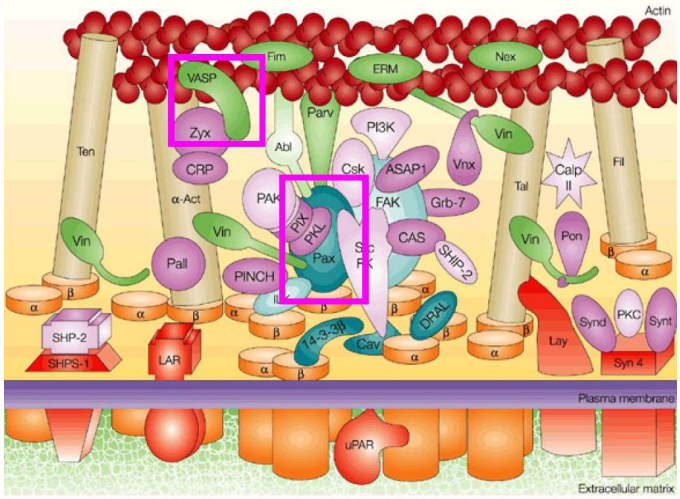
Analyzing the dynamics of focal adhesion proteins by FRAP using a TIRF microscope

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Background information

Focal adhesions (FAs) are specialized transient structures linking the extracellular matrix (ECM) to the intracellular cytoskeleton through a membrane-associated multi-protein complex. Broadly, they consist of 3 components, actin, integrin transmembrane receptors binding the ECM, and in between a large number of intracellular proteins. FAs are essential to cell adhesion, spreading and migration and as such are of key importance for many physiological processes as well as in many diseases.



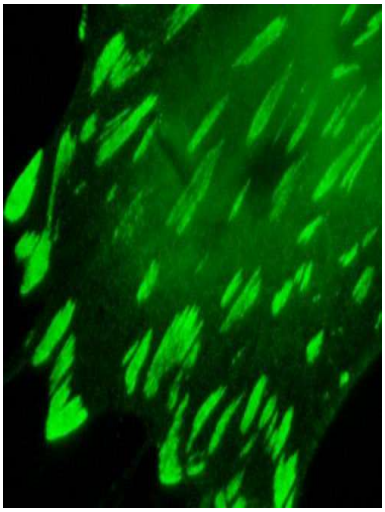


Fig 1 A: Focal adhesions are very large multi-protein complexes linking the ECM to the cytoskeleton, note that Paxillin is found at the very core of the complex while VASP directly binds to actin
B: Focal adhesions visualized by GFP-Paxillin.

Aim of the experiment

We will make use of fluorescence recovery after photo bleaching (FRAP) to examine the dynamics of Paxillin, a key FA core adaptor protein, and VASP, an actin-binding FA protein. To visualize Paxillin and VASP in the cell these proteins are fused to green fluorescent protein (GFP).
The research question is: Do you expect differences in the immobile fraction and/or recovery dynamics between Paxillin and VASP?

The set-up

We will image the focal adhesions of U2OS cells (human bone cancer cells) stably expressing GFP-Paxillin or GFP-VASP using Total Internal Reflection Fluorescence (TIRF) microscopy. In a TIRF microscope the laser beam used for excitation is reflected within the glass coverslip leaving only a very small fraction of the light, the evanescent wave, to actually illuminate the sample. This evanescent wave can only penetrate to a depth of about 200nm from the coverslip making this technique ideal to visualize objects close to the membrane such as FAs. We are using a Nikon inverted TIRF-microscope equipped with a 60x 1.45NA Plan APO TIRF objective and a Roper 3D FRAP unit.

Image acquisition

The cells are grown on a 24 mm round coverslips, which is placed between 2 metal rings, containing a small amount of medium. A Tokai Hit incubation system is used to provide a humidified atmosphere of about 37°C and 5% CO_2 during the whole experiment. The laser beam used for excitation is focused and then adjusted to the appropriate TIRF-angle. Before the experiment the FRAP laser is calibrated using an empty area on the coverslip. Once a good field of view with cell(s) is found small 20 by 20 pixel square regions of interest (ROIs) are drawn around ~15 FAs in the Metamorph software controlling the microscope. Subsequently these ROIs are added in the ILAS software, controlling the frap unit, as areas to be bleached. A Metamorph journal is run to start the experiment, images are taken every 500ms with 61 frames pre-bleach followed by 721 frames after bleaching of the selected FAs. The two separate sets of images (pre- and post-bleach) are saved as well as the ROIs marking the bleached FA to facilitate later analysis. For each protein at least 3 different FRAP- experiments should be conducted.

Image analysis

Broadly, the fluorescence intensity of focal adhesions over time is extracted from the saved image stacks using Fiji (or ImageJ). After subtraction of the background intensity this data is normalized in 3 different ways to highlight different aspects of the dynamics of the imaged proteins, and the averaged curves for Paxillin and VASP are compared. The steps in Fiji [the sequence of buttons to click]:

- Open both the pre-bleach and post-bleach image stacks of a single experiment in Fiji.
- Use concatenate to paste the two stacks together: [Image – Stacks – Tools - Concatenate], in the pop-up menu select your pre-bleach stack as image 1, your post-bleach stack as image 2 and ‘none’ as image 3 then press ok
- Think of a way, to use image processing, to specifically visualize the bleached focal adhesions. Hint1: Use the image calculator [Process > Image calculator …]. Hint2: You first need to duplicate two single images, which ones?.
- **Draw a rectangular ROI around each of the bleached FAs**. Use the zoom function and adjust the brightness/contrast ([Ctrl + Shift + c]) if desired. Try to **only include efficiently bleached areas** of your FA by scrolling to the last frame and **shrink the selected area to fit to the area where the FA is still present in the last frame.** Press **[Ctrl + t]** to add your region to the RoiManager.
- Also draw ROIs around two unbleached FAs (again scroll to first and last frame and adjust your selected areas accordingly) and two background regions (where there is no cell) and add these to your RoiManager as well. For easier analysis in Excel, rename these ROIs by selecting them in the RoiManager and then pressing [rename].

- Save all your ROIs: press [**deselect**] in the RoiManager and then click [more-save], to save all ROIs in a zip file. (If you are saving as a ".roi" file you probably forgot to press deselect).
- Measure the intensity of your ROIs in all frames: In Fiji menu [Analyze - Set measurements] make sure ONLY the Mean gray value box is ticked and set the number of decimals to 0. Then go to the ROIManager, press [deselect], followed by [more-Multi Measure]. Make sure in the pop-up menu the boxes "measure all slices" and "one row per slice" are activated.
- Save the Results table [Ctrl + s] as a ".csv" file (Comma separated values).

Analysis in excel

- Open the .csv file in Excel
- Now normalize your data in 3 ways using the following formula:
  - $\frac{I_t\text{-}BG_t}{I_{pre}\text{-}BG_{pre}}$ $(I_t\text{-}BG_t) / (I_{pre}\text{-}BG_{pre})$
  - $\frac{(I_t\text{-}BG_t)-(I_0\text{-}BG_0)}{(I_{pre}\text{-}BG_{pre})-(I_0\text{-}BG_0)}$ $((I_t\text{-}BG_t)\text{-}(I_0\text{-}BG_0)) / ((I_{pre}\text{-}BG_{pre})\text{-}(I_0\text{-}BG_0))$
  - $\frac{(I_t\text{-}BG_t)-(I_0\text{-}BG_0)}{(I_{post}\text{-}BG_{post})-(I_0\text{-}BG_0)}$ $((I_t\text{-}BG_t)\text{-}(I_0\text{-}BG_0)) / ((I_{post}\text{-}BG_{post})\text{-}(I_0\text{-}BG_0))$

  I_t is the measured intensity of the adhesion at time point t.
  BG_t is the measured background intensity at time point t.
  I_{pre} and **BG_{pre}** are the measured <u>average</u> intensity of the adhesion or the background signal over the <u>entire</u> <u>pre-bleach period.</u>
  I_0 and **BG_0** are the intensity of the adhesion or background signal at the first time point after the bleach pulse.
  I_{post} and **BG_{post}** are the measured <u>average</u> intensity of the adhesion or background signal of the <u>last 25 time points</u> of the post-bleach period.
- Plot the data (intensity versus time) of all individual focal adhesions for each cell and for each normalization method.
  Now critically look at your data to determine which FAs you want to include in your final analysis, for example if there is a strange kink in the recovery curve of an FA or if it is consistently losing signal (the lifetime of an FA is in the order of minutes so you may have chosen an FA to bleach which disassembles during your experiment) you may exclude these FAs. However, be very careful at this point, you should NOT be excluding FAs because they don't fit with your hypothesis!
- Also plot the average data (per movie and for all movies of the same protein together) and compare.
  Think about what the different methods of normalization show you. Which normalization is best to determine the bleach depths? Which method can be used best to compare the speed of the recovery? And which method shows you if there is a difference in the stably bound fraction of a protein?