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Reconstitution of LRRK2 membrane recruitment onto planar lipid bilayers

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Suzanne Pfeffer

Supported lipid bilayers have emerged as an ideal model system to study the interaction of proteins with cellular membranes. We describe here a method to monitor the recruitment of purified LRRK2 kinase onto planar lipid bilayers containing lipid-anchored Rab10 protein using Total Internal Reflection Fluorescence (TIRF) Microscopy.This method utilizes purified, FLAG-tagged, full length LRRK2 labeled with CF633 succinimidyl ester (Biotium) and bacterially expressed eGFP-Rab10-His tagged protein. LRRK2 recruitment is captured in real time at 25°C using a Nikon Ti-E inverted microscope with an Andor iXon+EMCCD camera model DU885, with PerfectFocus and a Nikon TIRF Apo 100X 1.46 NA oil immersion objective.

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1.
Protein LoBind Tubes Protein LoBind® 0.5 mL PCR clean colorless 1 bag × 500 tubes **Eppendorf Catalog #0030108434**
; Eppendorf LoRetention tips 0.1-10uL Catalog No. 022493018

2. Bacterially expressed pET21b C-terminal His-tagged eGFP-Rab10 full length Q63L mutant. This mutant displays decreased intrinsic GTP hydrolysis and helps retain fully active GTP bound Rab10 protein.

3. FLAG-tagged full length LRRK2 expressed in HEK 293T cells (HEK293T **ATCC Catalog #CRL-3216**). It is essential that the purified protein be prepared in a buffer that lacks primary amino groups and avoid freeze thawing.

Photostable CF633 succinimidyl
4. FLAG LRRK2 is fluorescently labeled with ester **Biotium Catalog #92217**). It should be used fresh or within ~2 days if kept on ice in a cold room.

5.
Nunc™ Lab-Tek™ II Chambered Coverglass, 8 well, 8-well; Working Volume: 0.2mL to 0.5mL **Thermo Fisher Catalog #155409**
are used for recruitment assays. The reaction chambers are washed with Piranha solution and then plasma cleaned before use.

Mini-hand extruder **Avanti Polar Lipids,**
6. Liposomes are produced using a **Inc. Catalog #610000-1EA** and are best used within 1 week after preparation when stored at 4°C.

7. As LRRK2 is a GTP-binding kinase that requires ATP, reactions include a nucleotide regeneration system to ensure adequate ATP and GTP levels throughout the incubation period.

InstantBlue® Coomassie Protein Stain (ISB1L) (ab119211) **Abcam Catalog #119211**
Photostable CF633 succinimidyl ester **Biotium Catalog #92217**
Pierce Dye Removal Columns **Thermo Scientific Catalog #22858**
Poly-D-lysine hydrobromide **Contributed by users Catalog #02150175-C**
ANTI-FLAG® M2 Affinity Gel **Millipore Sigma Catalog #A2220**
mouse anti-LRRK2 N241A/34 primary antibody **NeuroMab Catalog #75-253**
InstantBlue® Coomassie Protein Stain (ISB1L) (ab119211) **Abcam Catalog #119211**

Method for cleaning glass coverslips 40m

1 30m

Piranha Solution: 1:3 [vol/vol] ratio of [M]**30 % (v/v)** H_2O_2 and [M]**98 % (v/v)** H_2SO_4

Clean Lab-TeKII 8 chamber, No. 1.5 borosilicate coverglasses (Fisher) using Piranha solution by **00:30:00** incubation followed by extensive washing in Milli-Q water.

The reaction chambers are stored for up to 2 weeks in Milli-Q water.

2 Before the experiments, dry the chambers and clean further using a Harrick Plasma PDC-32C plasma cleaner for **00:10:00** at 18W under ambient air. 10m

Preparation of Large Unilamellar Vesicles (LUVs) 1h 0m 10s

3 Form the substrate supported lipid bilayers (SLB) with **65 % (v/v)** DOPC, **29 % (v/v)** DOPS, **5 % (v/v)** DOGS-NTA [Ni^2], **1 % (v/v)** PI(4)P, **0.1 % (v/v)** DiD (Avanti Polar Lipids; ThermoFisher) to mimic the composition of the trans Golgi network (Thomas et al., 2016).

4 1h

Dry the above-mentioned lipid mixture (in chloroform) under nitrogen flow in a glass vial and then maintained under house vacuum for at least **01:00:00** .

5 Resuspend the dried lipids in SLB buffer (**20 millimolar (mM)** Hepes **pH 8**, **150 millimolar (mM)** potassium acetate, **1 millimolar (mM)** $MgCl_2$) and vigorously vortex to produce multilamellar vesicles (MLVs).

6 Prepare the large unilamellar vesicles (LUVs) by two, **00:00:10** cycles of bath sonication followed by extrusion through a 100 nm polycarbonate membrane, 21 times (Avestin) and store at **-20 °C** . 10s

Preparation of Supported Lipid Bilayers 45m

7 Form the supported lipid bilayers in cleaned reaction chambers by addition of liposomes to the glass surfaces at a final concentration of **3 millimolar (mM)** liposomes in SLB buffer.

8 45m

Induce the fusion of the LUVs by addition of **1 millimolar (mM)** $CaCl_2$ followed by incubation for **00:45:00** at **37 °C** .

9

Remove the unfused vesicles by extensive washing with Milli-Q water and STD buffer (**20 millimolar (mM)** Hepes **pH 8**, **150 millimolar (mM)** NaCl, **5 millimolar (mM)** $MgCl_2$).

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FLAG-LRRK2 Purification 2d 0h 15m

10 Plate 293T cells onto 2 x 15cm dishes to achieve 60% confluency the next day.

11

Transfect cells with FLAG-LRRK2 expression construct:

11.1

Tube#1: Dilute **16 µg** of DNA into a final volume of **700 µL** of Optimem.

A 1:4 DNA:polyethylenimine (PEI) [wt/vol] ratio is used.

11.2

Tube #2: Add **64 µL** of **1 mg/mL** PEI and **636 µL** of Optimem.

11.3 15m

Add tube #1 to tube #2 and incubate for **00:15:00** at **Room temperature** .

11.4 2d

Add the mixture dropwise onto cells and incubate at **37 °C** for **48:00:00** .

12

Harvest cells by pipetting vigorously with ~ **10 mL** - **20 mL** of 1xPBS for each plate.

Use a plastic cell scraper to ensure maximum cell recovery.

13 Collect cells into a conical tube.

14 5m

Spin cells at **1000 rpm, 4°C, 00:05:00** .

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15 Remove supernatant and resuspend with PBS.

16 Repeat 3X to wash away serum.

16.1 5m

Spin cells at **1000 rpm, 4°C, 00:05:00** . Remove supernatant and resuspend with PBS. (1/3).

16.2 5m

Spin cells at **1000 rpm, 4°C, 00:05:00** . Remove supernatant and resuspend with PBS. (2/3).

16.3 5m

Spin cells at **1000 rpm, 4°C, 00:05:00** . Remove supernatant and resuspend with PBS. (3/3).

17 Resuspend the pellet using **30 mL** lysis buffer (**50 millimolar (mM)** Tris **8**, **150 millimolar (mM)** NaCl, **5 millimolar (mM)** $MgCl_2$, **20 micromolar (µM)** GTP, **0.5 % (v/v)** Triton X 100, **10 % (v/v)** glycerol, 1x protease inhibitor tablet).

18 Rotate lysate end-over-end for **00:10:00** at **4 °C** . 10m

19 20m

Spin down cell debris at **12.500 rpm, 00:20:00** using a Fiberlite F15 rotor.

20 Collect supernatant and filter through a 0.2 µm filter; hold **On ice** .

21 Meanwhile, equilibrate anti-FLAG M2 resin (~ **200 µL** resin, capacity > **0.6 mg/mL** Millipore Sigma #A2220) with 10 column volumes H_2O followed by 10 column volumes of lysis buffer.

Resin is suspended in a small column fitted with a frit.

22 Apply sample (~ **20 mL**) to resin and circulate lysate with resin for **04:00:00** in the cold with an open top column and peristaltic pump. 4h

23

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Wash resin 3 X **400 µL** lysis buffer.

24

Wash resin with 3 X **400 µL** Elution Buffer (NO PEPTIDE ADDED YET) (**50 millimolar (mM)** Tris **pH 8** , **150 millimolar (mM)** NaCl, **5 millimolar (mM)** $MgCl_2$, **20 micromolar (µM)** GTP, **10 % (v/v)** glycerol).

25 Prepare FLAG peptide-containing Elution Buffer (**0.25 mg/mL** peptide [stock is **5 mg/mL**] in 5 column volumes).

26 5m

Remove as much liquid as possible from resin, add **250 µL** FLAG peptide in elution buffer and incubate in the cold room for **00:05:00** . Then open the stopper and collect eluate.

27 Repeat 4X

27.1 5m

Remove as much liquid as possible from resin, add **250 µL** FLAG peptide in elution buffer and incubate in the cold room for **00:05:00** . Then open the stopper and collect eluate. (1/4).

27.2 5m

Remove as much liquid as possible from resin, add **250 µL** FLAG peptide in elution buffer and incubate in the cold room for **00:05:00** . Then open the stopper and collect eluate. (2/4).

27.3 5m

Remove as much liquid as possible from resin, add **250 µL** FLAG peptide in elution buffer and incubate in the cold room for **00:05:00** . Then open the stopper and collect eluate. (3/4).

27.4 5m

Remove as much liquid as possible from resin, add **250 µL** FLAG peptide in elution buffer and incubate in the cold room for **00:05:00** . Then open the stopper and collect eluate. (4/4).

28

Load **20 µL** of each fraction onto an SDS-PAGE gel.

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29 1h

Detect the protein by Immunoblot using mouse anti-LRRK2 N241A/34 primary antibody ((Neuromab #75-253) diluted 1:1000 in [M]**5 % (v/v)** milk/TBS-T (Tris buffered saline with [M]**0.05 % (v/v)** Tween-20) overnight at **4 °C** , followed by secondary antibody (donkey anti-mouse IRDye680 (LI-COR #926-68072) for **01:00:00** , and image using a LICOR detector.

30

Determine the yield by Bradford Assay (Bradford, 1976); assess purity by SDS PAGE in conjunction with Instant Blue stain (Abcam #ab119211).

Fluorescent Tagging and analysis of Fluorescent FLAG-LRRK2: 1h 6m

31 1h

Label the purified FLAG-LRRK2 protein (in a non-primary amine-containing buffer) with CF633 succinimidyl ester (Biotium # 92217). Incubate the protein in the dark with the dye for **01:00:00** at **Room temperature** followed by removal of free dye by Pierce Dye Removal Columns (Thermo Scientific #22858). Labeling efficiency is determined by multiplying the dye extinction coefficient by molar protein concentration and then dividing the sample absorbances at 633nm by the product (as per labeling kit instructions). Labeling efficiencies of 2-3 moles dye per mole protein were used in experiments.

32 30s

For a **100 µL** labeling reaction, use **300 µL** of 1:1 resin slurry. First add slurry to supplier-provided column and remove storage buffer by spinning at **1000 x g, 00:00:30** .

33 30s

Add sample to column and briefly vortex; centrifuge at **1000 x g, 00:00:30** to collect protein.

34

Check protein concentration after dye removal by Bradford Assay. Labeling efficiency is determined using the dye extinction coefficient;labeling efficiencies of 2-3 moles dye per mole LRRK2 are ideal.

35 Evaluate the possible aggregation of fluorescent LRRK2 by allowing the labeled protein to bind to Poly-D-lysine coated coverslips and visualizing the protein using TIRF microscopy as described in **Section -TIRF Microscopy**.

35.1 5m

Dissolve **10 mg** Poly-D-lysine (MPBio # SKU:02150175-CF) in **1 mL** sterile Milli-Q water as a [M]**1 % (v/v)** stock solution. Dilute the stock solution two fold in PBS to prepare a 1X coating solution. Add the coating solution (**200 µL**) to the reaction chamber and incubate for **00:05:00** at **37 °C** . Remove the coating solution by rinsing the chamber thoroughly with sterile Milli-Q water and equilibrate with reaction buffer.

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35.2 Apply FLAG-LRRK2 ([M] **14 nanomolar (nM)**) to a Poly-D-lysine coated chamber instead of a lipid bilayer, and evaluate behavior of the labeled protein by TIRF as described in Section 6 below. Conditions are otherwise identical to bilayer binding reactions including nucleotides and nucleotide regenerating system.

Rab10 dependent recruitment of LRRK2 onto membranes 30m

36 30m

Add the C-terminally His-tagged eGFP Rab10 to supported lipid bilayers at a final concentration of [M] **2.5 micromolar (µM)** in STD buffer and incubate for **00:30:00** at **37 °C**

37

Wash the Rab10-coated lipid surfaces horoughly with STD buffer and then equilibrate with **200 µL** of reaction buffer and nucleotide regeneration system ([M] **20 millimolar (mM)** Hepes pH **8** , [M] **150 millimolar (mM)** NaCl, [M] **5 millimolar (mM)** $MgCl_2$, [M] **4 millimolar (mM)** ATP, [M] **20 micromolar (µM)** GTP, [M] **20 millimolar (mM)** creatine phosphate, 30U creatine phosphokinase).

TIRF Microscopy 26m 50s

38 5m

Dilute CF633-FLAG-LRRK2 to [M] **14 nanomolar (nM)** concentration in reaction buffer and allow to equilibrate to **Room temperature** for **00:05:00** .

38.1 After **00:00:50** imaging, remove **100 µL** of the **200 µL** buffer in the reaction chamber. 50s

38.2 21m

At **00:01:00** , add **100 µL** of [M] **14 nanomolar (nM)** CF-FLAG-LRRK2 to a final concentration of [M] **7 nanomolar (nM)** and imaging continues for **00:20:00** .

38.3 Prepare control bilayers with Rab11-His that does not bind LRRK2.

39

Videos of LRRK2 recruitment onto Rab10-decorated, supported lipid bilayers are recorded at **25 °C** at a frame capture rate interval of 500ms using a Nikon Ti-E inverted microscope with an Andor iXon+EMCCD camera model DU885, with PerfectFocus and a Nikon TIRF Apo 100X 1.46 NA oil immersion objective. Imaging is done with 300 EM camera gain and 50 ms exposure times at 200µW laser intensity.

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40

Analyze the data from TIRF microscopy movies using TrackIt (Kuhn T., et al., 2021) to obtain spot densities of bilayer-bound LRRK2 from the videos over time. Export data from TrackIt using a Mathworks MATLAB (version R2021a) script (Appendix 1); normalize raw values by assigning the highest value as 1 in Excel to enable comparison of different videos.

Data are graphed using GraphPad Prism version 9.0.Fiji is also used to analyze spot intensities and sizes in static images (Schindelin et al., 2012).

Appendix

41

MATLAB script to extract data from TrackIt.

```
function T = spots_export(btch, outfilename)
%eval('btch = currentBatch')
spots = getfield(getfield(btch, 'results'),'spotsAll');
spots_total = 0;
for i=1:length(spots)
 n = size(spots{i});
 spots_total= spots_total + n(1);
end
out = zeros(spots_total, 8);
spots_total = 0;
for i=1:length(spots)
 %disp(i)
 n = size(spots{i});
 n = n(1);
 if n>0
    spt = spots{i};
      out((spots_total+1):(spots_total+n),1) = spots_total+(1:n);
      out((spots_total+1):(spots_total+n),2) = i;
      out((spots_total+1):(spots_total+n),3:7) = spt(:,1:5);
      out((spots_total+1):(spots_total+n),8) =  sum(spt(:,3));
    spots_total = spots_total+n;
 end
end
T = array2table(out, 'VariableNames',
{'SpotID','Frame','X','Y','A','BG','Sigma','Atotal_frame'})
writetable(T, outfilename)
end
```


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