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Confocal-based bead-binding

In 1 collection

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ABSTRACT

Method of quantifying in vitro binding.

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Protein preparation

1 In order to prepare proteins for a fluorescent bead-binding experiment, you need one protein that can bind a resin (in my case MBP-Rubicon RH domain, which can bind amylose resin) and a fluorescently labelled bait protein (in my case, Rab7-AlexaFluor 647).

To prepare my AlexaFluor 647, used an NHS ester salt, and followed standard manufacturer procedures for labelling, quenching, and cleaning the protein (https://www.sigmaaldrich.com/US/en/technical-documents/protocol/genomics/pcr/nhs-ester-oligonucleotide-conjugation)

Measure the degree of labelling of your sample. My degree of labelling was 0.9 fluorophores per Rab7 copy

2 Here, I wanted to assess the impact of phosphorylation on binding. In order to keep the samples as comparable as possible, I fluorescently labelled my Rab7 with 647, then separated the Rab7 into two pools. One pool I phosphorylated using TBK1, the other pool I treated with the same dilution and recovery steps, but omitted the TBK1. See the TBK1 phosphorylation protocol for details.

Bead-binding and confocal

3 Washed and prepared a 40 uL suspension of 25% amylose resin in a 50 mM HEPES 7.5, 150 mM NaCl, 2 mM MgCl2, and 10 mM TCEP buffer. Incubated this resin with 500 nM MBP-RH domain and ~ 4 uM fluorescently-labelled Rab7.

Prepare a negative control consisting of the experimental sample absent the resin-binding partner, in this case MBP-Rubicon RH

4 Incubate samples on rocker at room temperature for 1 H.

5 Collect resin at bottom of tube using a tabletop centrifuge, and aspirate and discard supernatant.

6 Wash resin 3 times with 1 mL of room temperature buffer

7 Resuspend resin in 200 uL of buffer, then transfer to an 8-well confocal imaging chamber.

8 Focus sample and image resin. Adjust imaging parameters if necessary.

Analysis

9 In order to measure the brightness of an individual bead, export the images to FIJI, and define an ROI that contains a bead edge. Measure this ROI, and record the maximum brightness. This brightness should correspond to the edge of the bead. Repeat this for ~20 cells, and average to acquire a single biological replicate.

Repeat experiment at least twice more to acquire two more biological replicates. Perform a T-test between the comparison groups in the experiment