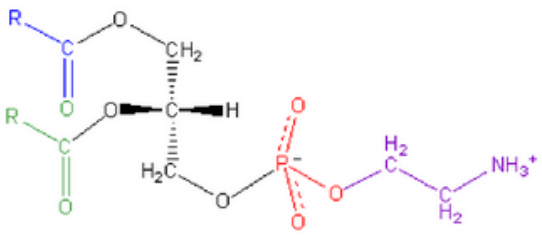
Systemic methods for capturing protein-lipid interactions

Despoina Goniotaki

Institute of Neuropathology, Aguzzi group

Lipids



- ✓ Large non-polar molecules
- ✓ They represent highly reduced forms of carbon.
- ✓ Upon oxidation in metabolism, yield large amounts of energy.

Lipids have a role in virually all biological processes:

- *Structural elements*
- *Scaffolds*
- *Signaling molecules*

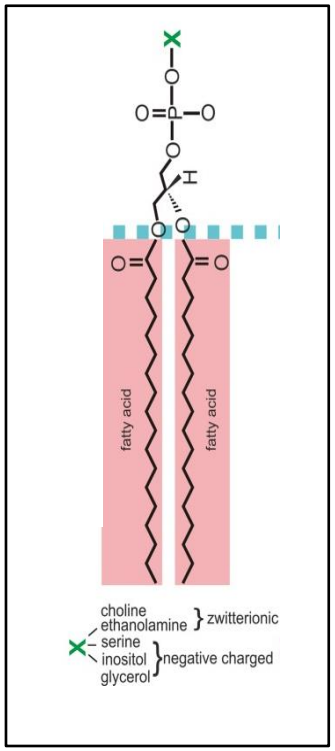
Examples of LIPIDS:

- FATS and OILS
- certain VITAMINS & HORMONES
- most NON-PROTEIN MEMBRANE COMPONENTS

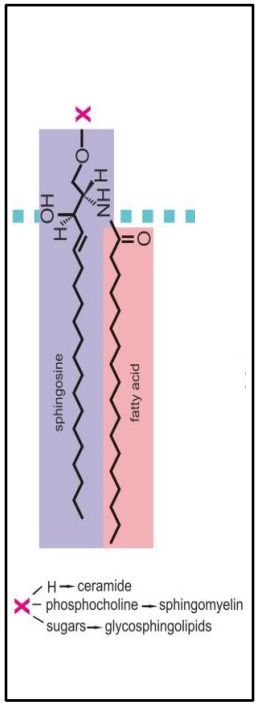
The repertoire of membrane lipids

Hydrophilic region

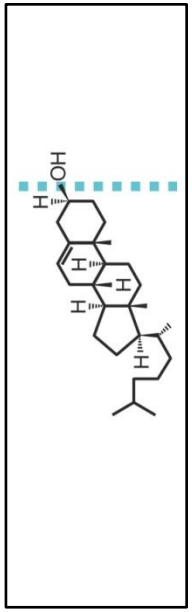
Hydrophobic region



Glycerolphospholipid



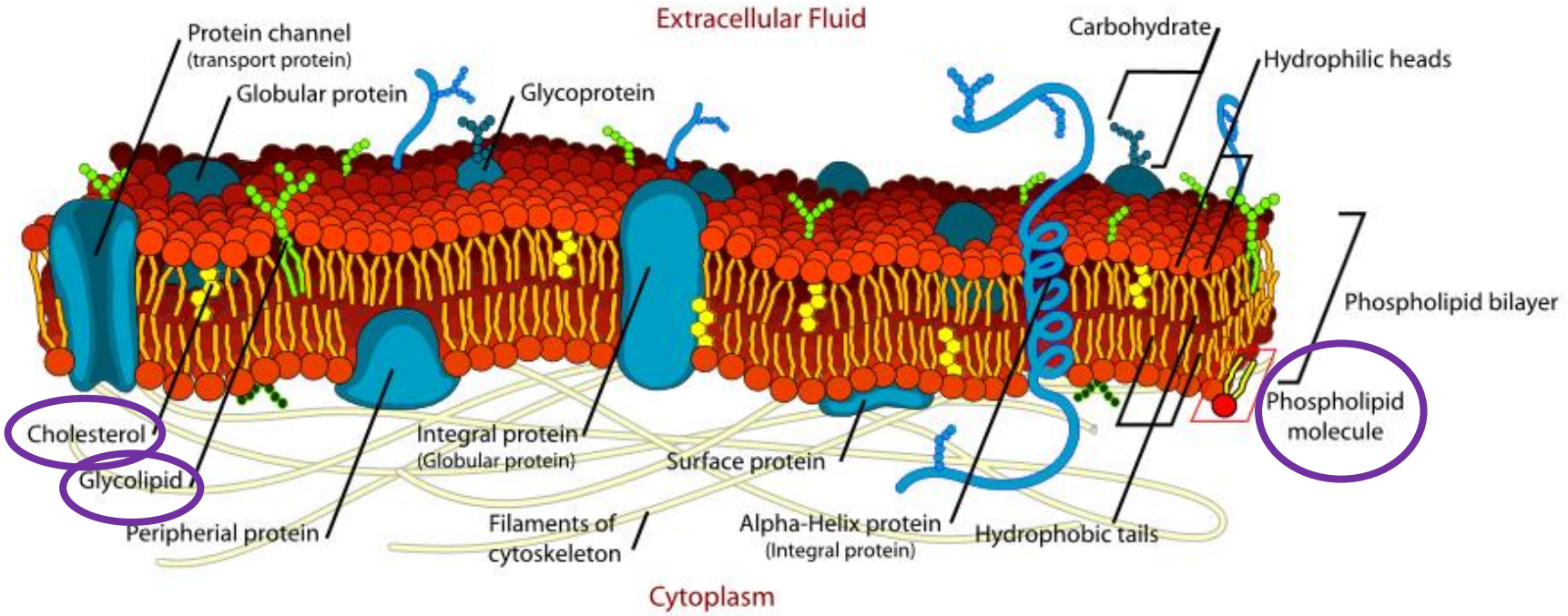
Sphingolipid



Cholesterol

Zhao and Lappalainen, Mol Biol Cell. 2012 Aug 1; 23(15): 2823–2830, doi: 10.1091/mbc.E11-07-0645

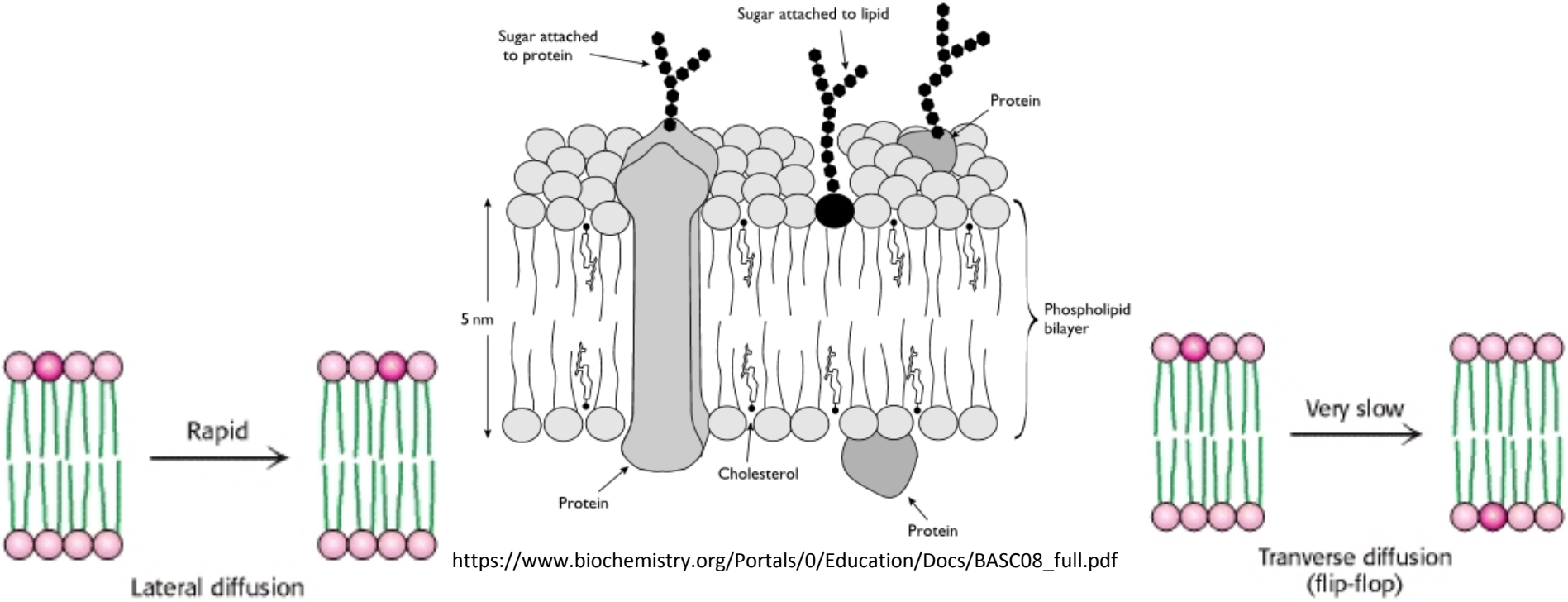
The repertoire of membrane lipids



https://upload.wikimedia.org/wikipedia/commons/d/da/Cell_membrane_detailed_diagram_en.svg

Lipids are complex and not static

The membrane structure is highly fluid and most of the lipid and protein molecules can move about in the plane of the membrane.



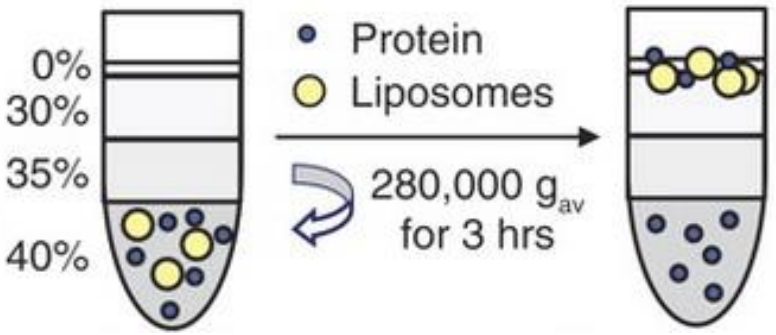
https://www.biochemistry.org/Portals/0/Education/Docs/BASC08_full.pdf

Section 12.6, Lipids and Many Membrane Proteins Diffuse Rapidly in the Plane of the Membrane, Biochemistry. 5th edition. Berg JM, Tymoczko JL, Stryer L. New York: W H Freeman; 2002.

Methods to study Protein-lipid interactions

Classical Methods

I. Flotation Assays



- Liposomes and proteins are mixed at the bottom of a sucrose gradient and ultracentrifuged.
- If proteins and lipids interact, the complex floats in the upper fractions of the centrifugation tube.

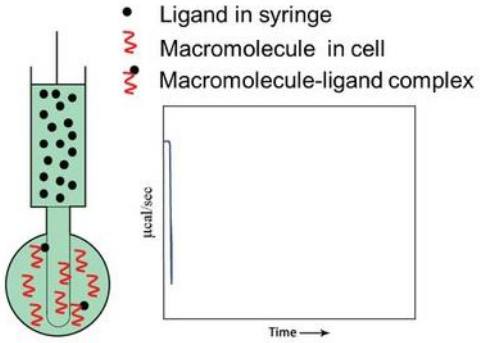
Burre et al., α-Synuclein Promotes SNARE-Complex Assembly in Vivo and in Vitro, Science (2010), DOI: 10.1126/science.1195227

Classical Methods

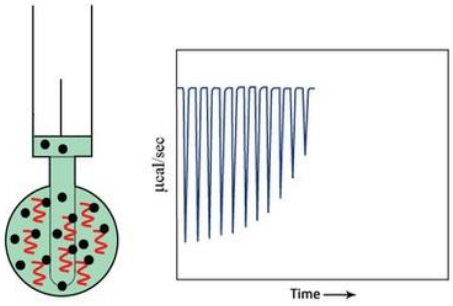
II. Isothermal Titration Calorimetry (ITC)

Set up

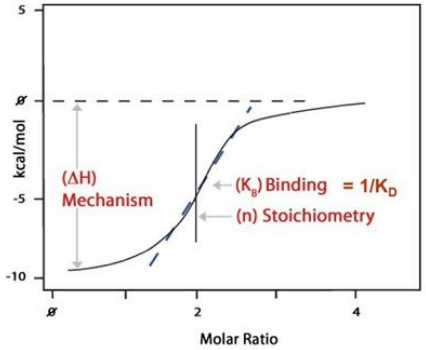
Ligand: protein
Analyte: liposomes



Binding



Titration Curve



- ITC measures the enthalpy change that occurs upon binding.
- Obtain thermodynamic parameters of protein lipid interactions.
- Identify molecular affinities of proteins and liposomes.

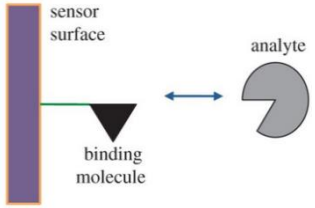
https://www.huck.psu.edu/content/instrumentation-facilities/automated-biological-calorimetry-facility/guides/itc

Classical Methods

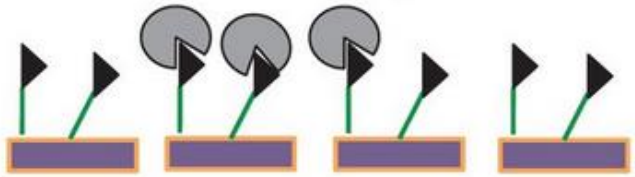
III. Surface Plasmon Resonance (SPR)

Set up

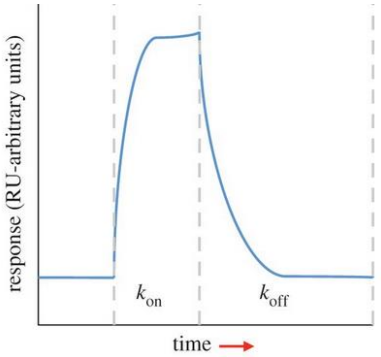
Ligand: protein
Analyte: liposomes



Liposome-Protein Binding



Sensogram:



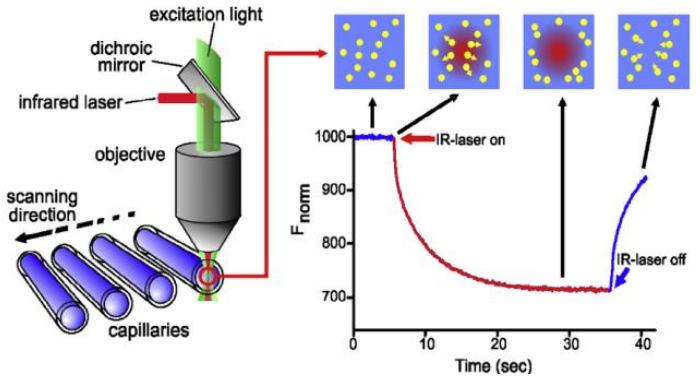
- The liposomes (ligand) are immobilised on top of a plasmon resonance sensor chip.
- The proteins (analyte) are added to the system
- As the analyte binds to/dissociates from the ligand a change in refractive index occurs.

Kastritis et al, `On the binding affinity of macromolecular interactions: daring to ask why proteins interact `, Interface (2012), DOI: 10.1098/rsif.2012.0835

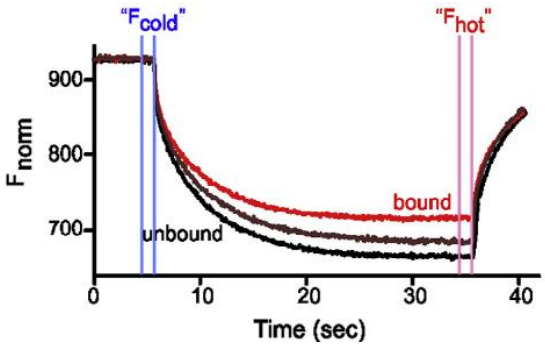
Classical Methods

IV. Microscale Thermophoresis (MST)

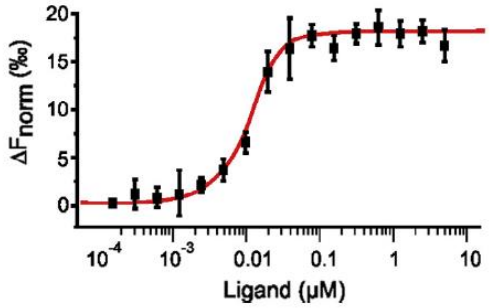
Set up



Typical Binding Experiment



Termogram



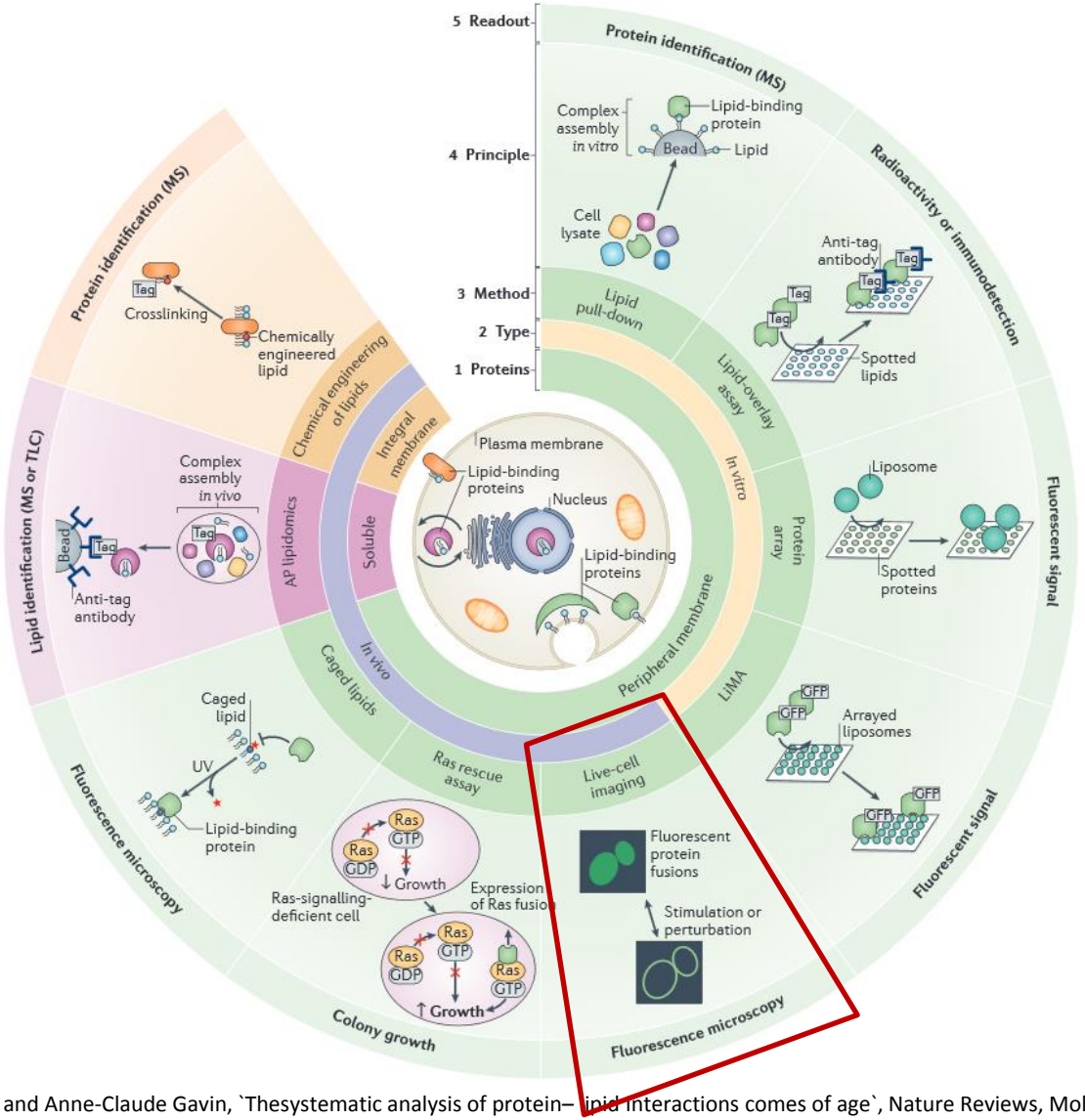
- MST measures the motion of molecules along microscopic temperature gradients.
- The fluorescence is used to monitor the motion of molecules along these temperature gradients.

Moran Jerabek-Willemsen et al, `MicroScale Thermophoresis: Interaction analysis and beyond `, Journal of Molecular structure (2014)

Classical Methods Overview

Advantages	Disadvantages
Quantitative	Fabrication,handling and storage of liposomes is difficult
Sensitive	Storage of liposomes for more than a few days is problematic
Large-Low sample volume	Use of nonphysiological buffers
Real time assay	Protocols cannot be scaled up
Quick and cheap	Large amount of lipids and purified proteins are required

Systemic Methods



Antoine-Emmanuel Saliba, Ivana Vonkova and Anne-Claude Gavin, `Thesystematic analysis of protein– lipid interactions comes of age`, Nature Reviews, Molecular Cell Biology, December 2015

Original Article

Cell Research (2014) **24**:959–976. doi:10.1038/cr.2014.89; published online 8 July 2014

Regulation of EGFR nanocluster formation by ionic protein-lipid interaction

Ye Wang[1,*], Jing Gao[2,6,*], Xingdong Guo[3,*], Ti Tong[4], Xiaoshan Shi[3], Lunyi Li[3], Miao Qi[5], Yajie Wang[7], Mingjun Cai[2], Junguang Jiang[2], Chenqi Xu[3], Hongbin Ji[1] and Hongda Wang[2]

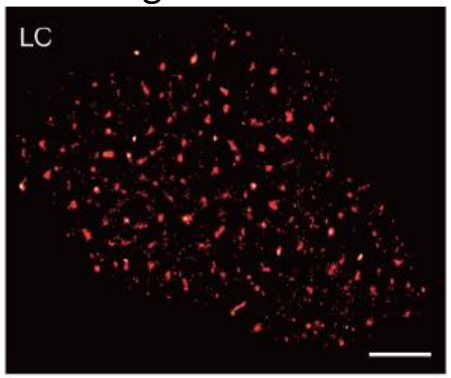
Aim: Identify the pattern of EGFR spatial distribution on the surface of living cells. Role of EGFR-lipid niche interaction in the activation/regulation of EGFR.

Method: Fluorescence Microscopy - Live cell imaging

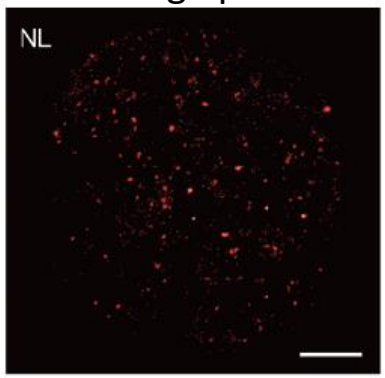
EGFR spatial distribution

EGFR cluster formation on the membrane

Lung Cancer cells

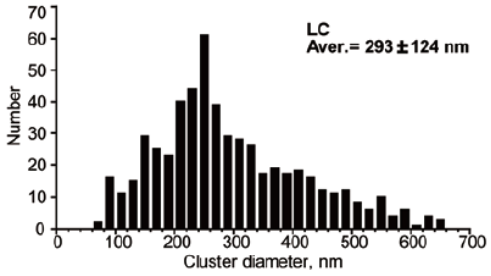


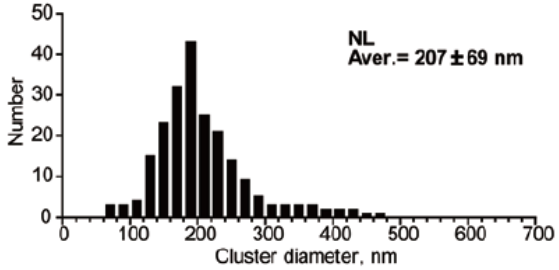
Normal Lung epithelial cells

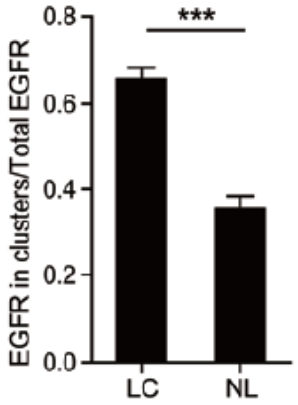


Reconstructed dSTORM images of labeled EGFR

Cluster Quantification





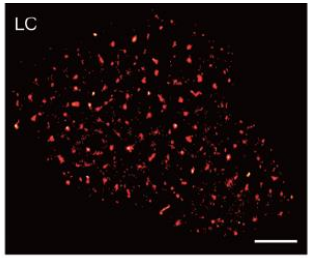


- Cluster number and diameter were significantly higher in LC cells

EGFR spatial distribution

EGFR cluster formation on the membrane

Lung Cancer cells



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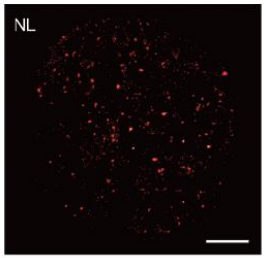
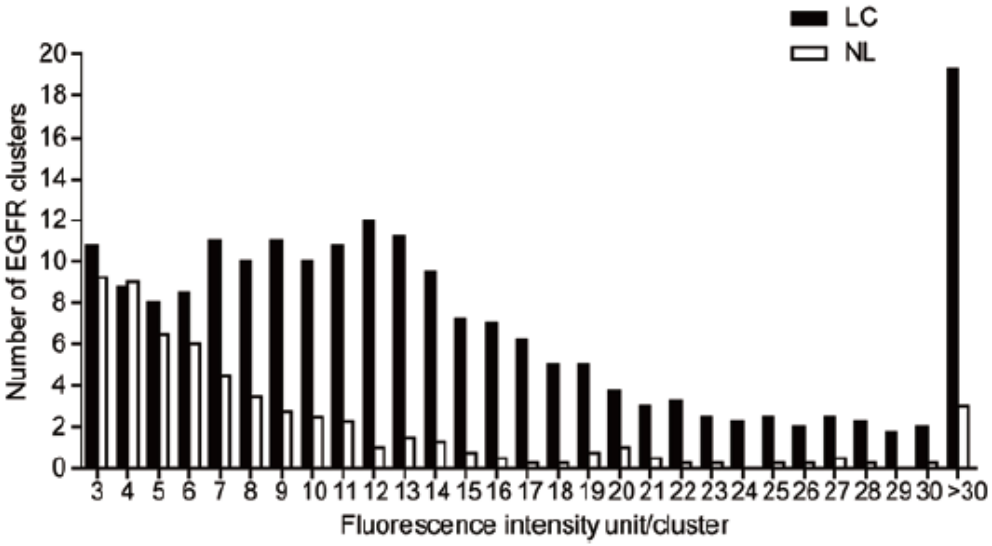


Reconstructed dSTORM images of labeled EGFR

Cluster Analysis

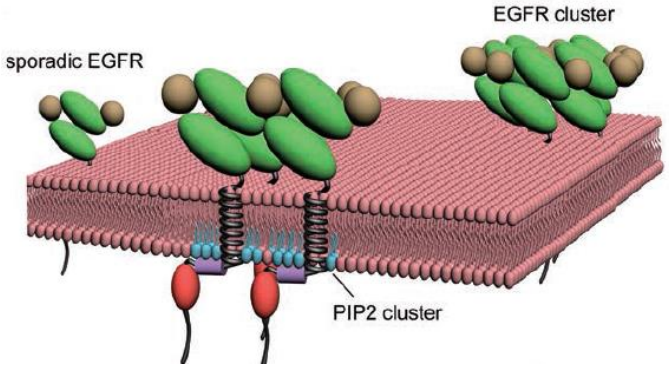


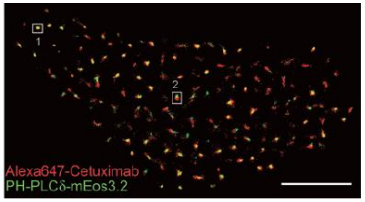
LC EGFR clusters are composed of significantly more moderate- and big-sized protein units (10-30).

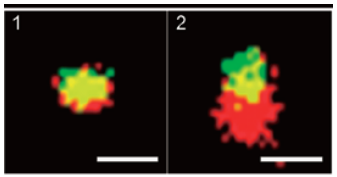
EGFR cluster formation: molecular mechanisms

Role of PIP2 in EGFP clustering in fixed COS-7 cells

1.EGFR and PIP2 colocalize in clusters

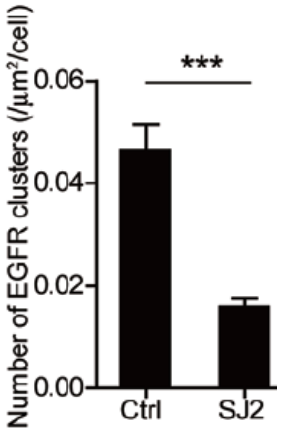


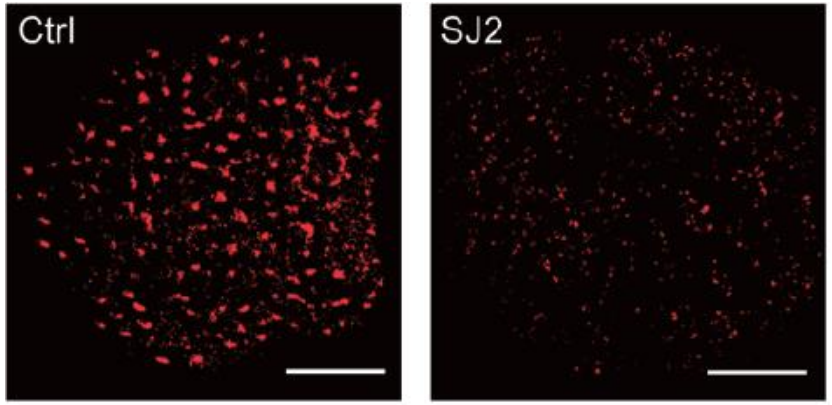


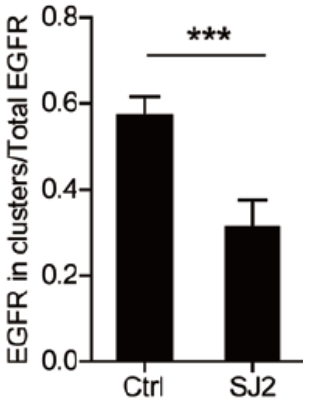


2.PIP2 depletion/Transfection with SJ2 dramatically decreases the surface density of EGFR clusters

SJ2: Inositol-polyphosphate 5-phosphatase



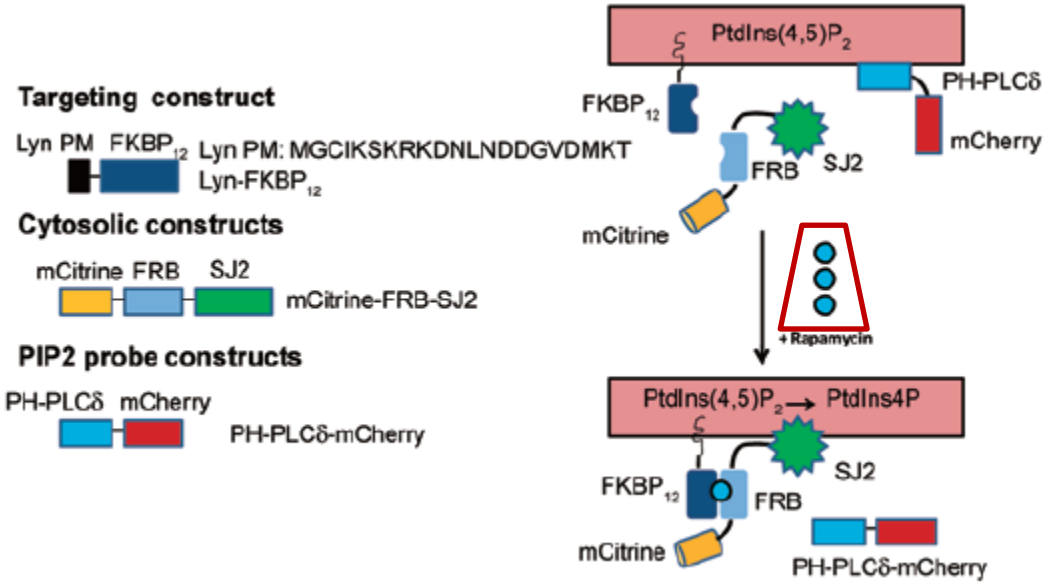


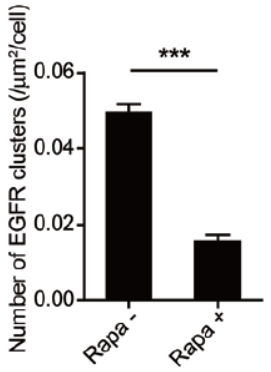


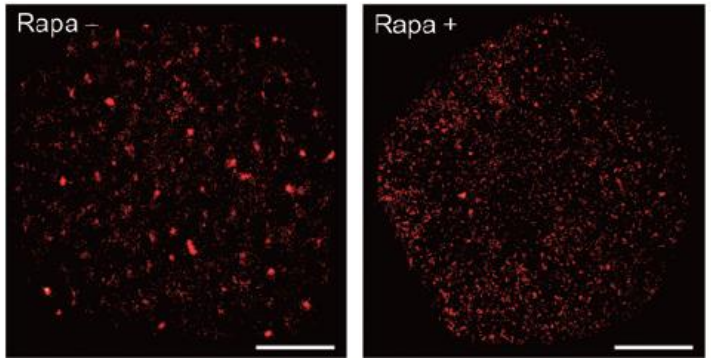
EGFR cluster formation: molecular mechanisms

Role of PIP2 in EGFP clustering in fixed COS-7 cells

PIP2 depletion/Inducible PIP2 depletion system



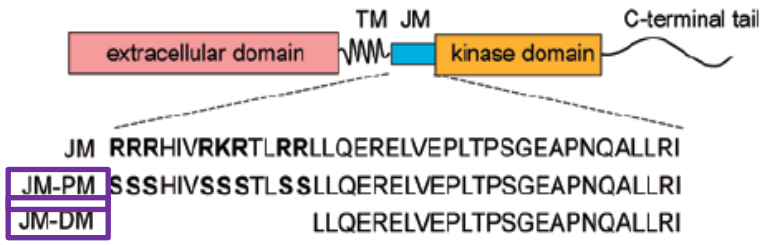


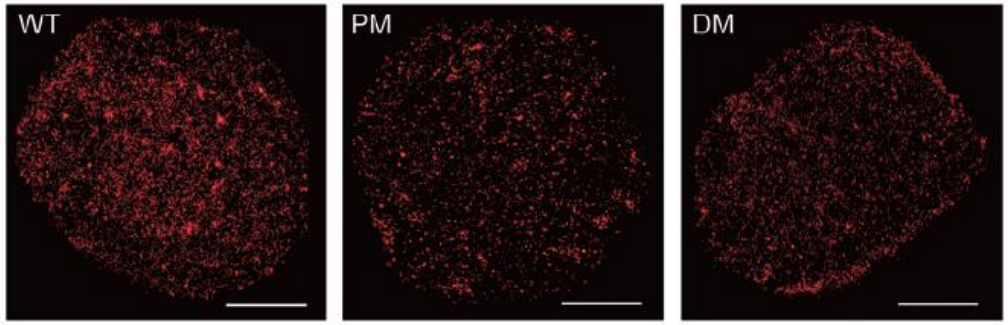


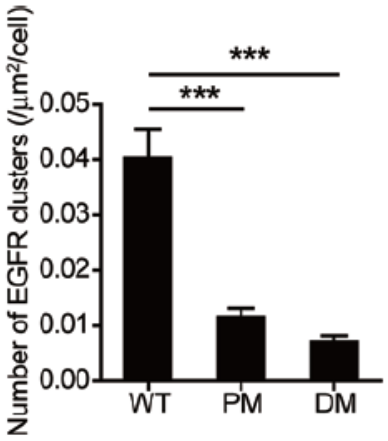
PIP2 depletion results in a significant reduction of EGFP clusters in the plasma membrane

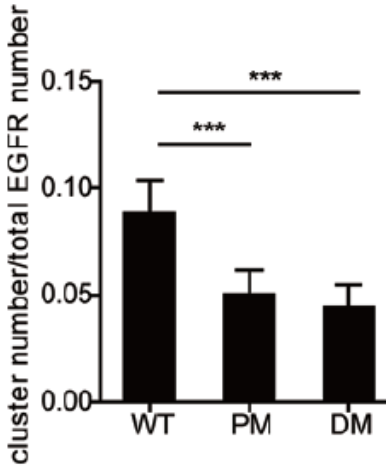
EGFR-PIP2 interaction: characterization

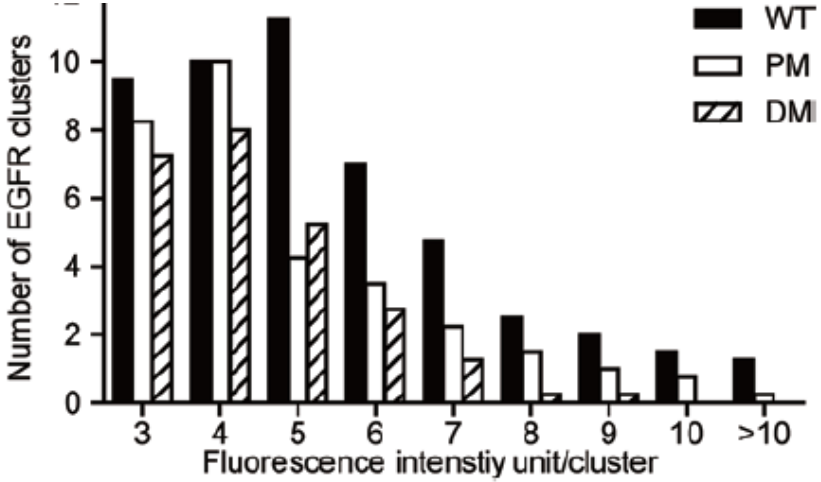
The JM region of EGFR is required for binding to the PIP2 phospholipid







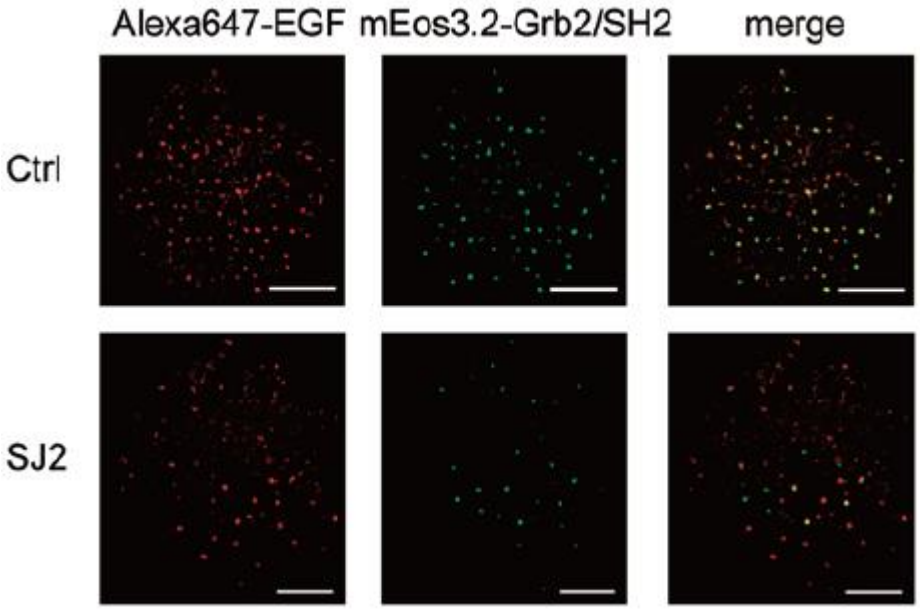


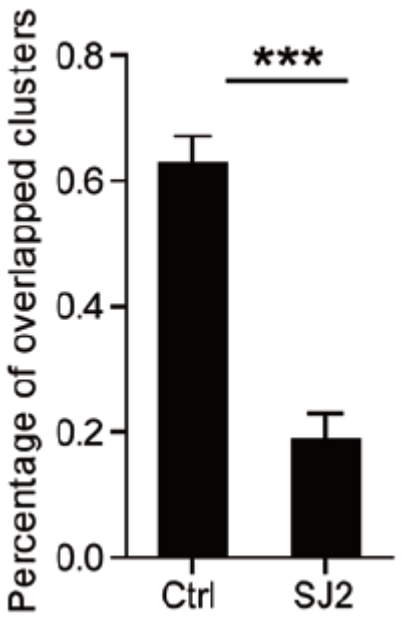


JM region depletion results in a significant reduction of EGFP clusters in the plasma membrane

EGFR-PIP2 interaction: characterization

The JM-PIP2 interaction regulates the EGFR activation, signaling and biological function





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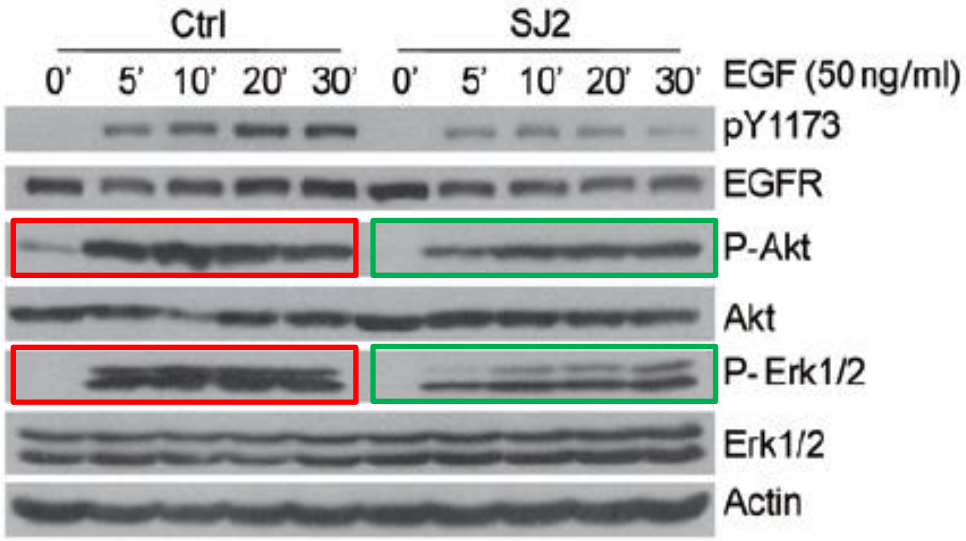
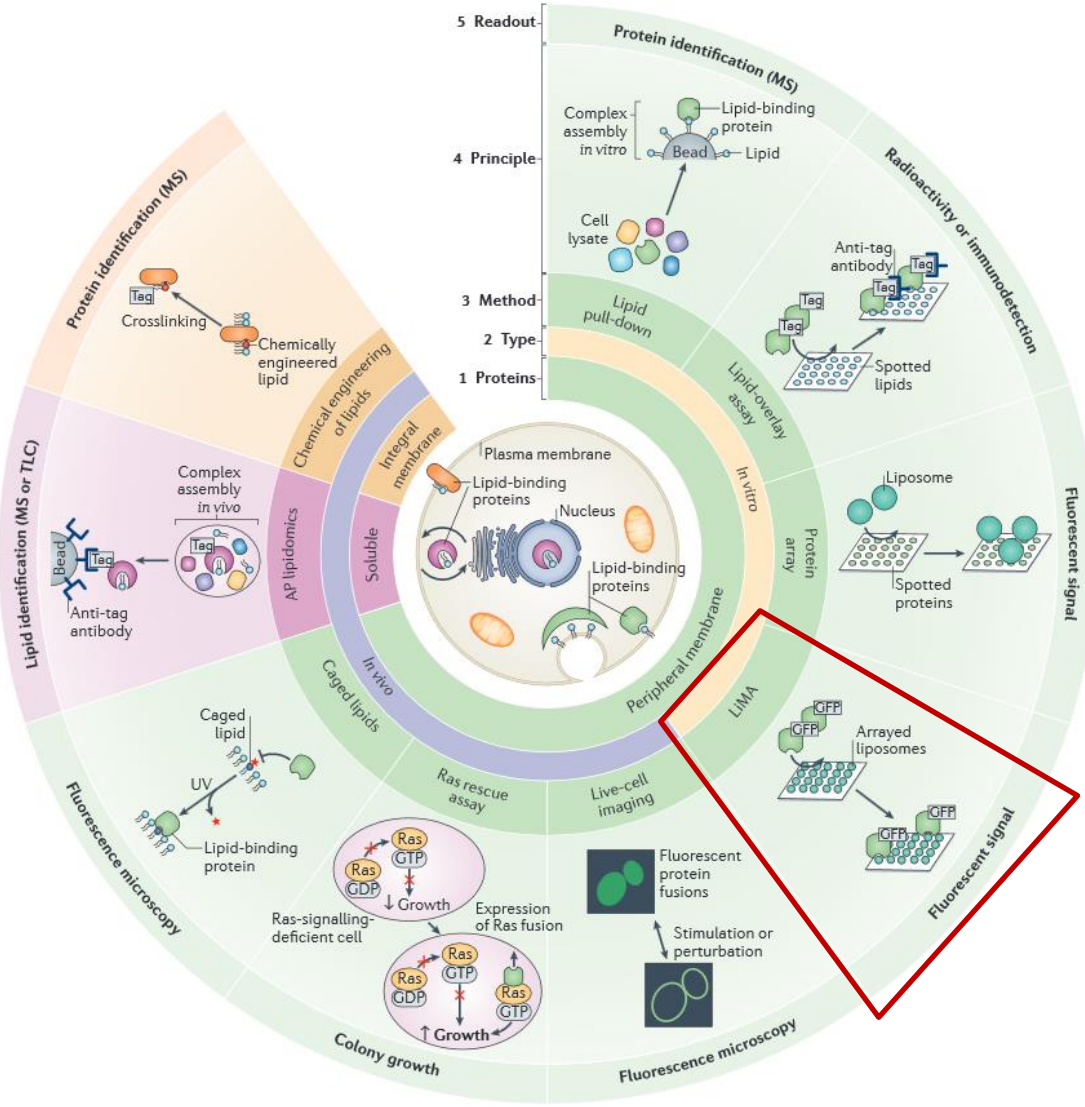


Role of EGFR-lipid niche interaction in the activation/regulation of EGFR

Systemic Methods



Antoine-Emmanuel Saliba, Ivana Vonkova and Anne-Claude Gavin, `Thesystematic analysis of protein– lipid interactions comes of age`, Nature Reviews, Molecular Cell Biology, December 2015

A quantitative liposome microarray to systematically characterize protein-lipid interactions

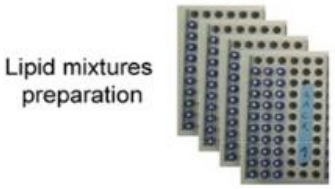
Antoine-Emmanuel Saliba[1,6], Ivana Vonkova[1,6], Stefano Ceschia[1], Greg M Findlay[2], Kenji Maeda[1], Christian Tischer[3], Samy Deghou[1], Vera van Noort[1], Peer Bork[1], Tony Pawson[2,5], Jan Ellenberg[4] & Anne-Claude Gavin[1]

Aim: create a simple set-up to measure protein recruitment to membranes in a quantitative, automated, multiplexed and high-throughput manner.

Method: Liposome Microarray-based Assay (LIMA)

Platform assembly

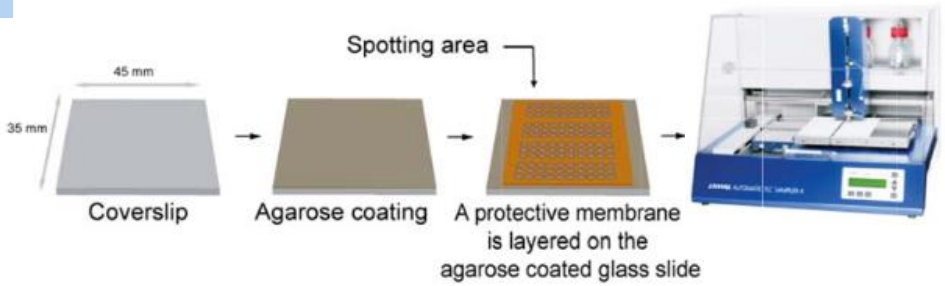
1. Lipid Mixture Preparation



- Glass vials containing lipids

- <u>Lipid Mixture</u>:
  - Carrier lipid , palmitoyl-oleyl-phosphatidylcholine (POPC)
  - Fluorescently labeled lipid, phosphatidylethanolamine (PE-Atto647)
  - Signaling lipids

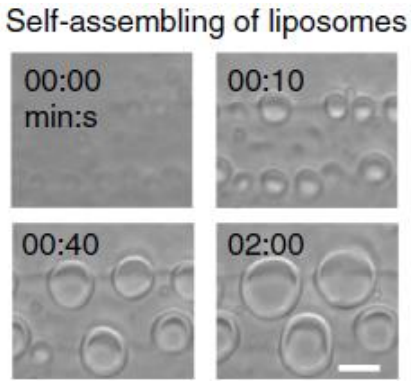
2. Thin Agarose Layer



- Automatic lipid spoting under inert atmosphere

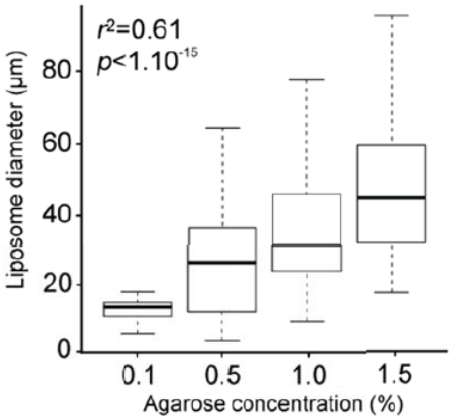
Assay validation

Liposome Formation and Characterization

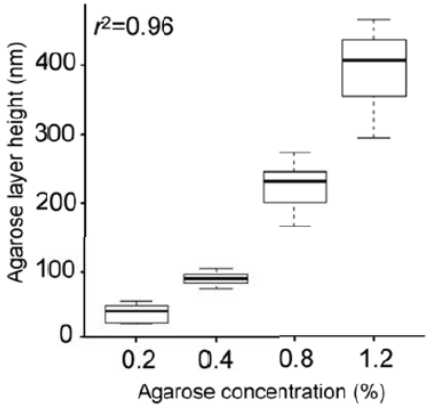


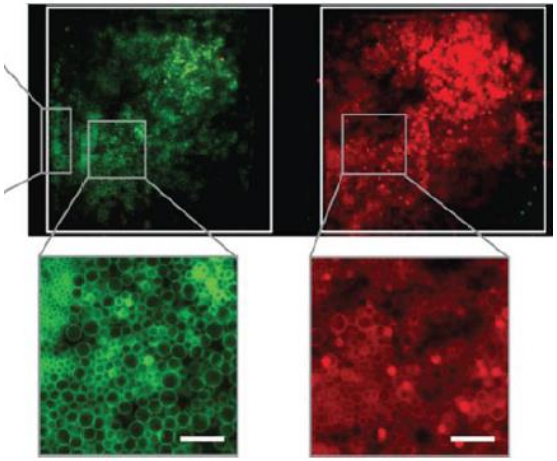
- Liposomes rapidly self-organize (within 2min) upon hydration of the agarose in a variety of physiological buffers.

- Liposome diameter is proportional to the agarose concentration.



TAL characterization

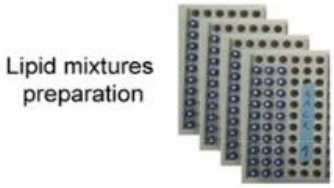




Liposomes are restricted to TAL areas

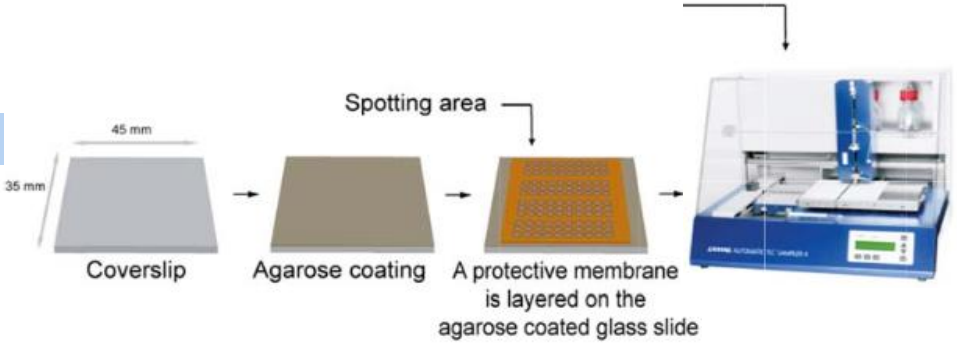
TAL integration into a miniaturized, fluorescence microscopy-based asssay

1. Lipid Mixture Preparation



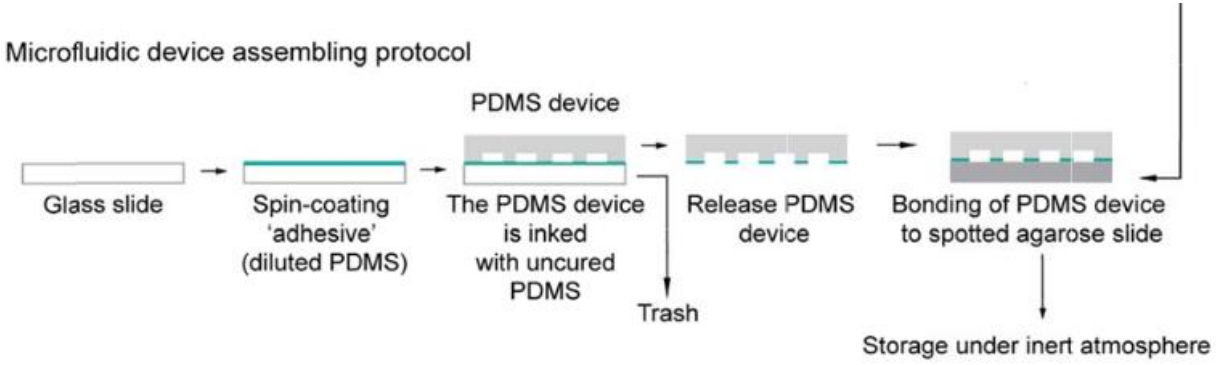
- Glass vials containing lipids

2. Thin Agarose Layer

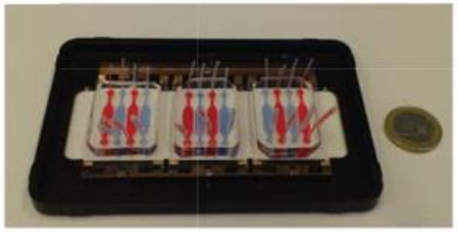


- Automatic lipid spoting under inert atmosphere

3. Microfluidic Device (PDMS)



c Microfluidic device plugged on a holder



20 devices/day

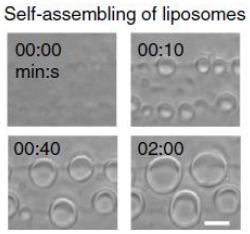
3 chambers/30 types of liposomes

LIMA applications - Lipid Binding Assay

TAL can support the formation of liposomes in lipid mixtures

Liposomes

Liposomes are giant (>5µm), thus amenable to quantitative analysis by microscopy.

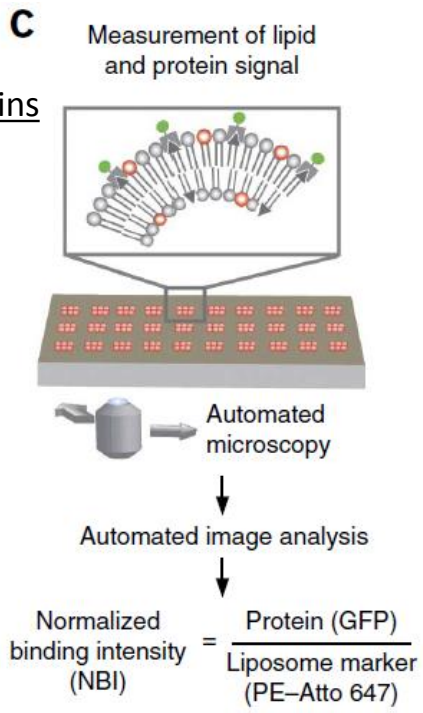


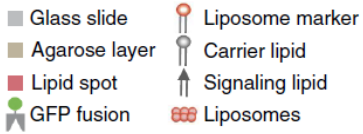
FunctionalMeasurements

Lipid Binding Assay

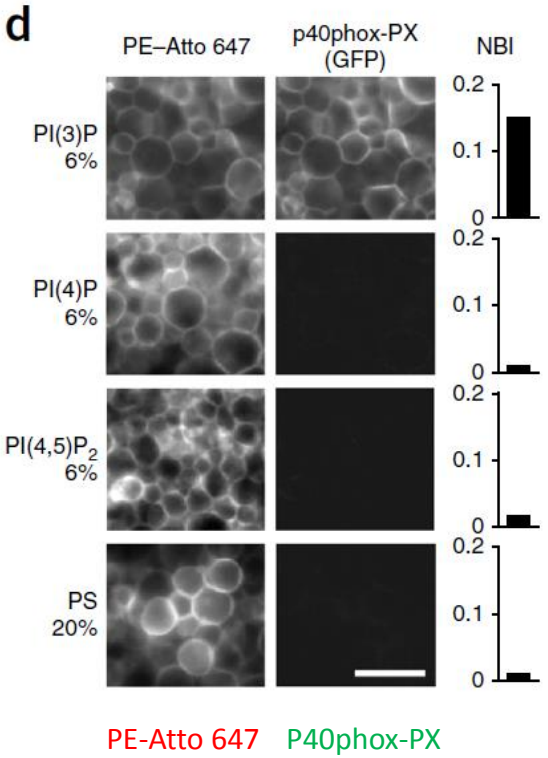
Common lipid binding domains (LBDs) in eukaryotes:

-PH, PX, C1, C2, C2-like, PROPPIN
-GFP-tagged recombinant proteins





Recruitment of LBDs to liposomes

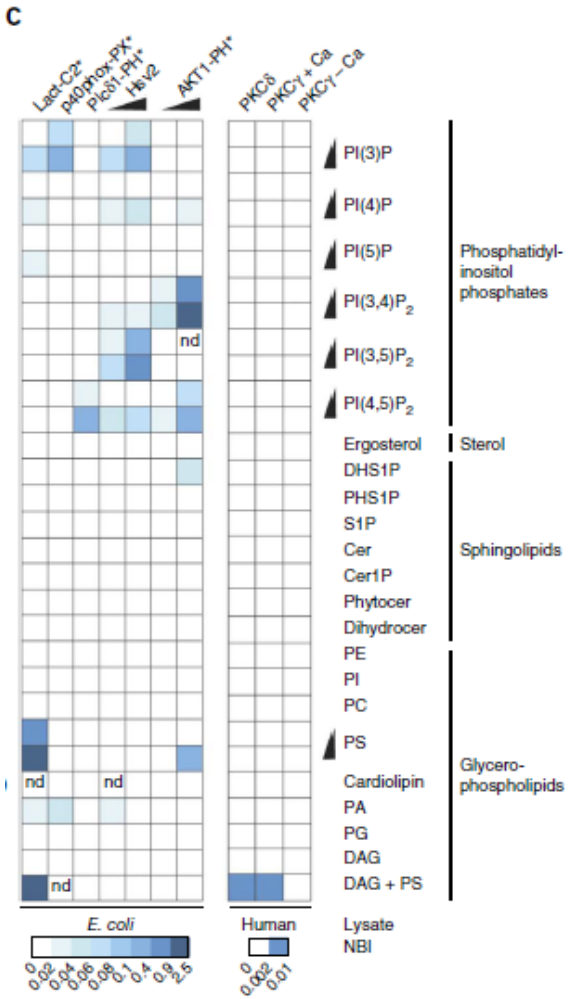


PE-Atto 647 P40phox-PX

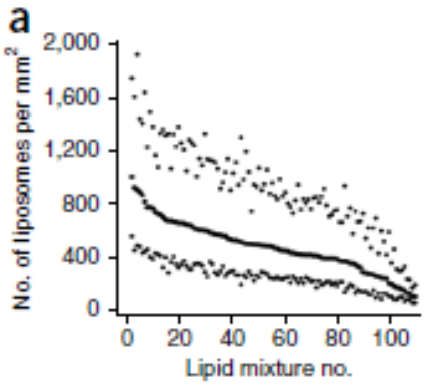
Recruitment of the PX domain of p40phox (NADPH oxidase subunit) to PI (3)P containing membranes.

LIMA applications - Lipid Binding Assay

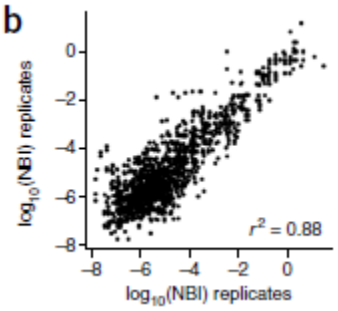
TAL can support the formation of liposomes in various (110) lipid mixtures



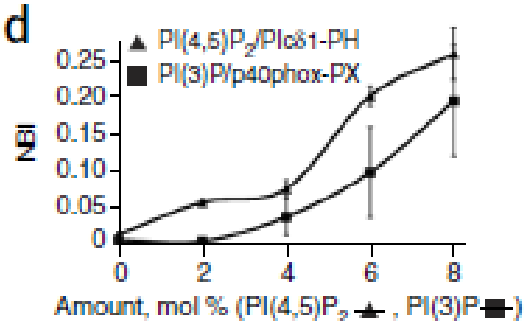
Efficiency of liposome formation

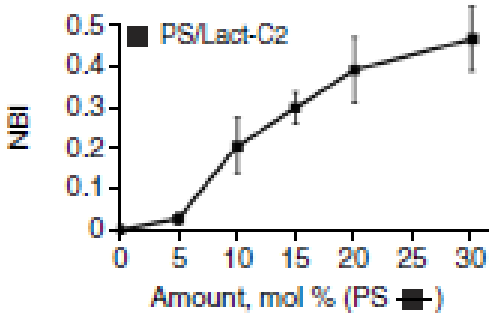


NBI correlation



Recruitment of LBDs to liposomes



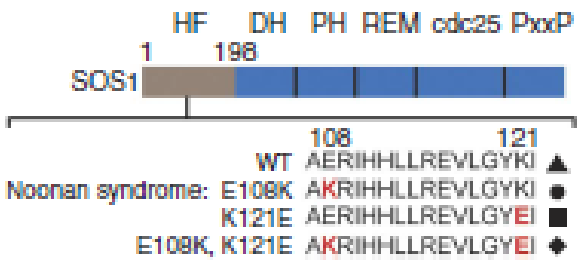


The higher signaling lipid concentration, the higher the recruitment of LBDs to liposomes

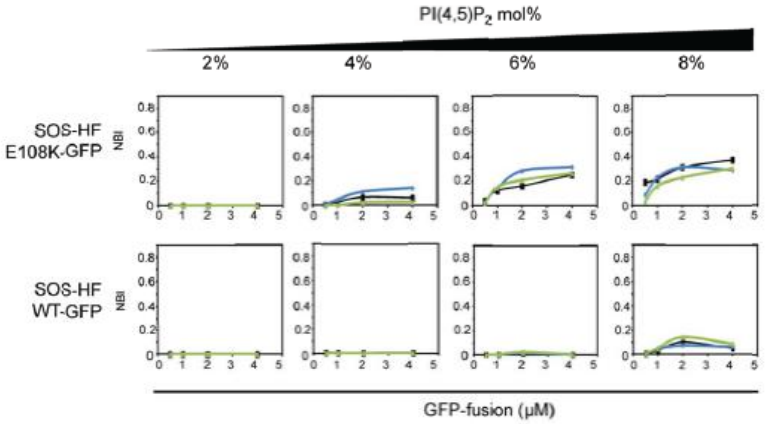
LIMA applications - Binding affinity modulations

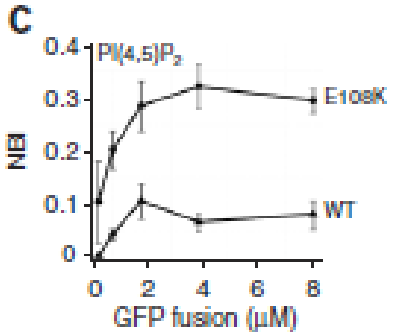
Detection of subtle changes in binding afffinity - the example of SOS1

Son-of-sevenless (SOS1)



- Wild type SOS1 binds to phosphatidic acid (PA) and phospatidylinositol 4,5-biphosphate (PI(4,5)P2).
- E108K increases SOS1 binding to PA and PI(4,5)P2 and causes Noonan syndrome.





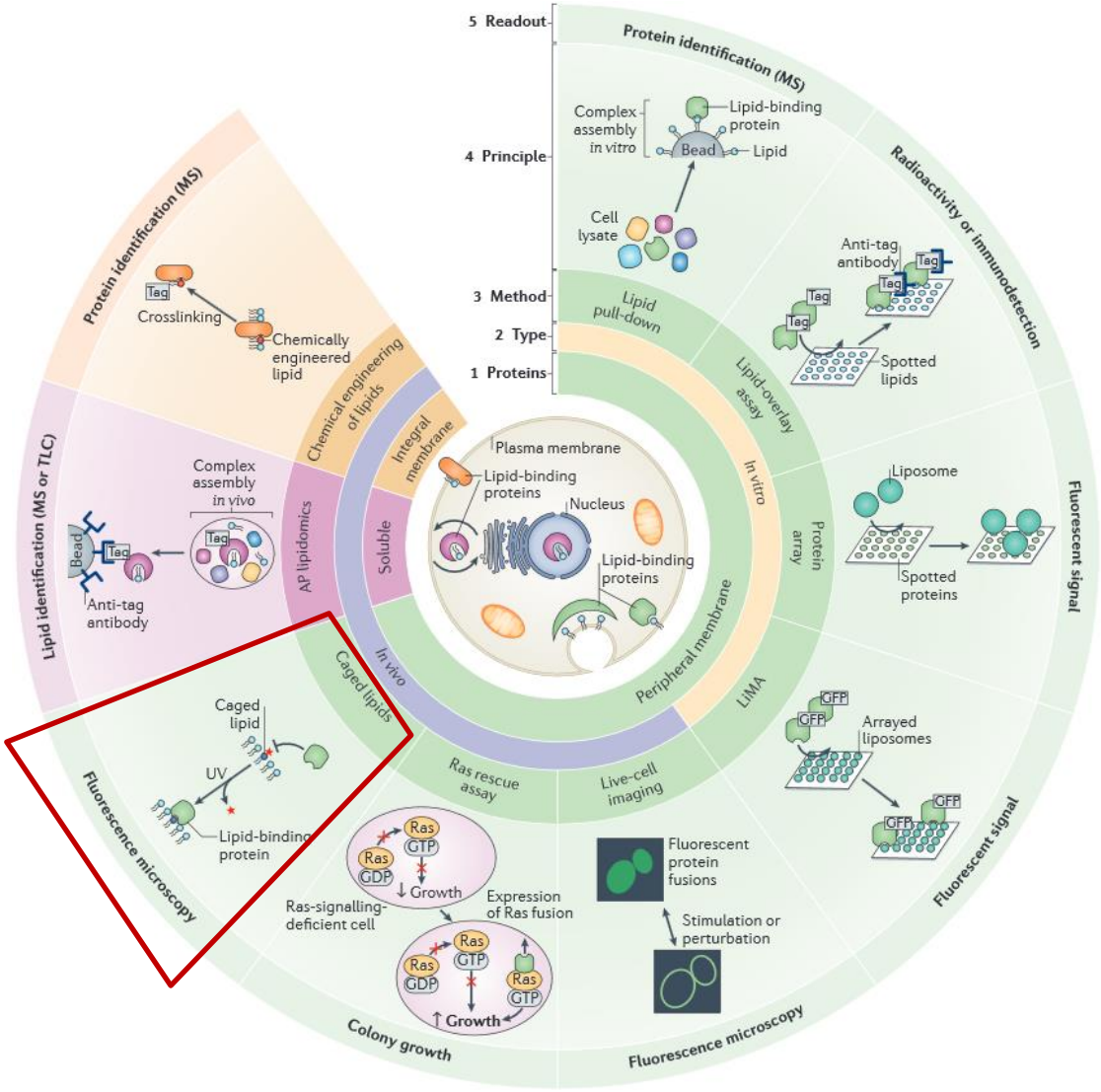
SOS-HF domain recruitment to PA and PI(4,5)P2 liposomes is higher upon presence of the E108K aminoterminal mutation

Outlook

LIMA

- **Sensitive** → measure interactions with <1pmol of protein
- **Quantitative** →NBIs for an interacting protein-lipid pair were proportional to the amount of lipid and protein present in the assay
- Allows the systemic mixing of lipids and probing for cooperative mechanisms
- Unlabeled proteins can be measured if LIMA is combined with mass spectrometry
- Integration with advanced optical methods is possible
- LIMA could allow studies on disruption of protein-lipid interactions by small molecules

Systemic Methods



Antoine-Emmanuel Saliba, Ivana Vonkova and Anne-Claude Gavin, `Thesystematic analysis of protein– lipid interactions comes of age`, Nature Reviews, Molecular Cell Biology, December 2015

Cell

Resource

A Global Map of Lipid-Binding Proteins and Their Ligandability in Cells

Micah J. Niphakis,[1,2,*] Kenneth M. Lum,[1,2] Armand B. Cognetta III,[1] Bruno E. Correia,[1] Taka-Aki Ichu,[1] Jose Olucha,[1] Steven J. Brown,[1] Soumajit Kundu,[1] Fabiana Piscitelli,[1] Hugh Rosen,[1] and Benjamin F. Cravatt[1,*]

[1]The Skaggs Institute for Chemical Biology and Department of Chemical Physiology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA

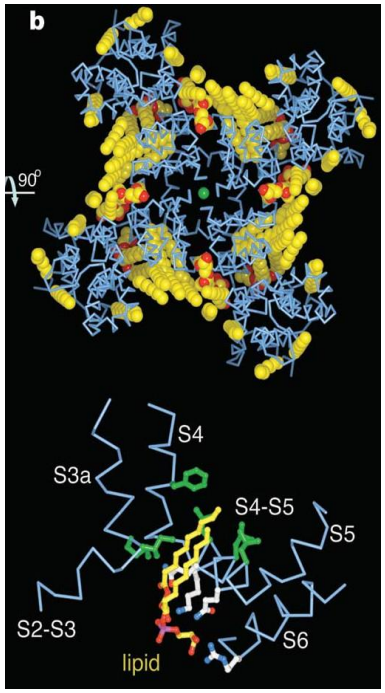
Aim: Mapping of Lipid-Protein Interactions in cells so as to uncover new modes of signaling that are amenable to pharmacological perturbation

Method: Caged-Lipids / Fluorescent Microscopy

Role of Lipids in Physiology and Pathophysiology

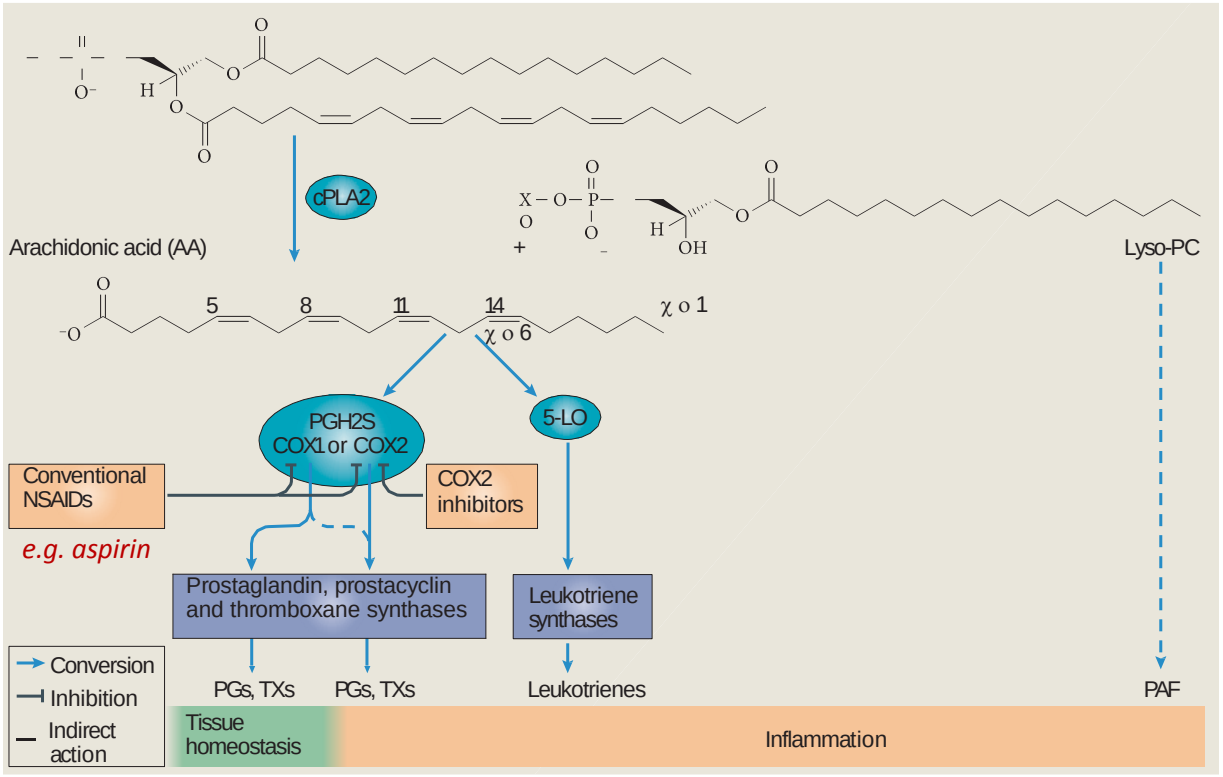
Lipids can have structural (e.g. stabilizing membranes or proteins) or signaling roles (e.g. eicosanoids)

Structural



Unusually positioned lipids hypothesized to influence structure and function of KcsA channel

Signaling

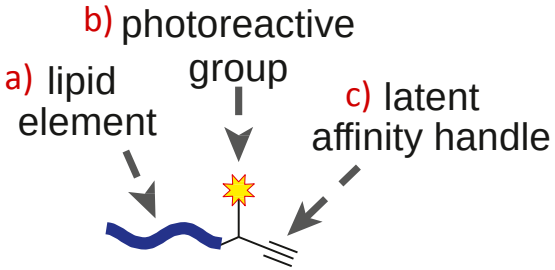


Arachidonic acid derived molecules mediate both physiological and pathophysiological signaling pathways

MacKinnon, R. *et. al. Nature* **2007**, *450*, 376; Wymann, M. P., Schneiter, R. *Nat. Rev. Mol. Cell Biol.* **2008**, *9*, 162

Design of novel chemical proteomic probes
to identify proteins that interact with fatty-acid-derived lipids

Probe design based on small molecule protein binding affinity and light - induced crosslinking to capture protein



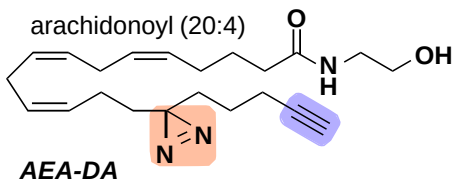
Design elements:

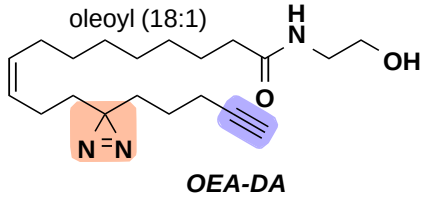
a) Small molecule to be recognized by protein ("lipid element").
b) Photoreactive element that covalently links lipid element and protein upon UV irradiation.
c) Alkyne to allow late-stage conjugation to azide tag via Cu-catalyzed alkyne-azide cycloaddition ('click' chemistry)

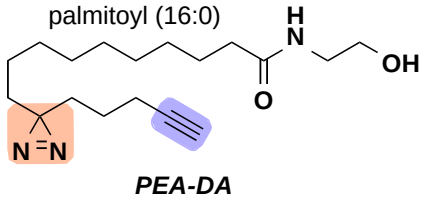
Set of lipid probes:

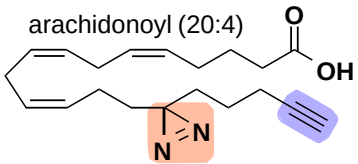
Diazirine photocroslinking group

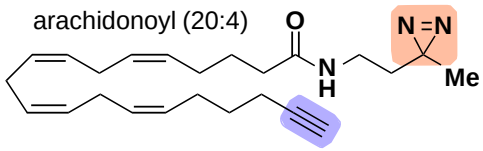
Alkyne affinity handle

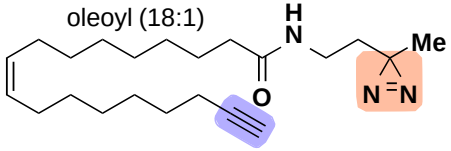


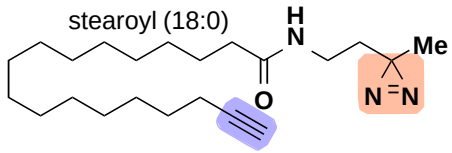




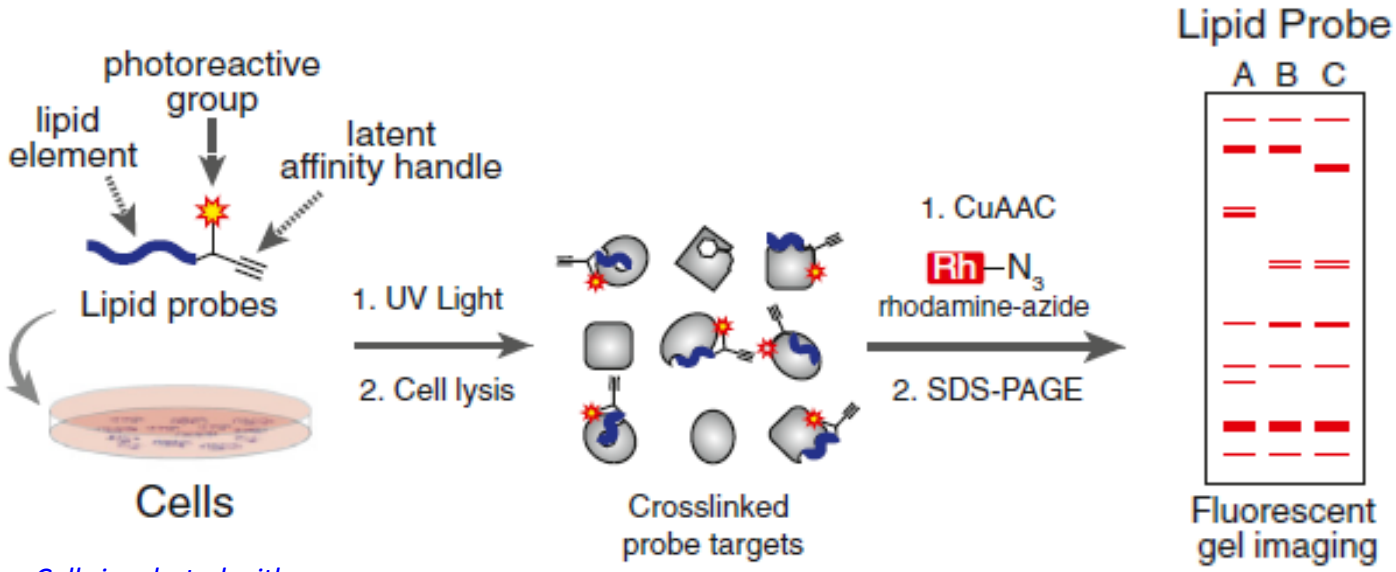








Characterization of lipid probe targets



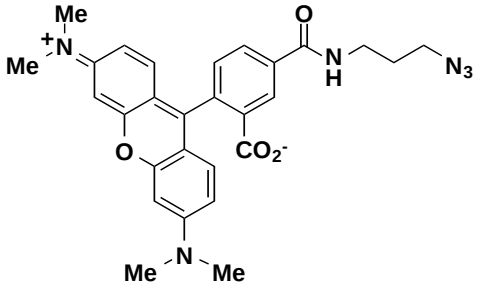
Cells incubated with probe for 30 min before UV

Cell proteome fractioned into membrane and soluble components

Identification of target proteins

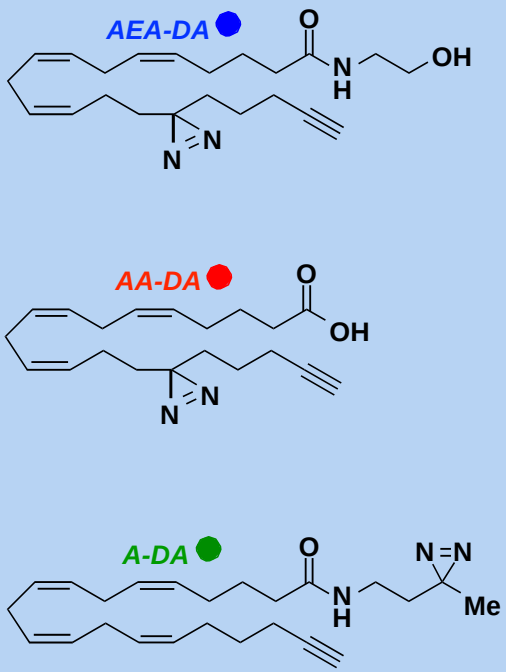
Rhodamine azide fluorescent reporter tag

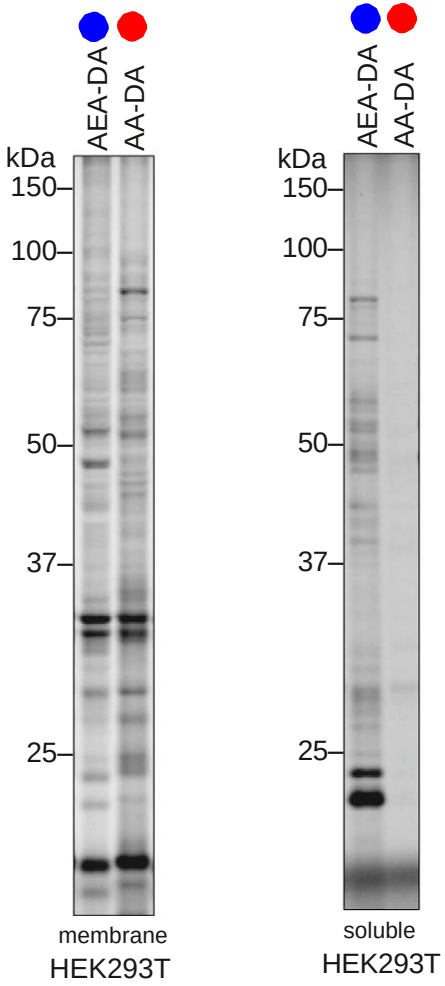
Rh–N_3



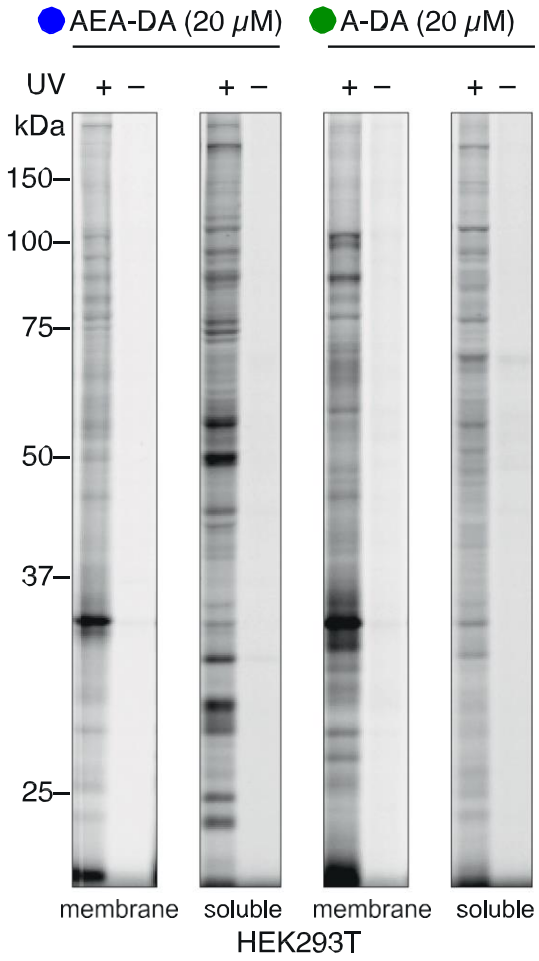
Lipid probes differentially label proteins

Lipid probes:





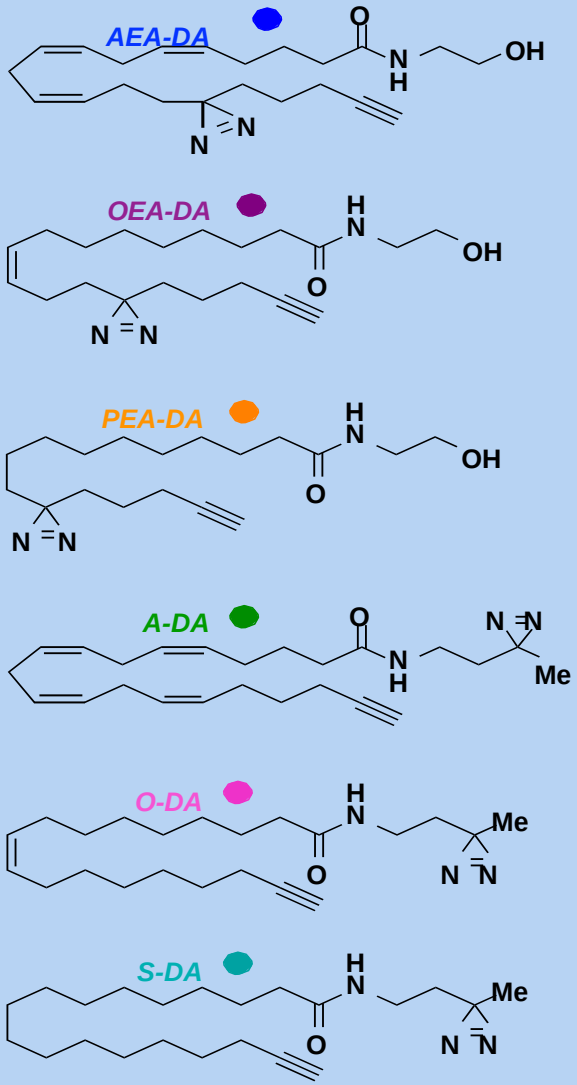
AA-DA almost exclusively labels membrane proteins

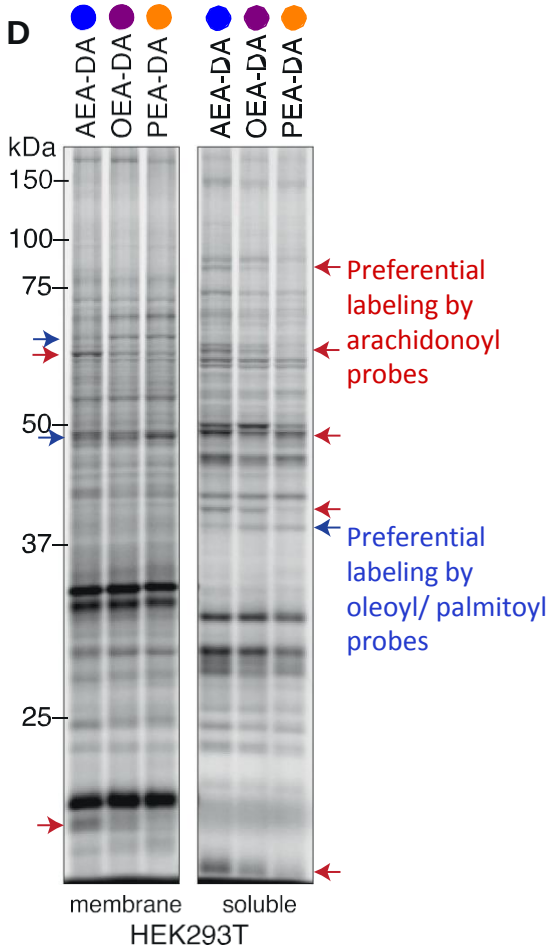


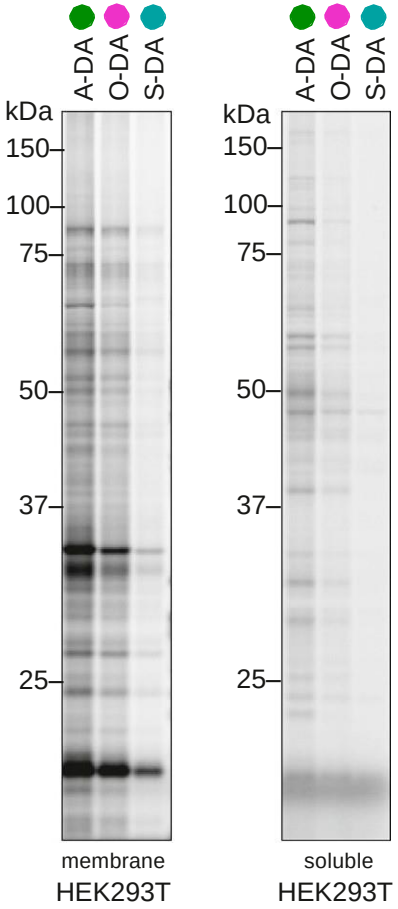
Protein Labeling is UV dependent

Lipid probes differentially label proteins

Lipid probes:







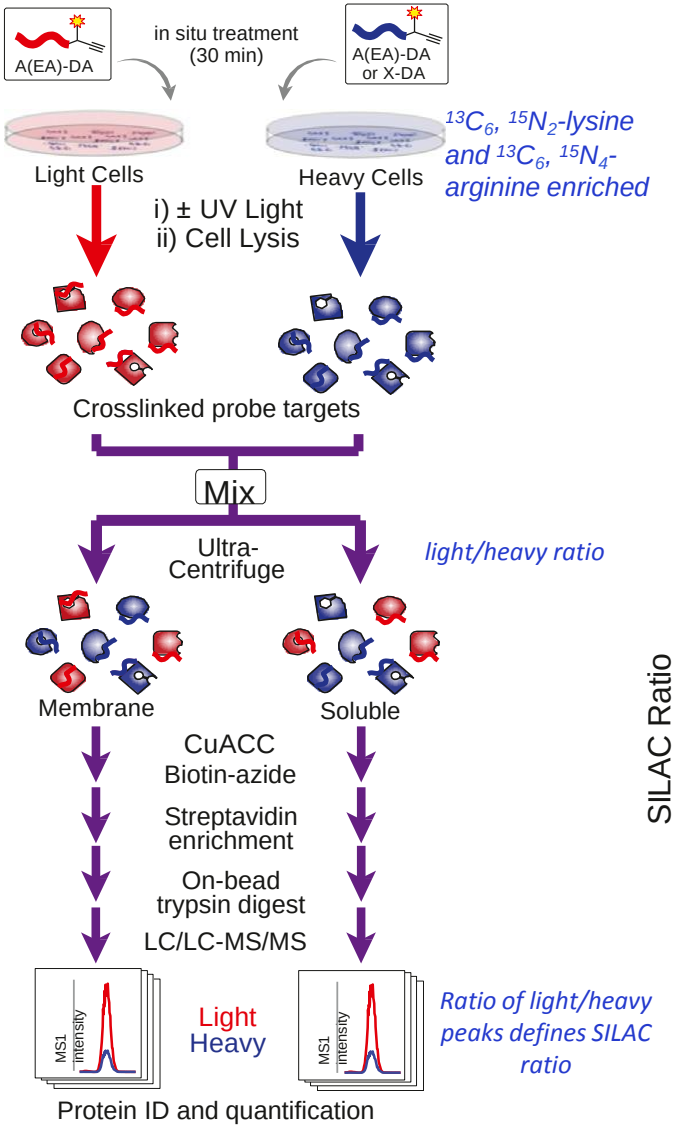
Polysaturated arachidonoyl probes (AEA-DA, A-DA) demonstrate more extensive protein labeling than monosaturated (OEA-DA, O-DA) or saturated probes (PEA-DA, S-DA)

Identification of Protein Targets

Stable isotope labeling by amino acids in cell culture (SILAC) and LC-tandem MS (LC-MS/MS)

SILAC

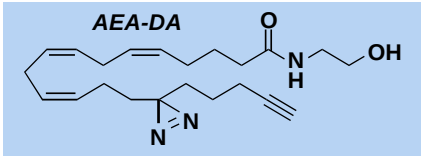
LC-MS/MS

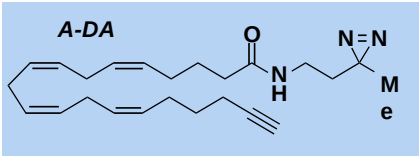


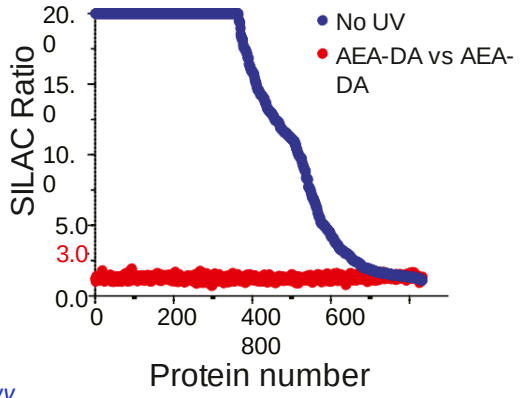
- **Light cells** are control and treated with arachidonoyl probe and UV
- Heavy cells are comparison

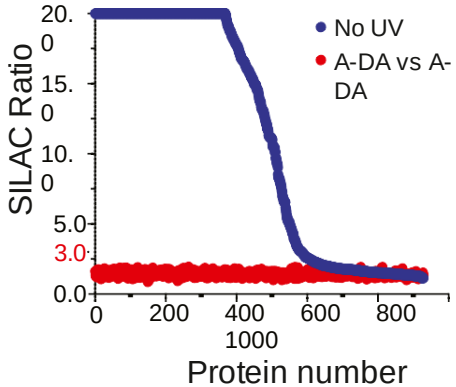
Heavy cell group:

a) Same condition as light cells (probe (vs) probe control)
b) Same probe as light cells but no UV (probe (vs) no UV)
c) Other lipid probe (OEA-DA, O-DA, PEA-DA, S-DA)





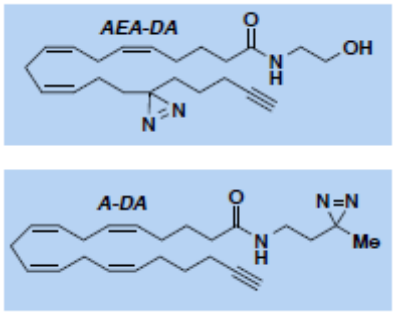




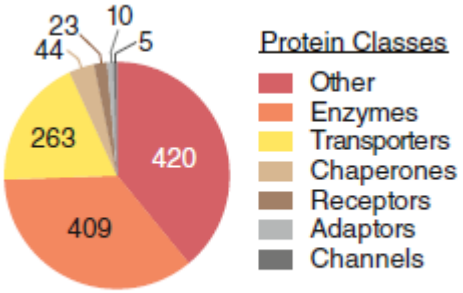
- Lipid probe protein targets are defined as proteins labeled in UV dependent manner (SILAC ratio > 3.0 in probe (vs) no UV) and not enriched in probe (vs) probe control (SILAC ratio < 2.0)

Classsification of identified proteins

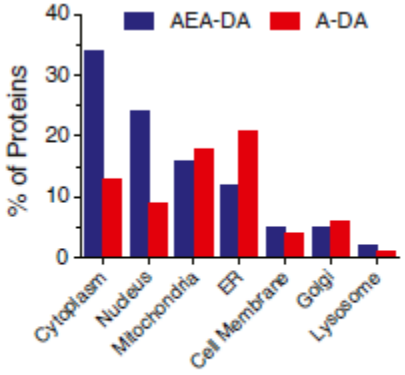
Identified protein targets include many known candidate (e.g. enzyme and lipid carriers involved in fatty acid uptake, transport, biosynthesis, catabolism), but also novel candidates.



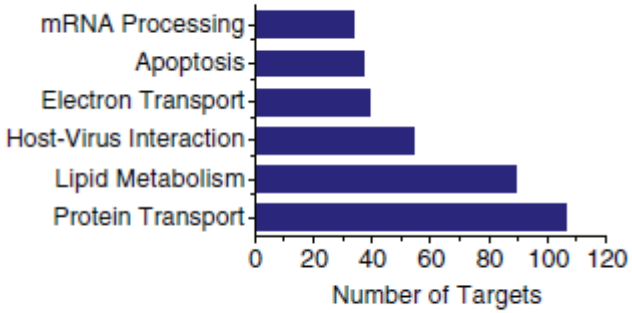
Proteins differentially enriched by two probes (from HEK293T (human) and Neuro2a (mouse) cells)



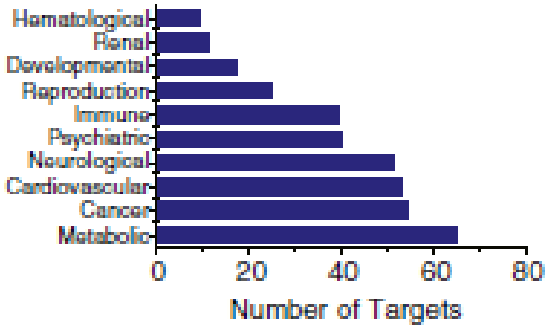
Protein class distribution



Known or predicted subcellular distribution

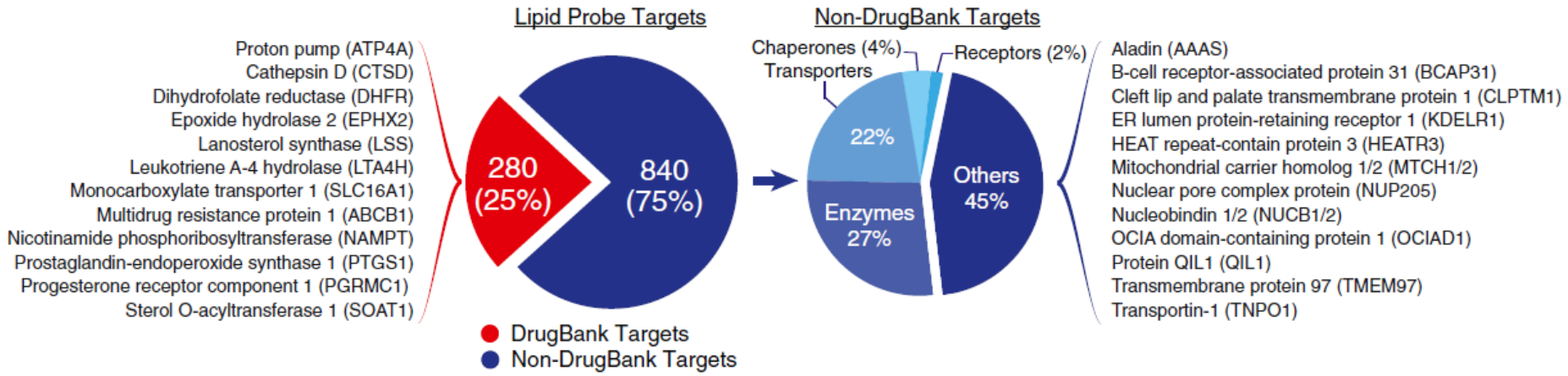


Protein involvement in biological processes



Protein links to disease

Lipid interaction proteome enriched in known drug targets

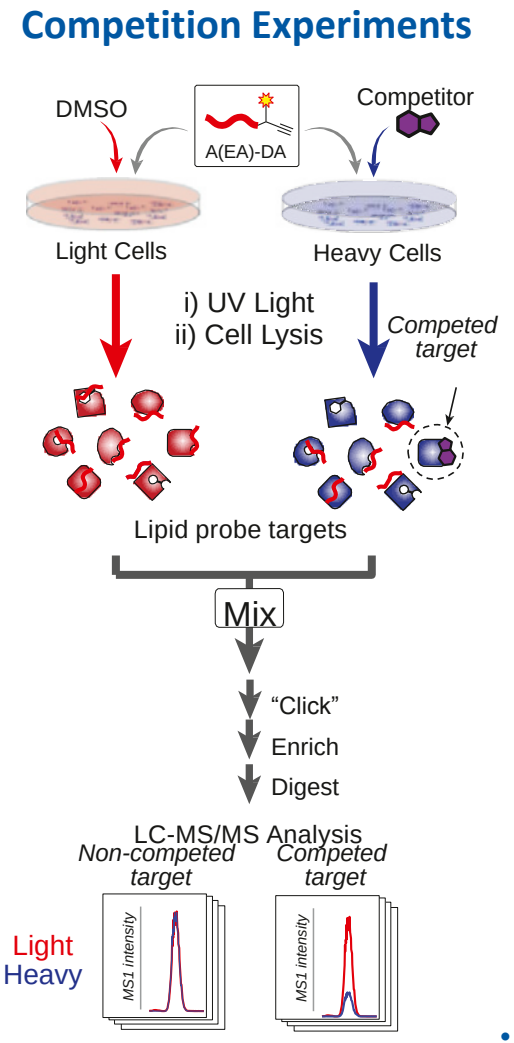


- *25% of the identified lipid interaction proteome is enriched in drug targets, while 12% of total human proteome is drugged.*

→ *lipid probes may preferentially interact with proteins that can bind other small molecule ligands*

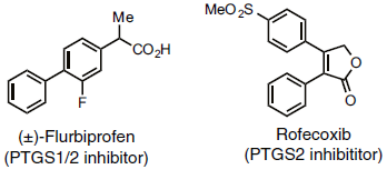
- *Hypothesize that lipid probes can provide methods to determine drug target engagement and selectivity*

Lipid probes as screening tools for novel ligands

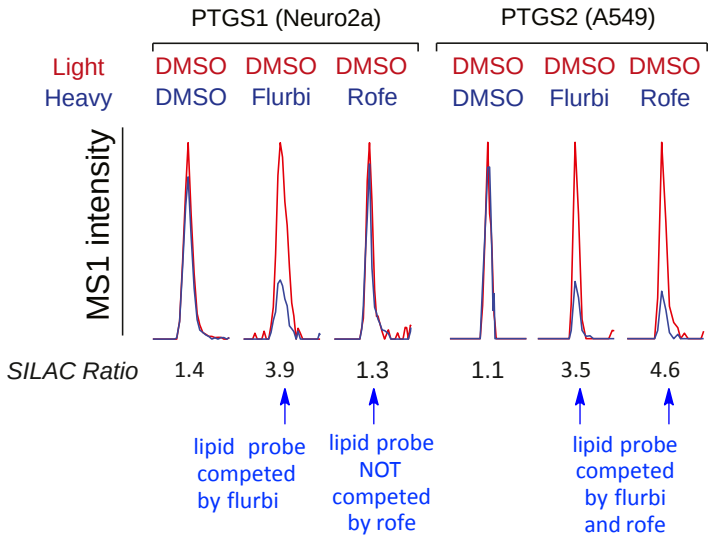


EXAMPLE

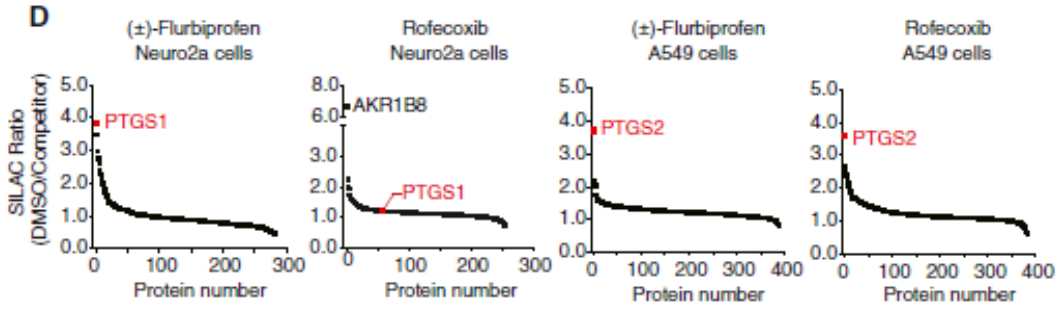
Prostaglandin biosynthesis enzymes (PTGS1, PTGS2)



Known Inhibitors



Competition consistent with known inhibitor selectivity



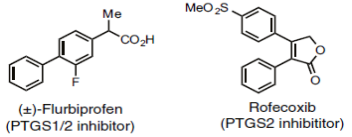
- *PTGS enzymes are among the most competed A-DA target proteins, indicating good selectivity*
- *AKR1B8 is mouse ortholog of human aldo-keto reductase which is modified/inhibited by prostaglandins*

Lipid probes as screening tools for novel ligands

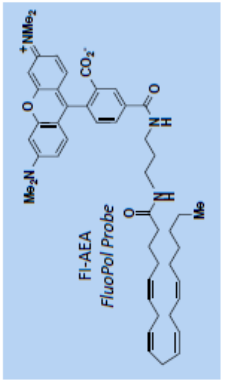
Nucleobindin protein NUCB1 *known to interact with PTGS1 and PTGS2 and enhance PTGS2-mediated prostaglandin synthesis (plays a role in lipid metabolism), but not before known to bind small molecule ligands.*

EXAMPLE

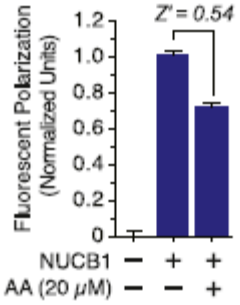
Prostaglandin biosynthesis enzymes (PTGS1, PTGS2)



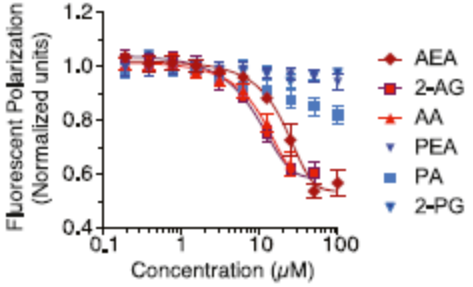
Known Inhibitors



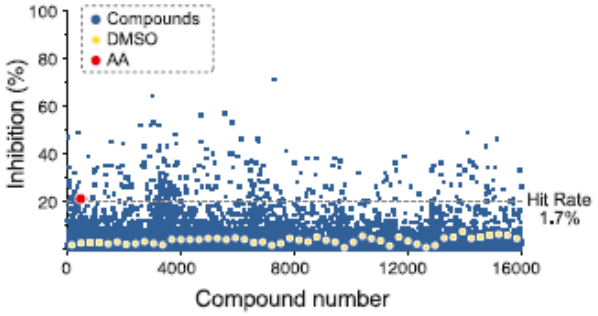
Synthesis of a fluorescent arachidonoyl lipid probe (FI-AEA)



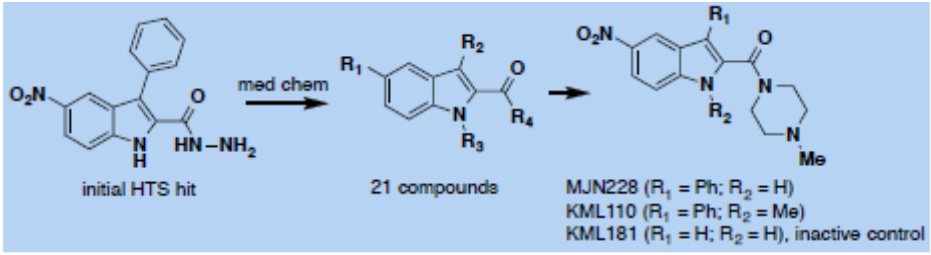
Increase in fluorescence polarization upon FI-AEA binding to purified recombinant NUCB1



Fluorescence polarization decreased by arachidonoyl but not palmitoyl competitor lipids



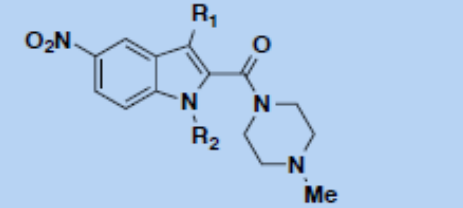
16,000 compounds screened for competitive binding to NUCB1 relative to FI-AEA probe



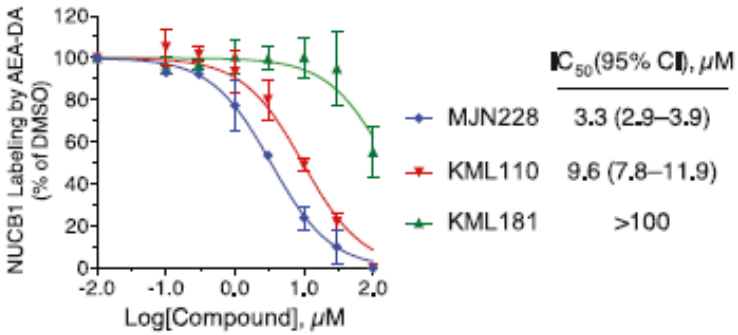
Optimization of initial screen hit to generate more potent NUCB1 binding ligands

MJN228 Competes Arachidonoyl Probe for NUCB1 Binding

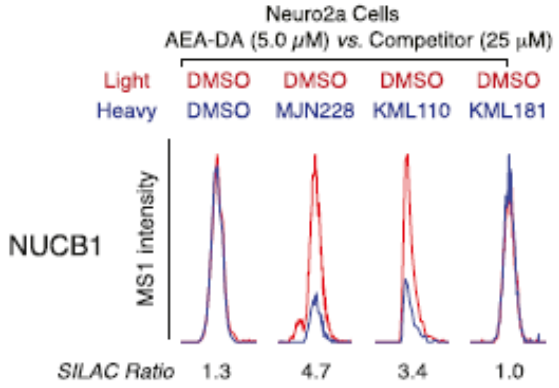
NUC1B ligands:



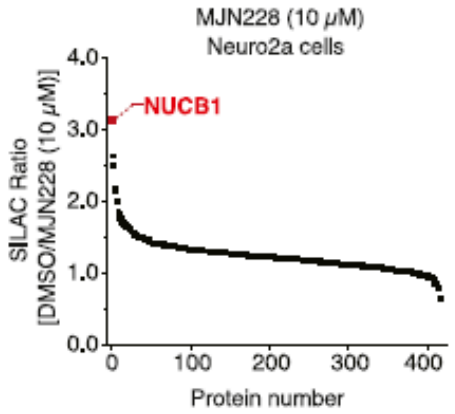
MJN228 (R_1 = Ph; R_2 = H)
KML110 (R_1 = Ph; R_2 = Me)
KML181 (R_1 = H; R_2 = H), inactive control



Competitive binding of optimized ligands to purified NUCB1 relative to FI-AEA probe



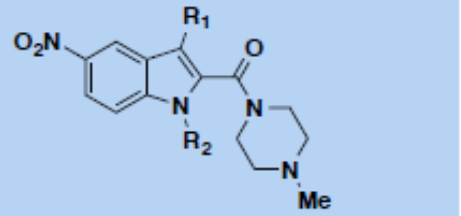
Inhibition of AEA-DA probe labeling of NUCB1 in cells by new ligands



MJN228 selectively inhibits AEA-DA probe binding to NUCB1 over ~400 other probe targets

Metabolic effects of NUCB1-ligand interraction

NUC1B ligands:



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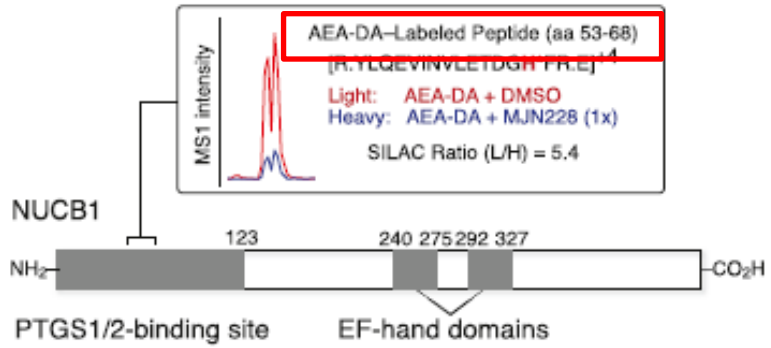
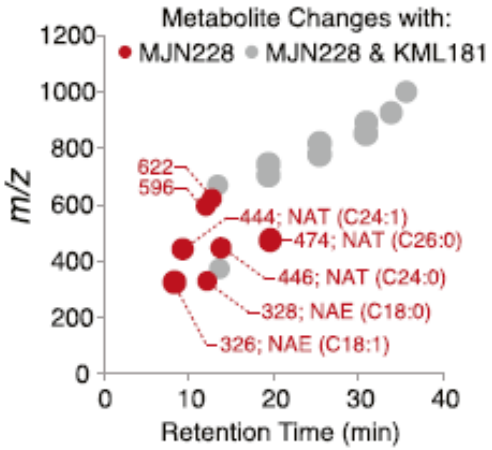
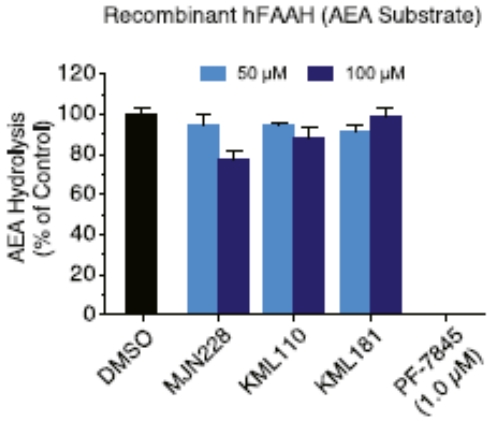


- *Site on NUCB1 of both ADA-DA probe and ligand MJN228 binding mapped to PTGS1/2 binding domain*
- *Suggests common region for NUC1B lipid-protein and protein-protein interactions*

Identifying metabolic consequences of NUCB1-MJN228 interaction:



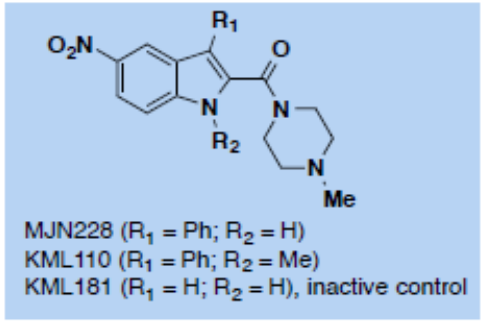
Cell treatment with MJN228 leads to elevated levels of N-acyl ethanolamines (NAEs) and N-acyl taurines (NATs), two classes of fatty acid amides



NAEs and NATs are both metabolized by the enzyme fatty acid amide hydrolase (FAAH), but neither MJN228 or KML110 inhibit FAAH (PF-7845 is known FAAH inhibitor)

Metabolic effects of NUCB1-ligand interraction

NUC1B ligands:



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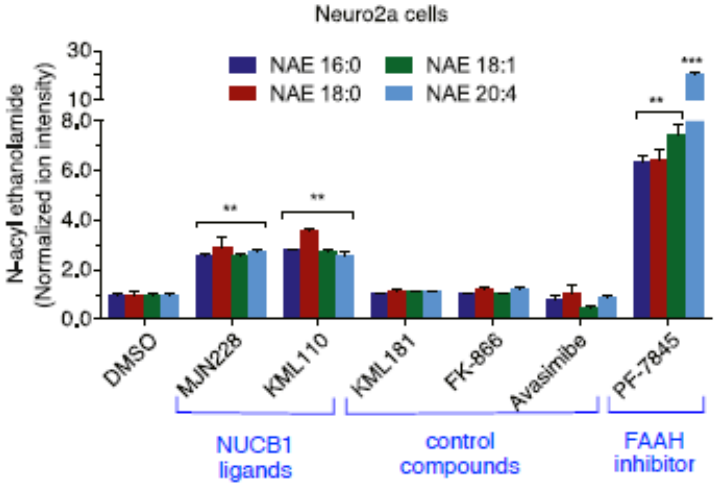
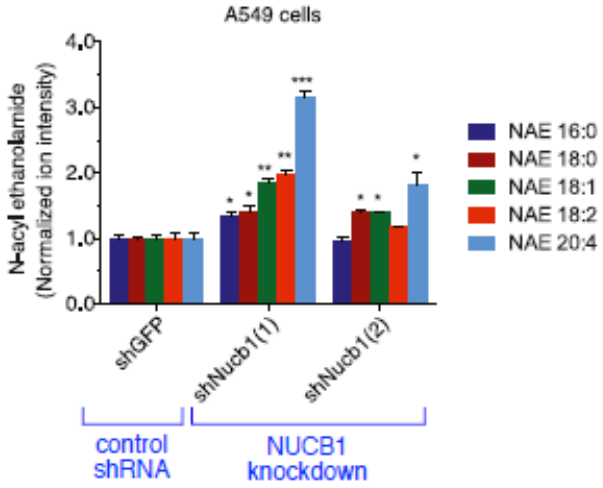
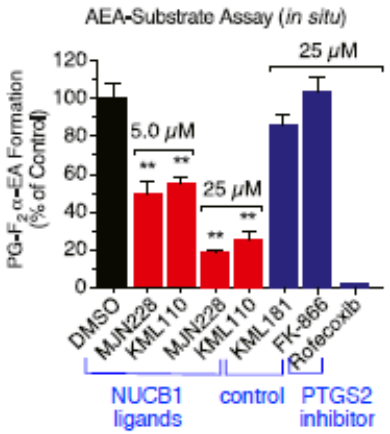


Cell treatment with MJN228 and KML110 leads to elevated levels of N-acyl ethanolamines



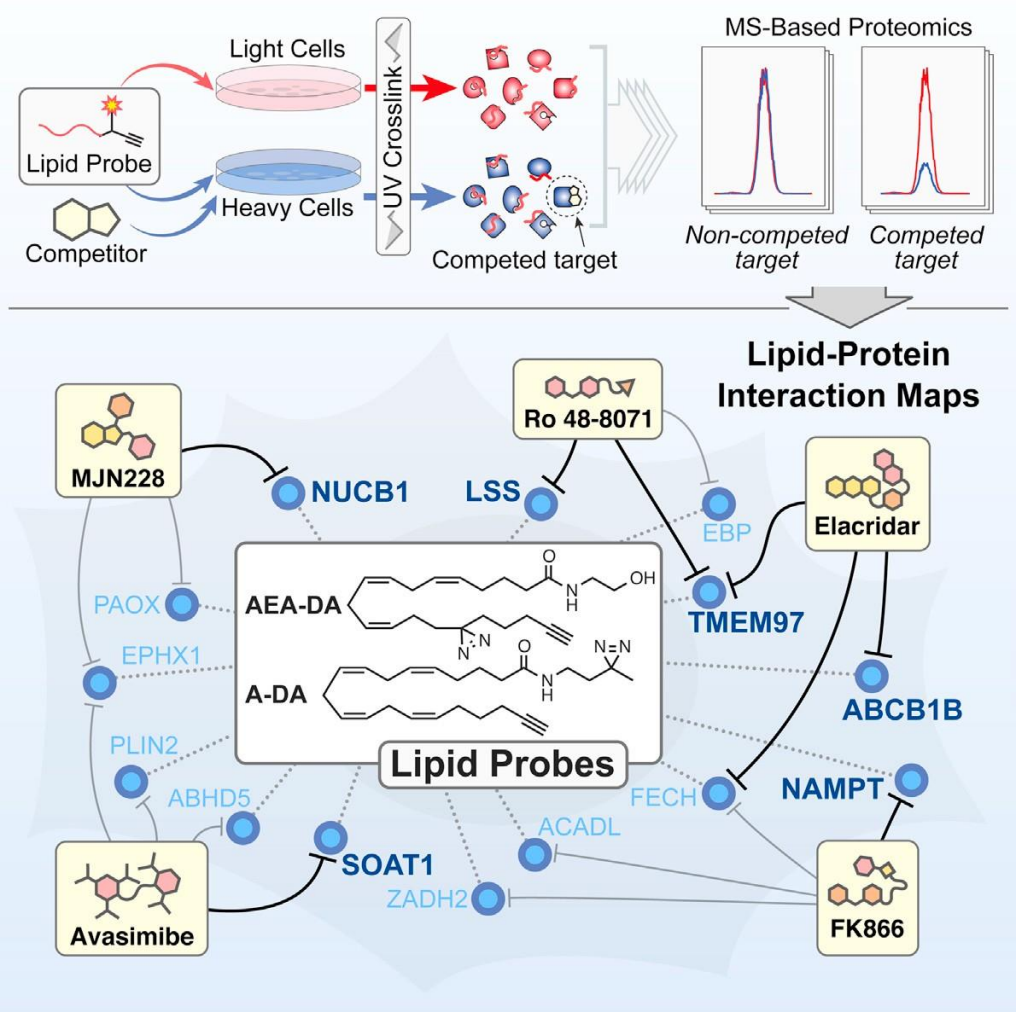
Knocking down expression of NUC1B leads to elevated levels of fatty acid amides



NUCB1 ligands also inhibit the oxidative metabolism of exogenously added arachidonylethanolamine

Data collectively suggests that NUCB1 plays indirect role in facillitating fatty acid amide metabolism, e.g. serving as intracellular carrier to deliver lipids to fatty acid amide hydrolase (FAAH)

Mapping Lipid-Binding Proteins and their Lingability



Thank you!

Questions?