**Changes made 5/19/15:**

**2) Please re-write second sentence of step 1.2 in the imperative tense, such as:  
  
1.2) Tag the proteins of interest to the PAmCherry1 fragments, for example, by using cloning plasmids listed in the materials table…**

The sentence now reads, “As one option, use the cloning plasmids listed in the materials table….”

**3) The protocol section should be composed almost entirely of discreet steps, without large paragraphs of text or “NOTES” between sections. Please move much of the text in the notes following steps 2.4.2.1, and 2.4.4 to the Discussion or a section other than the protocol.**

These “Notes” have been moved to the discussion section. A sentence has been added to direct readers to the Discussion section.

**4) Please include a reference to the Supplemental Code File in step 3.3.2.**

This code is not included in the Supplemental Code File, hence the reference to other software. Added some clarification and re-worded the sentence to make it clearer. The tracking package is in development and will likely be included in future versions of the code that will be made available on our website. **5) Please add more details describing how to perform 3.3.3 or add a citation where this process is described.**

The analysis of the tracking data would still be done with other code as referenced in the previous step (for processing). It now specifies to “use an SPT package.” A citation is also provided here for vbSPT (previously it was only in the Results section). **6) Please modify your references section to comply with JoVE instructions for authors, specifically when there are more than 6 authors, list only the first author then "et al." Currently, two authors are listed, then et al.**

Good catch. All citations with “et al.” should now only have the first author. The JoVE CSL does not seem to work well with Mendeley and Microsoft Word 2010. **7) The highlighting must include all relevant details that are required to perform the step. Step 1.1 is highlighted but the details of how to perform this step are in the subsequent steps, which are not highlighted. Editor added the words “as described below” to step 1.1. Please either remove the highlighting from step 1.1 or ensure that all relevant details are highlighted as well.**

The cloning part was left out to leave room for the rest, but we kept that first step to maintain a “cohesive story.” It has been unhighlighted since adding more steps would likely take us over the 2.75 page limit. **8) After you have made all of the recommended changes to your protocol (listed above), if required, please adjust the highlighting to identify 2.75 pages or less of text which are most important to include in the video.**

The highlighted text should now be 2.75 pages long and is reproduced below.

**2. Imaging fixed cells**

2.1.2) Plate about 5.5 x 104 of the U2OS stable expression cells per well in 350 µL of phenol red-free DMEM with 10% FBS so that cells are healthy and not over confluent when imaging.

2.1.4) Fix the cells immediately before imaging.

2.1.6) Vortex 100 nm gold particles to break up aggregates and add 35 µL per well (10x final dilution) for tracking stage drift during imaging.

2.2) Acquire images.

2.2.1) Turn on the microscope. Power on the 405 and 561 nm lasers, but keep the shutters (internal or external) closed at this point. Turn on the EMCCD camera and allow it to cool down. Ensure the 561 nm filters are in place.

2.2.2) Open the acquisition software and set the exposure time to 100 ms and the EMCCD gain to 300 (range 1 – 1000).

2.2.3) Add immersion oil to the objective and secure the sample to the microscope stage.

2.2.4) With either bright field or the 561 nm laser on (~1 kW/cm2), bring the sample into focus.

2.2.5) For imaging Ras and other membrane proteins, use a 60X apochromat TIRF objective with 1.49 numerical aperture and bring the microscope into TIRF configuration. Adjust the excitation laser so that it is off-centered when hitting the back aperture of the TIRF objective; this causes the laser to deflect upon reaching the sample. Keep adjusting the laser until the critical angle is reached and the laser is being reflected back. Search for a cell to image with several gold particles in view. Set a region of interest that encloses the cell (or a region within the cell) and the gold particles.

2.2.7) Begin acquisition with the 405 nm laser off and the 561 nm laser on (~1 kW/cm2) in case sufficient activation is occurring already (typically if expression levels are high). Otherwise, turn on the 405 nm laser at the lowest factory power setting (0.02 mW or 0.01 W/cm2) and increase gradually (0.1 mW at a time) as necessary until there are several tens of molecules per frame or so that single molecules are well separated.

2.2.8) As data acquisition continues, gradually increase 405 nm laser power to keep the spot density roughly constant.

2.2.9) Continue image acquisition until high 405 nm power (2.5 – 10 W/cm2) does not activate more activation events.

2.3) Process the images.

2.3.1) Download the image processing software below (Supplemental Code File) or the latest version at <http://www.ohsu.edu/nan>. Open Matlab and load the wfiread software (which is already put in the default path).

2.3.2) View the raw image sequence and determine the region of interest. Select the area of the stack to be processed by left clicking and dragging a box around the desired area. If the region of interest was not reduced during acquisition (to about 256x256 pixels), select a smaller area to lessen the compute time. To deselect an area and processes the entire frame, right click anywhere in the image.

2.3.4) Select a gold particle (one that is isolated and uniform in shape) by left clicking on the image and dragging a small box around it. Under Particle Tracking, click the Track button. A graph will appear that shows the position of the selected gold particle across the stack of images, depicting the extent of the drift.

2.3.5) Repeat this process to track as many gold particles as possible and determine the ones that track together (the particles will be color coded). Ideally the overall drift is on the order of one pixel at most.

2.3.6) Individually select the gold particles that tracked together one at a time and click the Add Marker button under Particle Tracking. A green cross will appear signifying that it has been added as a marker and will be used to correct for drift.

2.3.7) Adjust the Sigma Range, Smoothing Range and Threshold as necessary by changing the values slightly. Click the Find Particle button to test the settings. The particles that will be processed will be boxed and those that are not will be left out. The PAmCherry1 molecules, particles that are relatively round and bright, should be selected.

2.3.8) Start processing the images by clicking the Make Coord File button and save the file.

2.4) Post-process the images and render the PALM image.

2.4.1) In Matlab, launch the ‘palm’ package and load the .cor file just created.

2.4.2) Before rendering the PALM image using the coordinates, sort the individual coordinates (each from a ‘localization event’). The optimal sorting values may vary depending on the setup and fluorophore.

2.4.2.1) For PAmCherry1, use the following values: Combining Frame - 8; Combining Distance (nm) - 100; Minimum RMS - 4; Minimum Fit Goodness - 0.25; and Max. Eccentricity - 1.4. See the Discussion section for a additional explanation of these parameters.

2.4.3) Click the Sort button to generate a new set of coordinates ready for rendering high resolution images.

2.4.4) Enter values for proper rendering of the final image such as the raw pixel size (nm), the desired feature size (nm) in the rendered image, and the pixel size for the rendered image. See the Discussion section for more details.

2.4.5) Click the Render button to generate the PALM image. Use the +/- magnifying icons in the tool bar of the figure window to zoom in and out.

**3. Single molecule tracking in live cells**

3.1) Treat the tissue culture surface and plate the cells as described in Protocol 2. Since the temperature and/or CO2 controlled stage may require a certain culture dish, ensure sure that the coverglass has the appropriate thickness (0.17 mm) for the microscopy setup.

3.1.2) Place the culture dish on an on-stage incubator. Let the dish settle on the stage for a few minutes until the temperature and CO2 concentration stabilize every time after mounting the culture dish or moving to a new region of interest. Block lasers at this step. If CO2 control is not available, change to a CO2 independent media right before imaging, such as Leibovitz’s L-15.

Note: Phenol-red-free medium is recommended for depressing background fluorescence.

3.2) Acquire images as in Protocol 2. However, set an appropriate exposure time (typically 25-50 ms for PAmCherry1).

3.3) Process the images.

3.3.1) Extract coordinates of single molecules with the wfiread package as described in Protocol 2, or with another package.

3.3.2) Reconstruct single particle trajectories with home built Matlab package (not included in the Supplemental Code File, but may be available in future revisions), or choose among various single particle tracking (SPT) packages based on different algorithms found elsewhere23.