the reviewer for this comment and agree that the introduction could be further developed. To address this comment, we have added the following to the introduction:

Line 90: ***"****Therefore, implementing a molecular force sensor capable of accurately measuring selectin mediated interactions is crucial to improving our understanding of rolling adhesion. Here, we describe the protocol for the adhesion footprint assay where PSGL-1 coated beads are rolled on a surface presenting p-selectin functionalized tension gauge tethers (TGTs)[11]. These TGTs are irreversible DNA-based force sensors that result in a permanent history of rupture events in the form of fluorescence readout. This is achieved through the rupturing of the TGT (dsDNA) and then subsequent labeling of the ruptured TGT (ssDNA) with a fluorescently labelled complementary strand. One major advantage of this system is its compatibility with both diffraction limited and super-resolution imaging. The fluorescently labelled complementary strand can either be permanently bound (> 12 bp) for diffraction limited imaging, or transiently bound (7-9 bp) for super-resolution imaging through DNA PAINT. This is an ideal system to study rolling adhesion as the TGTs are ruptured during active rolling, but the fluorescence readout is analyzed post-rolling. The two imaging methods also provide the user with more freedom to investigate rolling adhesion. Typically, diffraction limited imaging is useful for extracting molecular rupture force through fluorescence intensity[11], whereas super-resolution imaging allows for quantitative analysis of receptor density. With the ability to investigate these properties of rolling adhesion, this approach provides a promising platform for understanding the force-regulation mechanism on the molecular adhesion of rolling cells under shear flow."*

The authors should discuss what can be learned from this assay. A specific discussion of readouts, and what can be interpreted from the fluorescent traces (super-res and diffraction limited). Why would DNAPAINT or widefield be chosen as the readout? How does intensity relate to forces?

Define the tension threshold of the TGTs. The ability of cells to roll on the surface is based on transient adhesions. However, the signal is obtained at regions where TGT is ruptured, terminating the interaction with the surface. This is directly dependent on the underlying force threshold which can influence rolling dynamics. Hence a discussion of the threshold for the TGT employed, and how is critical.

Response: We thank the reviewer for these suggestions and have added the following to the text. (please refer to the line XXX)

Line 557: *"The TGT and its corresponding force dependent lifetime is an important factor in the results of this assay. During rolling adhesion, the force on the tether is transmitted through both the TGT and the P-selectin:PSGL-1 interaction. Each of these individual components has a unique force dependent lifetime, and depending on the applied force, the rupture probability will favor one over the other. For example, it has been shown that when using the TGT described in this article, at forces below 13.6 pN, P-selectin:PSGL-1 primarily dissociates whereas above 13.6 pN, the TGT primarily dissociates 13. This is important to understand when performing this assay because if the shear stress is too low, or the beads are rolling too slow, the rupture events will primarily be the P-selectin:PSGL-1 interaction and there will be minimal, or no measurable fluorescence signal from the TGTs. The tension threshold of the TGT will also influence the results. If the TGT ruptures at too high of a force, the rupture events will primarily be P-selectin:PSGL-1 and there will be minimal fluorescence signal.*

The method described here allows for the analysis of the molecular rupture forces, as well as the locations of molecular adhesion events involved in rolling adhesion. Instead of real-time detection of adhesion, the most significant advantage of this method is that it allows for post-experiment imaging and analysis. Once the adhesion footprint has been left on the surface in the form of ssDNA, the tracks can still be imaged after 12 hours if the flow channels are maintained in 4C fridge in a dark humidity chamber with both inlets and outlets blocked to extend the lifetime of the track up to 12 hours. The interpretation of the fluorescence readout for this assay is dependent on the chosen imaging method. Through super-resolution imaging, this assay achieves high spatial resolution (<50 nm) that allows for quantitative analysis of the density of ruptured TGTs 13. The analysis of receptor density or ruptured TGT density would be useful in the investigation of rolling adhesion behaviour under different conditions. Contrarily, diffraction limited imaging does not provide high spatial resolution, however, it allows a large surface area to be imaged to analyze the fluorescence tracks of multiple beads over hundreds of fields of view. This is advantageous as the fluorescence intensity of a track can be analyzed for a single bead over a large distance providing information on changes in rolling behaviour over time. Such an example is changing the shear stress over time and observing the corresponding changes in fluorescence intensity. Recently, it has been shown that through a simple model, the fluorescence intensity of the tracks can be used to estimate the molecular force distribution 13. There is also potential application of ratiometric methods to achieve force quantification with this assay 23.

Because cell rolling happens rapidly (10s of μm/s) and over an extended distance (1000s of μm), studying their molecular tension has been challenging with traditional real-time molecular tension sensors. The adhesion footprint assay breaks this demanding constraint to allow for post-event imaging. Although the TGT rupture event does not directly report the

magnitude of tension experienced prior to rupture, promising developments have been made in the analysis of the fluorescence tracks to allow for the quantitative investigation of the molecular forces involved in rolling adhesion 13, 23, 24."

Technical steps that need more description/clarification
1) Describe the algorithm and/or make available the custom MATLAB script to estimate the conjugation efficiency and purity of the ProtG-ssDNA (Line 135).

Response: We appreciate the reviewer's comments. The algorithm for estimation of ProtG-ssDNA conjugation efficiency has been described in protocol step 1.4 as shown below. The custom MATLAB script will also be made available.

Line 164: "1.4.4. *To determine the conjugation efficiency and ratio of ProtG to ssDNA, a custom-written MATLAB script is used to decompose the final product spectrum based on the three spectra collected previously (ProtG, SMCC-strand, Ni-NTA bead elution buffer), briefly, the code works as described below. The typical concentration is 4 µM of ProtG-ssDNA with ProtG and ssDNA at a ~1:1 molar ratio. (Figure 2A)*

a. *ProtG, SMCC-strand, Ni-NTA bead elution buffer, and the ProtG-ssDNA UV/Vis spectra are input into the MATLAB script*
b. *A multidimensional unconstrained nonlinear minimization is performed to reconstruct the ProtG-ssDNA spectra from the source spectra (ProtG, SMCC-strand and Ni-NTA bead elution buffer spectra)*
c. *The minimization function outputs three transformation factors, one for each source spectra.*
d. *Upon multiplying the spectra by its corresponding factor and combining the transformed source spectra, the ProtG-ssDNA spectra is reconstructed*
e. *The initial concentration of the ProtG and SMCC-strand is multiplied by the corresponding transformation factors to determine the concentrations of SMCC-strand and ProtG in ProtG-ssDNA product."*

2) Are there any guidelines or tips for using Picasso to generate the super-resolution images? (Line 313)

Response: We have incorporated the additional guideline for using Picasso:

Line 376: "5.3.4. *Use Picasso software package to localize and render the super-resolution images (Figure 4D).*

a. *Load DNA-PAINT movie into Localize program to determine the localization of each fluorophore in every frame.*
   NOTE: Optimizing box side length and Min. Net Gradient parameters until only fluorophores are accurately tracked. Min. Net Gradient parameter can often go above 100000 to achieve optimal tracking. Fit setting: MLE, integrated Gaussian method produces the best result. Lastly, if the movie is too long, split it into stacks of 10000 frames in order for the preview tracking in Localize to work properly before recombining them into a final hdf5 file.

b. *The resulting hdf5 file is then loaded into Render program where drift correction and rendering are performed.*
NOTE: Multiple drift correction via "Undrift by RCC" can be done to improve the final result."

3) Include parameters for image acquisition of longer tracks with permanent labeling and image stitching. How many images are stitched (abstract mentions thousands)? Are there issues with acquiring or managing such large image files? (Line 315)

Response: We have included the detailed image acquisition of longer tracks:

Line 397: *"Program the microscope to scan over the area of 400 x 50 images (20000 images in total). Using FIJI program, split raw data into individual tiff files, each containing a maximum of 10000 images."*

4) How is the illumination profile image acquired and applied?

Response: We have mentioned this in the text (please refer to reviewer 3 comments).

5) "Tracks remain visible for 12 hours if appropriately kept" What are appropriate conditions? (476)

Response: We have added the appropriate conditions for extending the visibility of tracks to the manuscript.

Line 573: *"Once the adhesion footprint has been left on the surface in the form of ssDNA, the tracks can still be imaged after 12 hours if the flow channels are maintained in 4°C fridge in a dark humidity chamber with both inlets and outlets blocked to prevent drying."*

Minor Concerns:
Figure 4D: The figure legend claims that the same track is imaged by TIRF and DNA-PAINT but there seems to be a discrepancy in the orientation of the tracks? Please verify.

Response: Sorry about the confusion. The image is showing a continuous track from the left (TIRF) to the right (DNA-PAINT), they are not the same image. We edited the figure caption to ensure this won't confuse readers.

Line 505: ***"(D)*** *A fluorescent track shown in diffraction limited (left half) and DNA-PAINT (right half) imaging."*

The authors briefly introduce the failure of conventional mechanobiology tools to measure individual adhesion events with temporal precision and sensitivity when studying cell rolling. Expand on this.

Response: We have expanded the introduction with the following text:

Line 74: ***"****However, because cell rolling is fast and dynamic, conventional experimental*

mechanobiology methods are unsuitable for studying molecular interactions during cell rolling. While single-cell and single-molecule manipulation methods like the atomic force microscopy and optical tweezer were able to study the molecular interactions such as P-selectin's force-dependent interaction with PSGL-1 at the single-molecule level 9, they are unsuitable for investigating live adhesion events during cell rolling. Additionally, the interaction characterized in vitro cannot directly answer the question about molecular adhesion in vivo. For instance, what molecular tension range is biologically relevant when cells are functioning in their native environment? Computational methods such as adhesive dynamics simulation 10 or simple steady state model 11 have captured certain molecular details and how they influence the rolling behaviour, but are highly dependent on the accuracy of the modelling parameters and assumptions. Other techniques such as traction force microscopy can detect forces during cell migration, but do not provide sufficient spatial resolution or quantitative information on molecular tension. and None of these techniques can provide direct experimental observations of the temporal dynamics, spatial distribution, and magnitude heterogeneity of molecular forces, which directly relate to cell function and behaviour in their native environment."

Reviewer #2:

Manuscript Summary:

This manuscript described detailed experimental protocol for an adhesion footprint assay that enables visualization of the molecular adhesion events between PSGL-1 and P-selectin during cell rolling adhesion. The protocol described here utilizes an irreversible DNA-based tension gauge tethers to produce fluorescence tracks of molecular adhesion events, therefore provides a method for quantitative investigation of cell rolling and adhesion events.

Major Concerns:

The manuscript provided detailed protocol description. I didn't have any major concerns.

Minor Concerns:

Two minor concerns should be addressed on this manuscript prior to publication:

1) The authors should provide full description of all the abbreviations at the first appearance. Many of them are missing. While some of them are common (i.e., PS, PSGL-1 etc.), the others such as TGT, DNA-PAINT, TCEP etc., may not be familiar to all the readers. Additionally, providing vendor of the key reagents may help readers to replicate the experiment.

Response: As suggested by the reviewer, we added the full description of all the abbreviations. Also, materials needed are provided in the Table of Materials.

2) The authors mentioned that they used customized MATLAB program to conduct data analysis. Is this program available to other researchers as well? If not, I think it would be helpful to provide an overall workflow on the program, so the other researchers can better follow this protocol.

Response: We have already mentioned this in the text (please refer to response to reviewer 1 comments).

Reviewer #3:

Manuscript Summary:

This paper presents a method to visualise cell rolling adhesion by recording fluorescent tracks resulting from PSGL-1 and P-selectin interactions. Detailed procedures for biomolecule and buffer **preparation and troubleshooting measures are provided.**

Major Concerns:

1. The manuscript would benefit from more details on image processing. For flattening images, authors averaged 'subtracted the camera noise from both raw and illumination profiles and then normalising by the illumination profile'. How was camera noise recorded? Is it recorded prior to every experiment? Is normalising by the illumination profile the same as dividing by the maximum illumination? From the caption of Figure 3C: "Corrected images by flattening the illumination profile." Is this histogram equalisation?

Response: We thank the reviewer for the suggestion and have added some more details on image processing. To clarify, normalizing by the illumination profile is not the same as dividing by the maximum illumination. We obtain an illumination profile by averaging the thousands of images collected for the stitched image. Each frame is then divided by this profile, therefore the value in which each pixel is divided by is dependent on the underlying illumination profile. The caption stating “Corrected images by flattening the illumination profile.” is not the same as histogram equalization. The term “flattening the illumination profile” is equivalent to normalizing by the illumination profile.

The following text was added to the manuscript:

Lines 394: *“5.4.5. Flatten all images using the illumination profile (Figure 3A-C).*

a. *Subtract the background camera noise from every frame.*
b. *Obtain the mean stack projection (illumination profile) of every background subtracted frame.*
c. *Normalize the illumination profile by its max value.*
d. *Divide every background subtracted frame by the normalized illumination profile.*
e. *Rescale the corrected frames to the appropriate range for the corresponding bit depth.”*

Minor Concerns:

2. Custom-written MATLAB scripts are referred to at least twice. It may be beneficial to readers to make this script public.

Response: Custom-written MATLAB scripts will be made public. Please see response to reviewer 1.

3. With regards to the use of concentrated sulphuric acid in step 2.1, should this step be done in a fume hood?

Response: We appreciate the reviewer’s careful comment. Preparation of piranha solution with concentrated sulphuric acid should be done in the fume hood. This concern has been

addressed as follows:

Line 198: *"In a fume hood, freshly prepare a piranha solution by adding 30 mL of 30% hydrogen peroxide to 90 mL concentrated (95-98%) sulfuric acid in a 250 mL beaker."*

4. Step 2.1.4 What should the temperature of the piranha solution be?

Response: We appreciate the reviewer's comment. Freshly made piranha solution may be over 100℃ in temperature, therefore, it is recommended to allow the solution to cool to approximately 80℃ prior to handling. This concern has been addressed in text as follows:

Line 204: *"Cool down the piranha solution to no more than 80℃ before pouring to prevent cracking the staining jar."*

5. Do steps 5.2 and 5.3 need to be done at a certain temperature for cell health?

Response: We appreciate the reviewer's comment. For step 5.2, growth and differentiation of cells should be done in 37℃ as stated in 5.2.1. Room temperature is sufficient for maintaining cell health during cell preparation and the rolling experiment. Cells are removed prior to imaging tracks described in step 5.3, so there are no concerns for cell health in this step.

6. Step 5.2.8 Should the pipetting of the 40 ul of cell suspension be done at a certain speed? Does it affect the speed of cell rolling?

Response: We appreciate the reviewer's comment. The pipetting speed should not affect cell rolling as most cells require a brief moment to settle onto the surface once introduced into the channel. The cell rolling velocity is controlled strictly via a syringe pump after the cells are introduced.

7. Step 5.2.10 Please specify the recommended magnification.

Response: We appreciate the reviewer's careful comment.This concern has been addressed in text as follows:

Line 366: *"Use a darkfield microscope with a 10X objective to observe cell rolling behaviour."*

8. Is the 'straightness' of cell tracks in Fig 4 representative of a typical experiment? If not, perhaps a troubleshooting step should be added.

Response: We appreciate the reviewer's comment. The straightness of the cell tracks is representative of a typical experiment.

Supplemental Coding File 1

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Supplemental Coding Files

spectraOverlap.m