Imaging lipids using lipid-binding toxins

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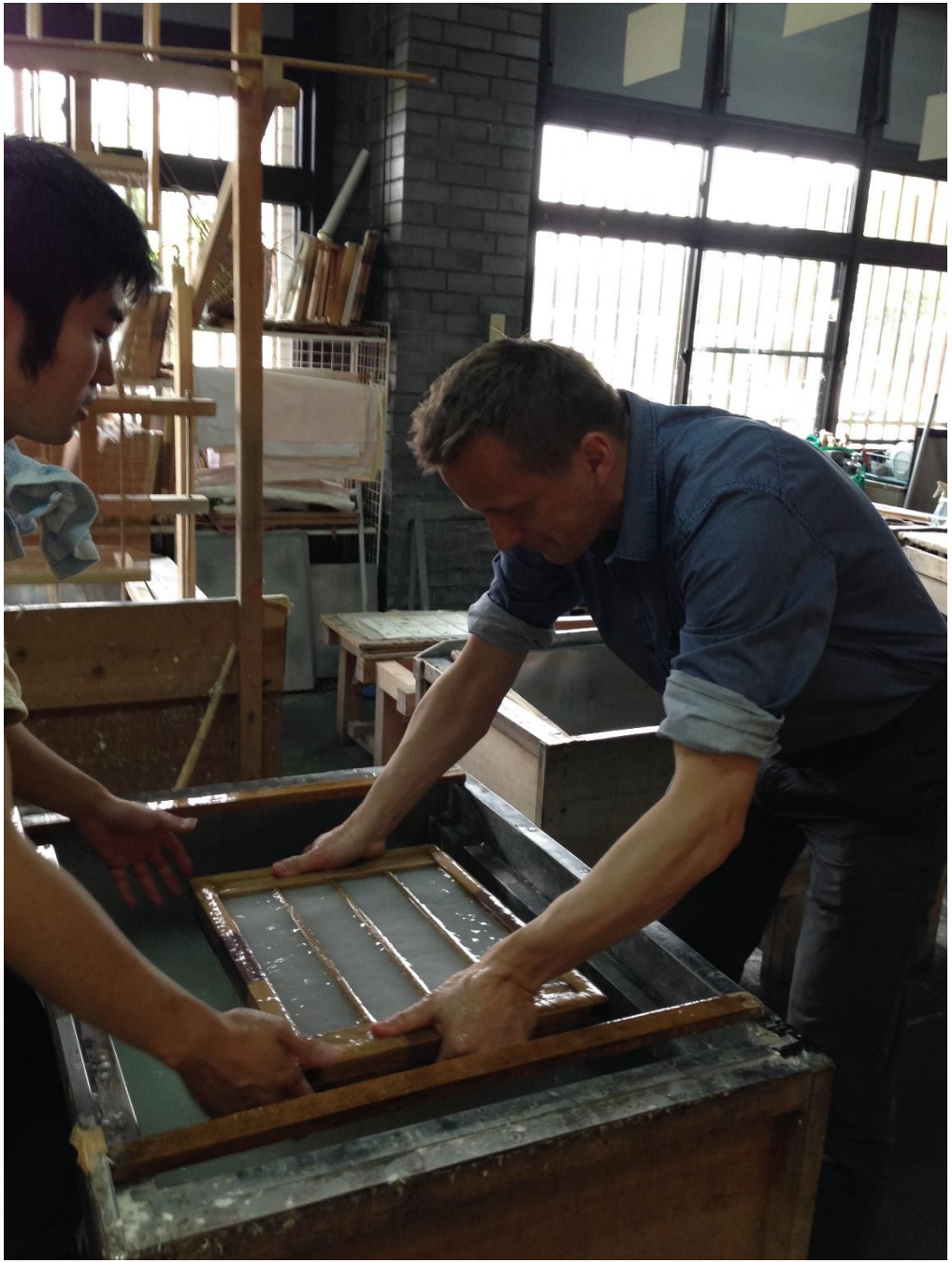
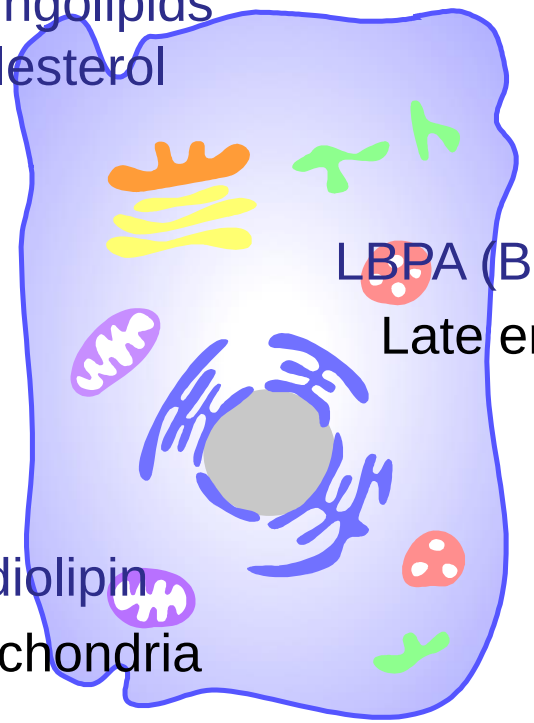
仙元山 山頂
298.9m



Lipids are not randomly distributed in the cell

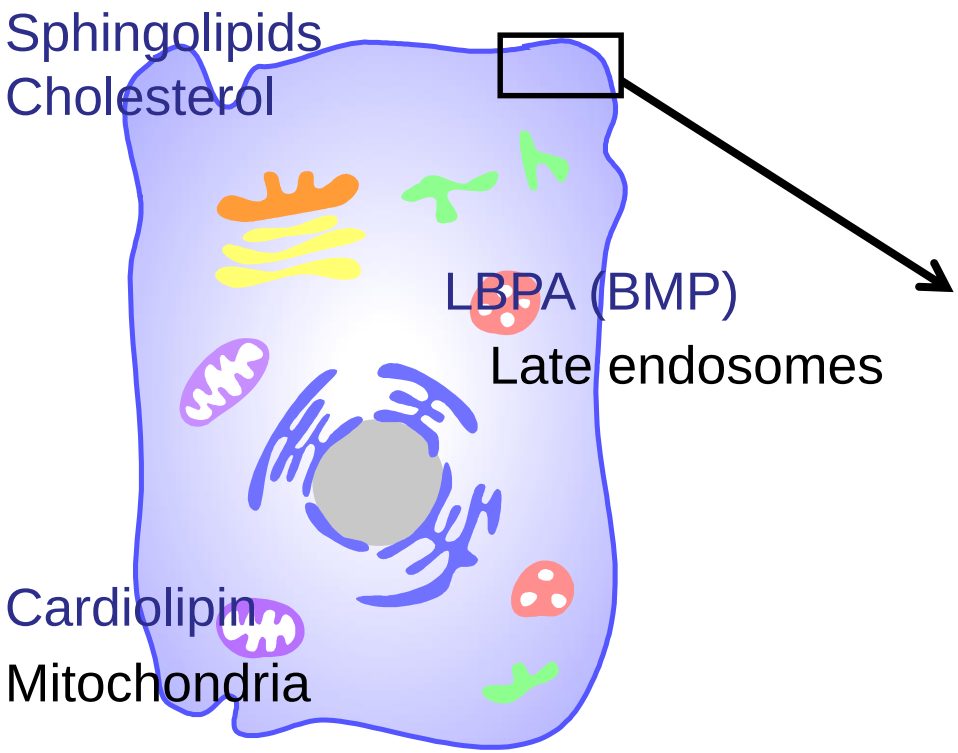
Sphingolipids
Cholesterol



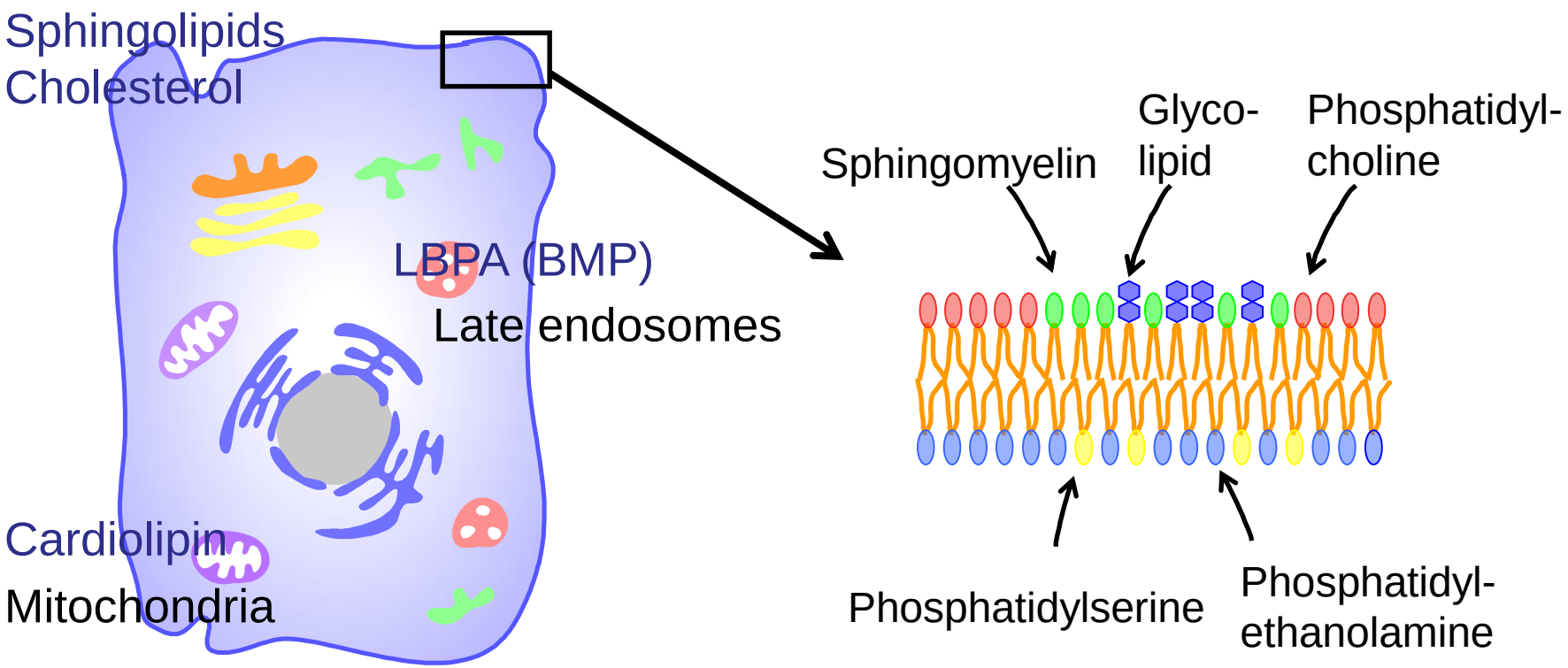
LBPA (BMP)
Late endosomes

Cardiolipin
Mitochondria

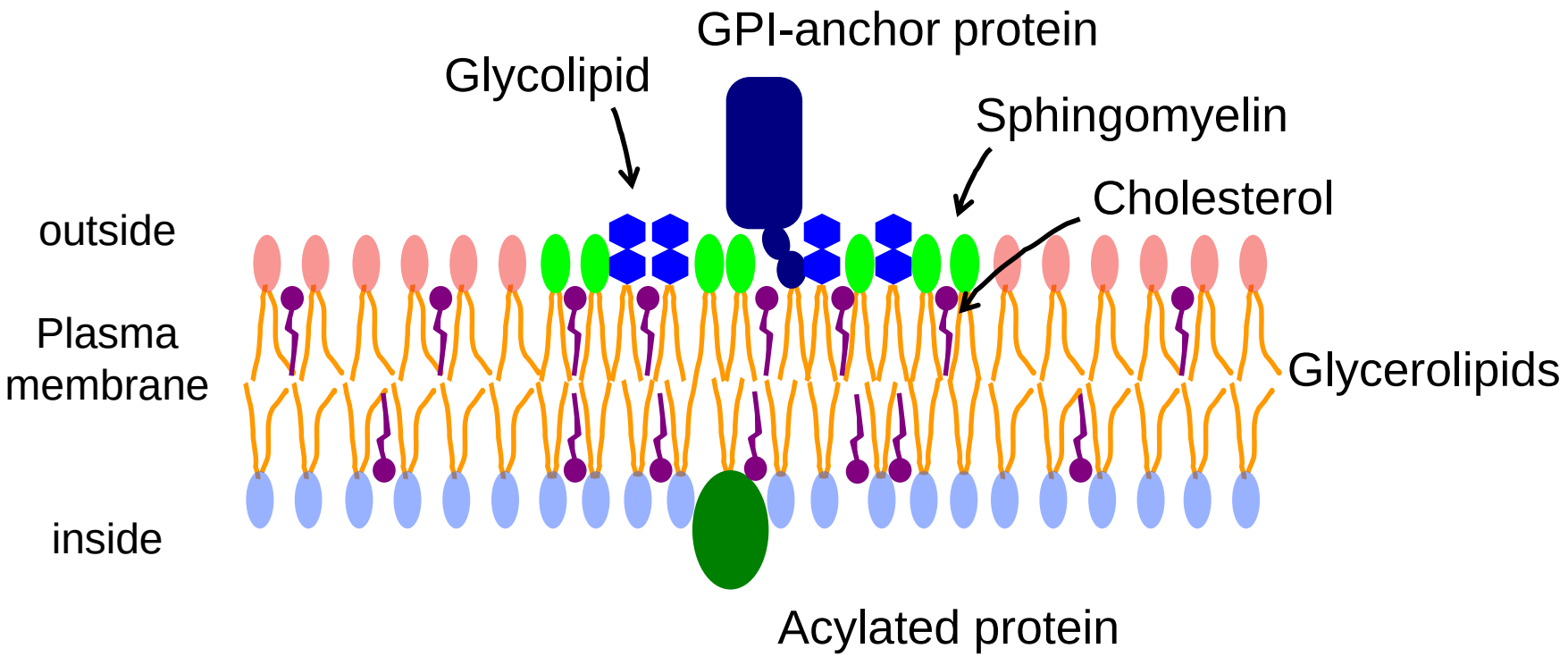
Lipids are not randomly distributed in the cell



Lipids are not randomly distributed in the cell

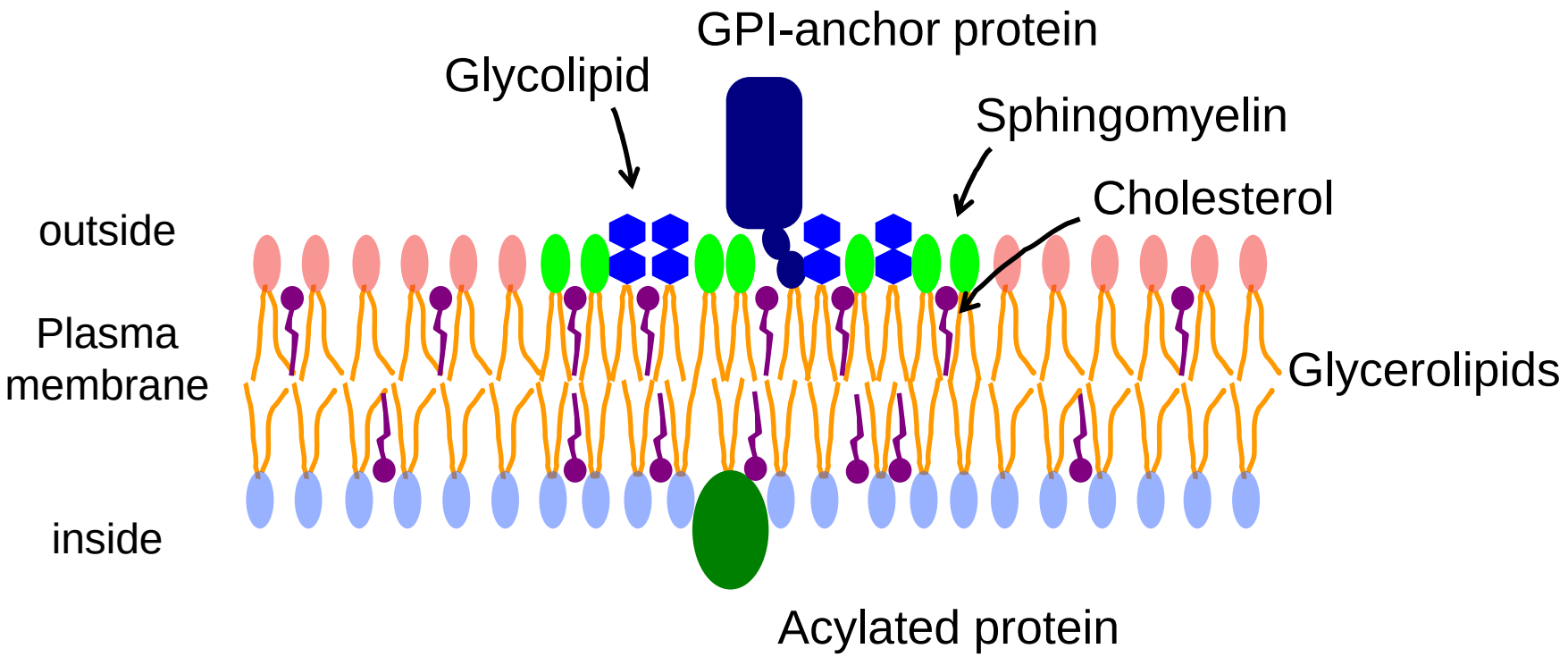


Lipid raft hypothesis



Simons, K. and Ikonen, E. (1997) *Nature*, **387**, 569-72
Munro, S. (2003) Cell, 115, 377-388

Lipid raft hypothesis



Proposed function:
Signal transduction
Membrane traffic
Virus and bacteria infection

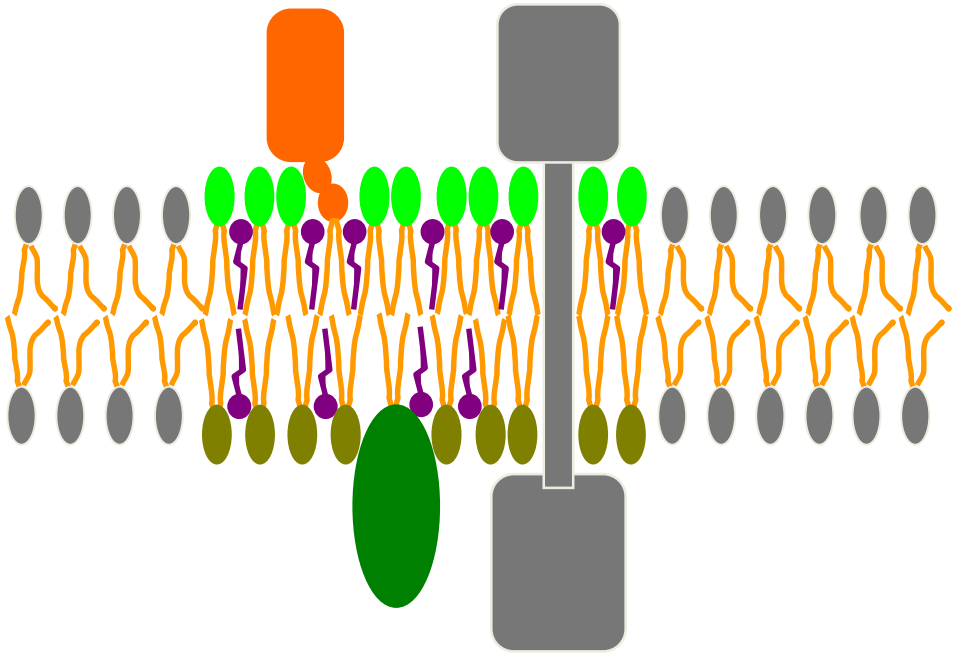
Simons, K. and Ikonen, E. (1997) *Nature*, **387**, 569-72
Munro, S. (2003) Cell, 115, 377-388

Our goal

Understanding the function of lipids and lipid domains by imaging them

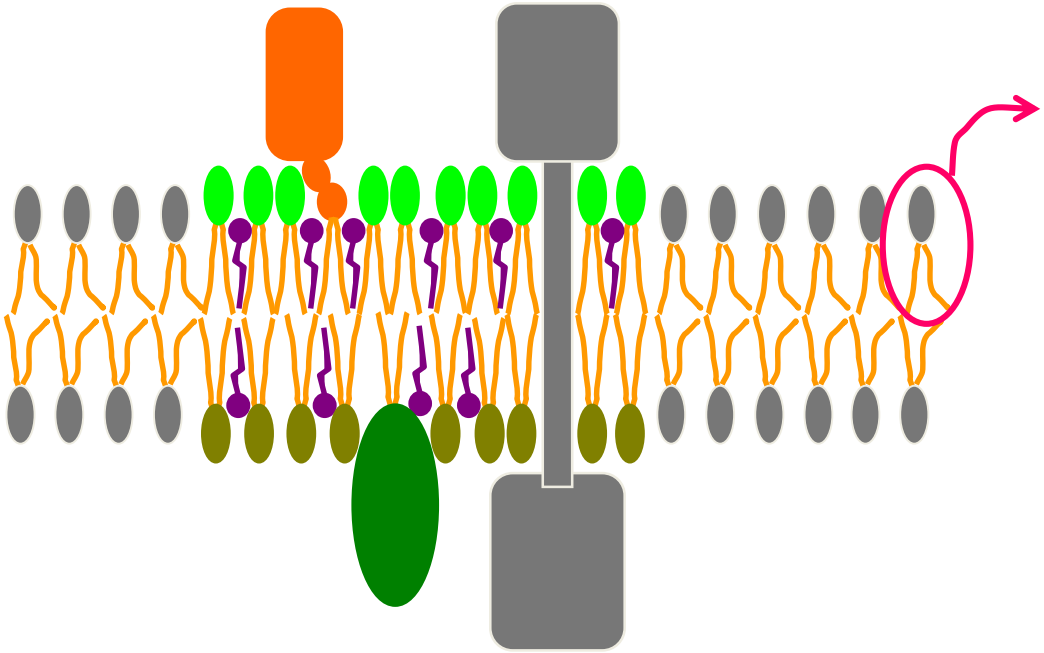
Difficulty of imaging lipids

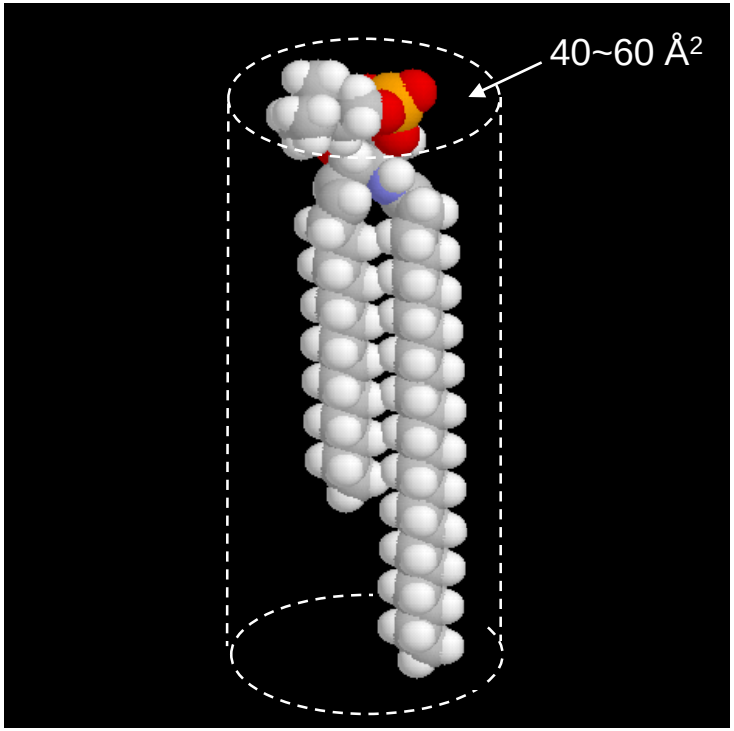
1. Small size domains



Difficulty of imaging lipids

1. Small size domains



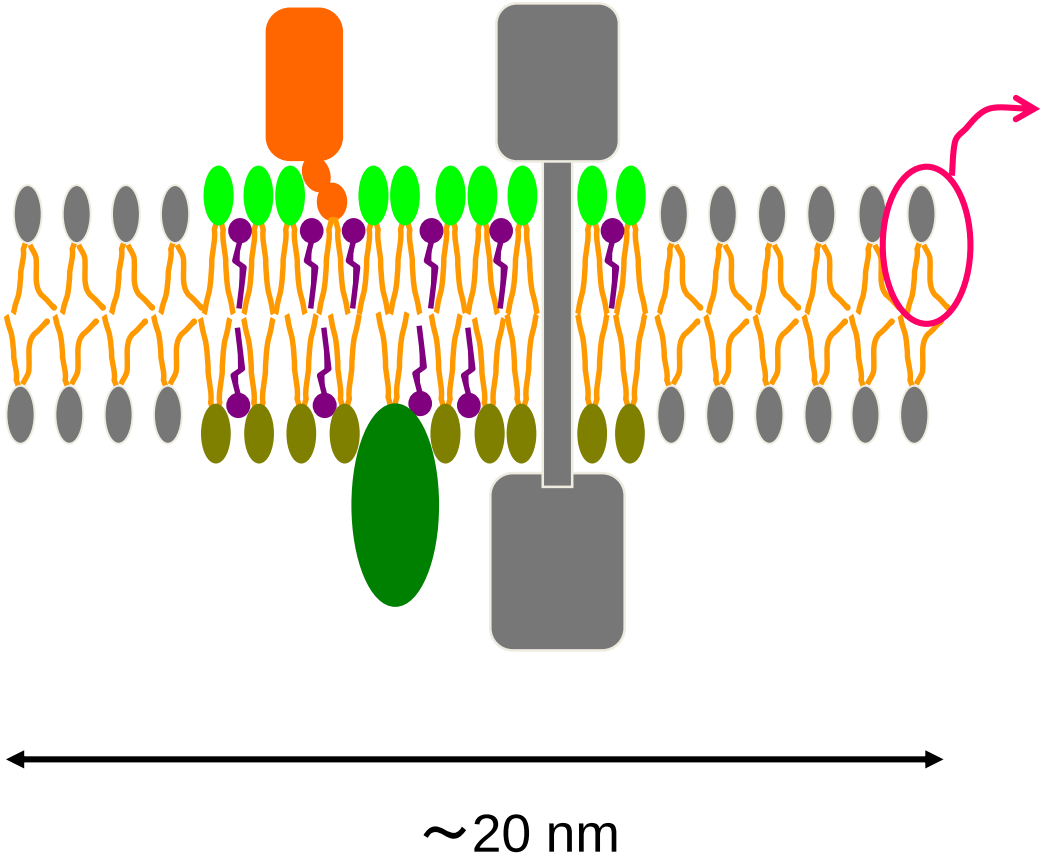


100 nm x 100 nm = 10^4 nm^2
= 10^6 A^2

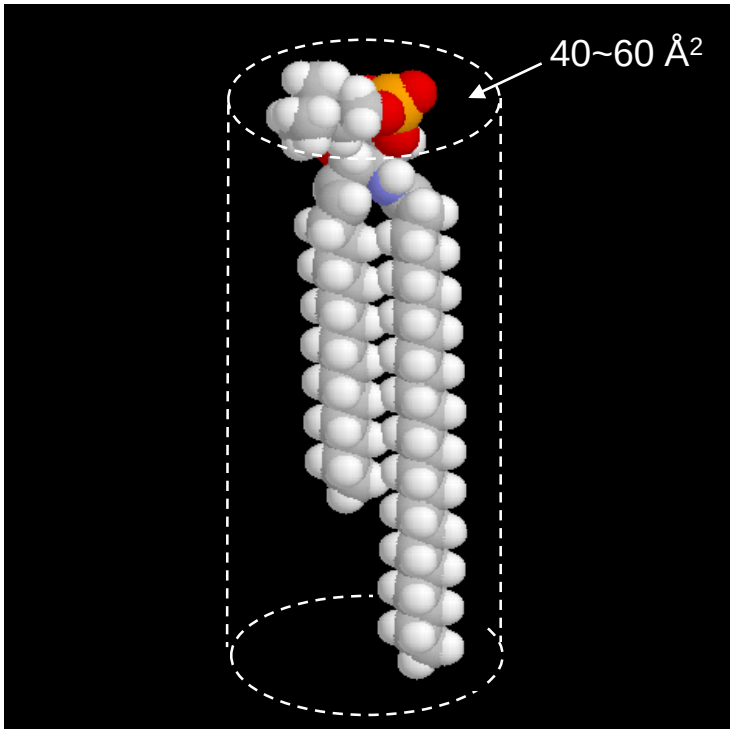
20,000 lipid molecules in 100 nm^2

Difficulty of imaging lipids

1. Small size domains



~20 nm

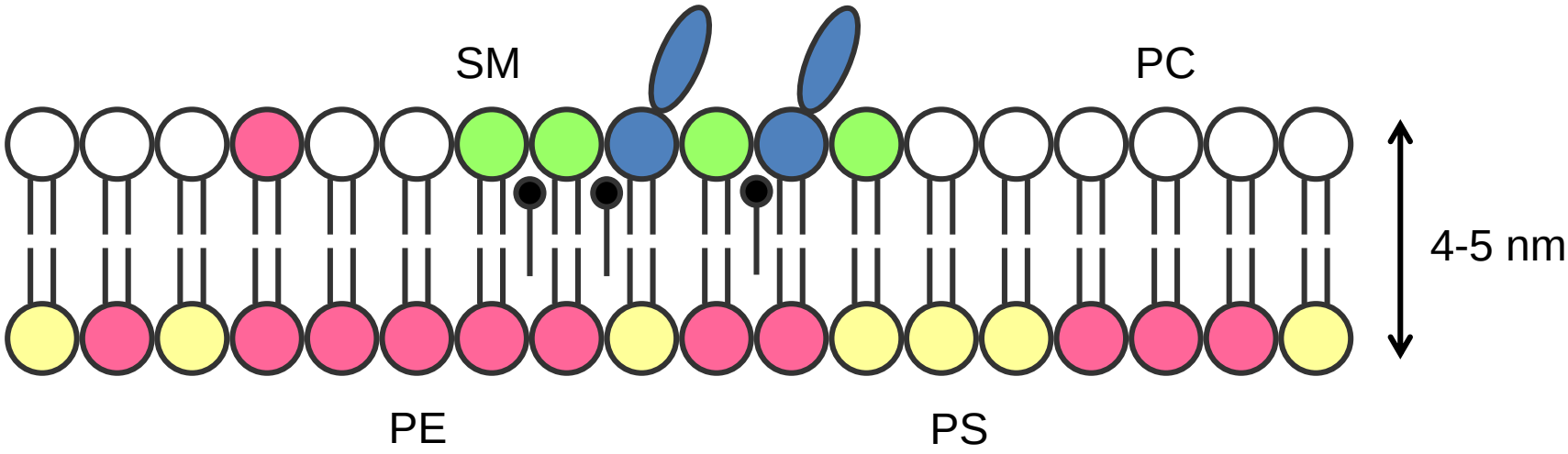


100 nm x 100 nm = 10^4 nm^2
= 10^6 A^2

20,000 lipid molecules in 100 nm^2

Difficulty of imaging lipids

2. Asymmetric distribution between outer and inner leaflet of the membrane



Sphingolipids are distributed in the outer leaflet of lipid rafts.

Lipid distribution of the inner leaflet of lipid rafts is not well understood.

Our attempt

1. Developing and characterizing lipid-specific probes.

2. Introducing state-of-the-art imaging techniques.

A toxin-based probe reveals cytoplasmic exposure of Golgi sphingomyelin.

Bakrac B, Kladnik A, Macek P, McHaffie G, Werner A, Lakey JH, Anderluh G.

J Biol Chem. 2010 Jul 16;285(29):22186-95. doi: 10.1074/jbc.M110.105122. Epub 2010 May 12

Tracking cholesterol/sphingomyelin-rich membrane domains with the ostreolysin A-mCherry protein.

Skočaj M, Resnik N, Grundner M, Ota K, Rojko N, Hodnik V, Anderluh G, Sobota A, Maček P, Veranič P, Sepčić K.

PLoS One. 2014 Mar 24;9(3):e92783. doi: 10.1371/journal.pone.0092783. eCollection 2014.

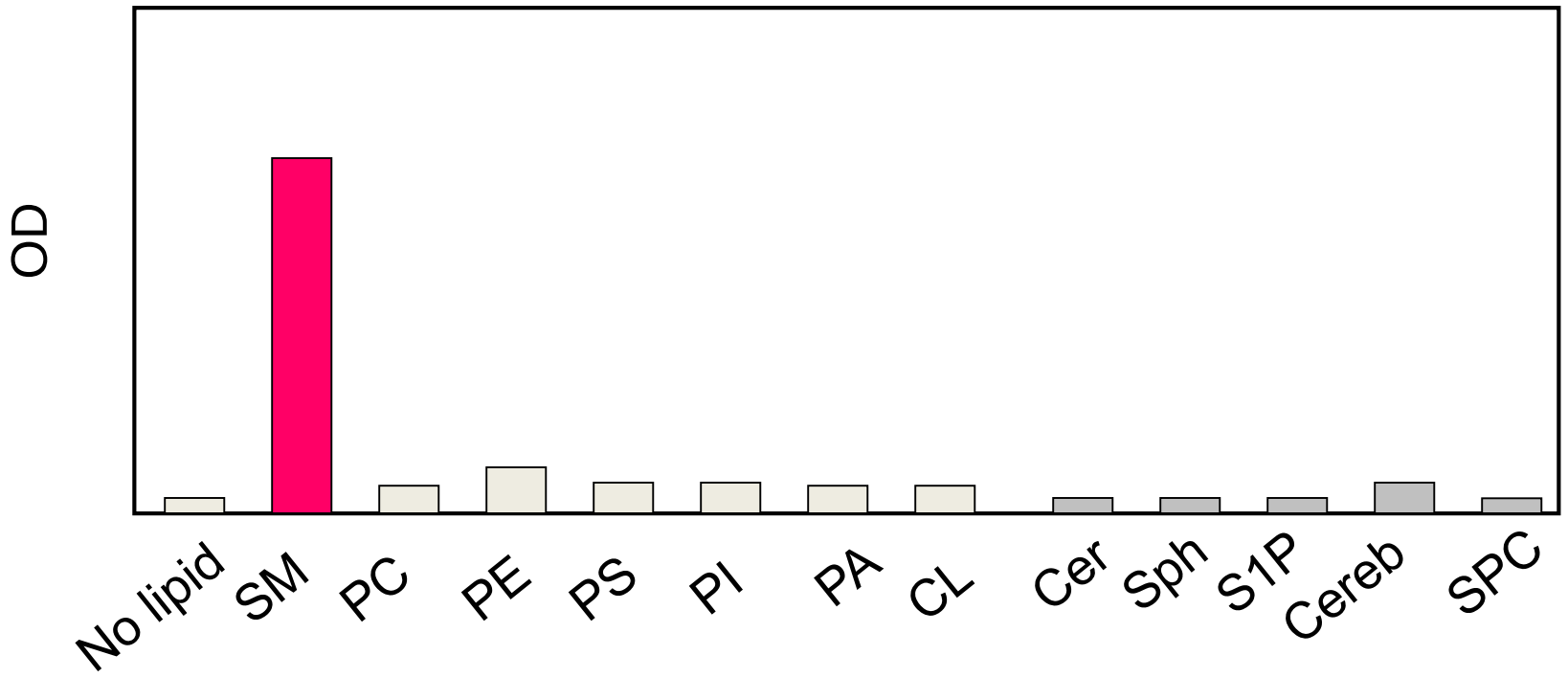
Imaging sphingomyelin

Lysenin

1. is an earthworm toxin.
2. is 41kDa protein.
3. causes contraction of vascular smooth muscle.
4. specifically recognizes sphingomyelin.
5. induces hemolysis and cell death.

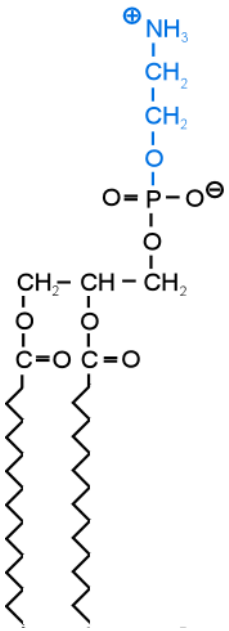
Yamaji A, Sekizawa Y, Emoto K, Sakuraba H, Inoue K, Kobayashi H, Umeda M
Lysenin, a novel sphingomyelin-specific binding protein
J Biol Chem. 273, 5300 (1998)

MBP(Maltose-binding protein)-Lysenin specifically recognizes sphingomyelin in ELISA

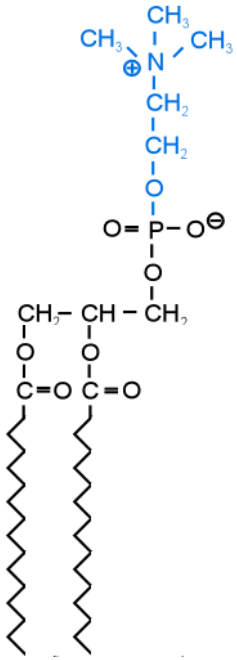


Kiyokawa 2004

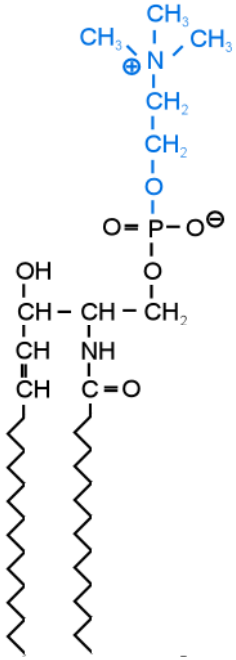
Structure of Membrane Lipids



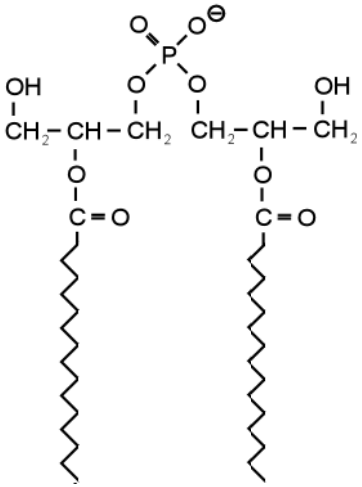
Phosphatidyl-ethanolamine



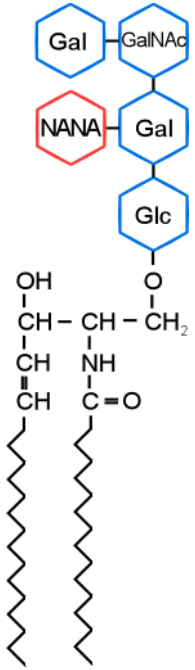
Phosphatidyl-choline



Sphingomyelin

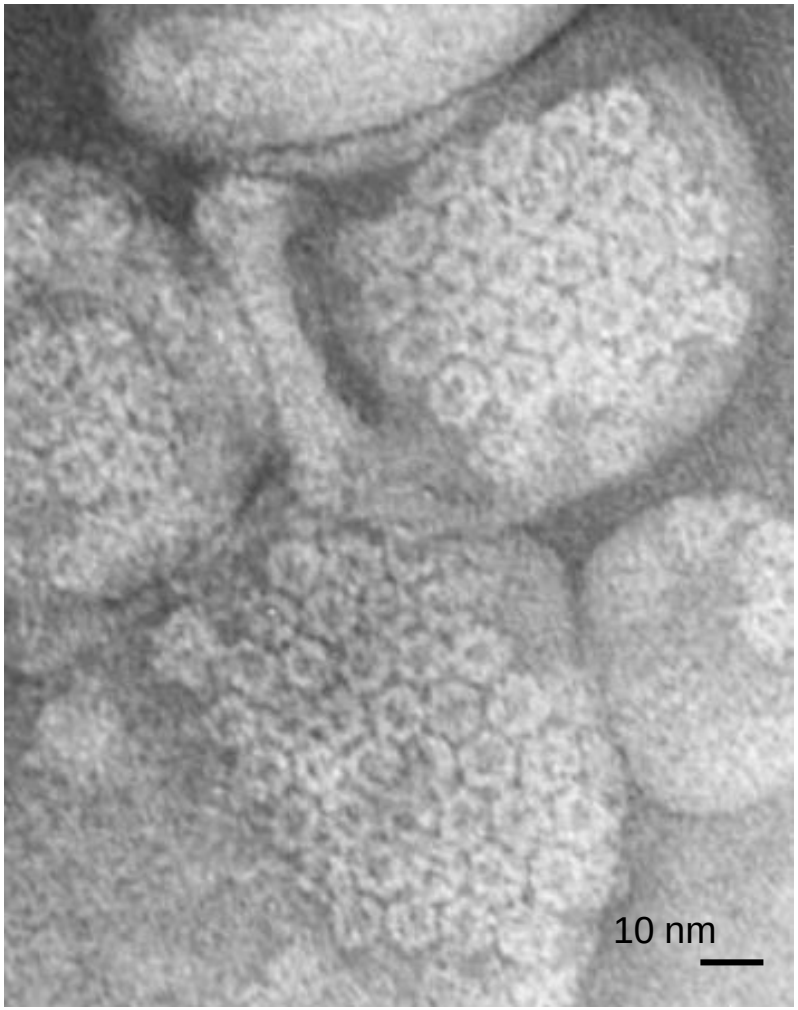


Lysobisphos-phatidic acid



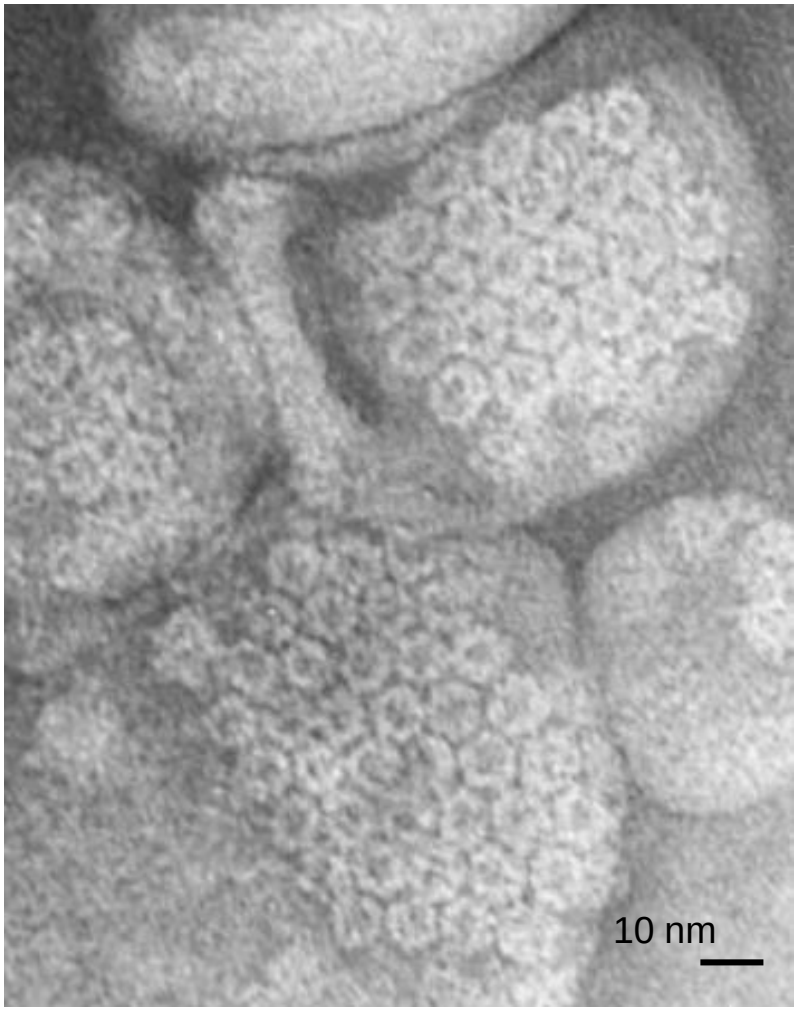
Ganglioside (GM1)

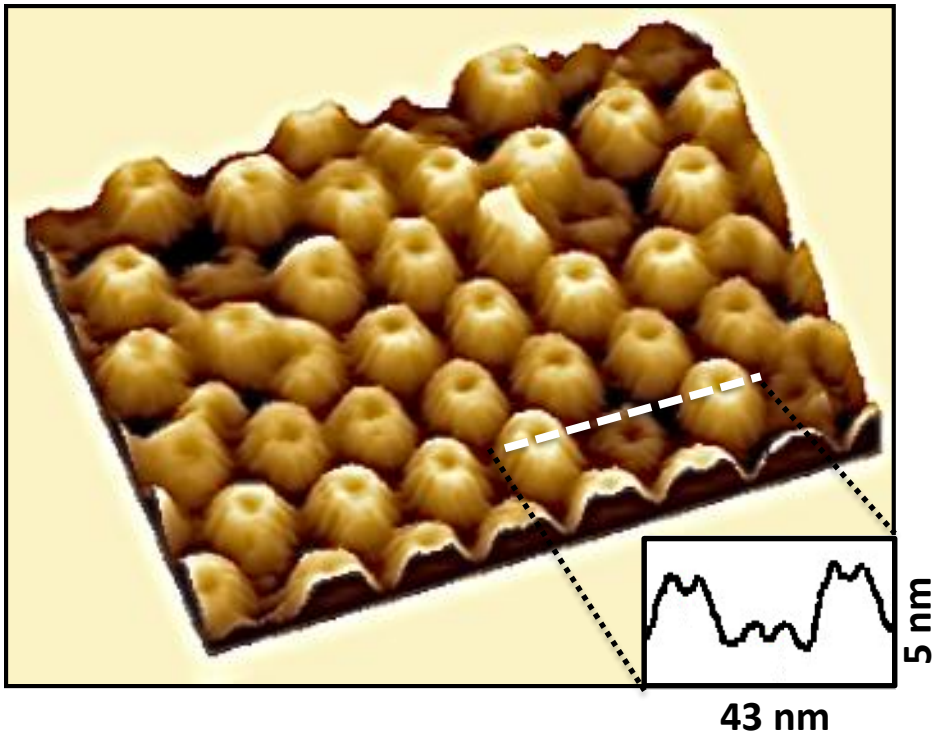
Lysenin induces characteristic honeycomb structure in sphingomyelin-containing membranes



Yamaji-Hasegawa 2003; Yilmaz 2013

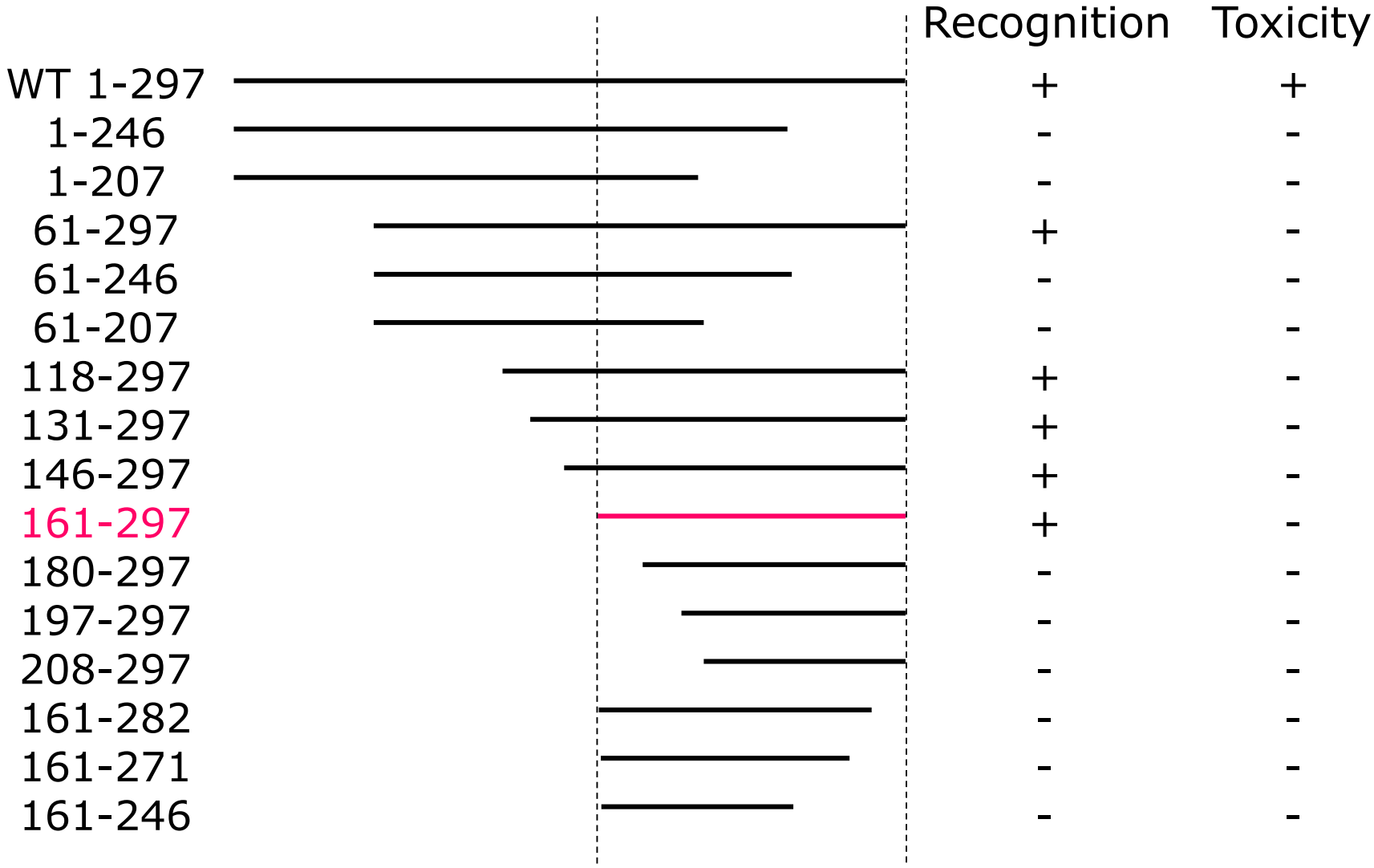
Lysenin induces characteristic honeycomb structure in sphingomyelin-containing membranes





Yamaji-Hasegawa 2003; Yilmaz 2013

Recognition of sphingomyelin by lysenin mutants

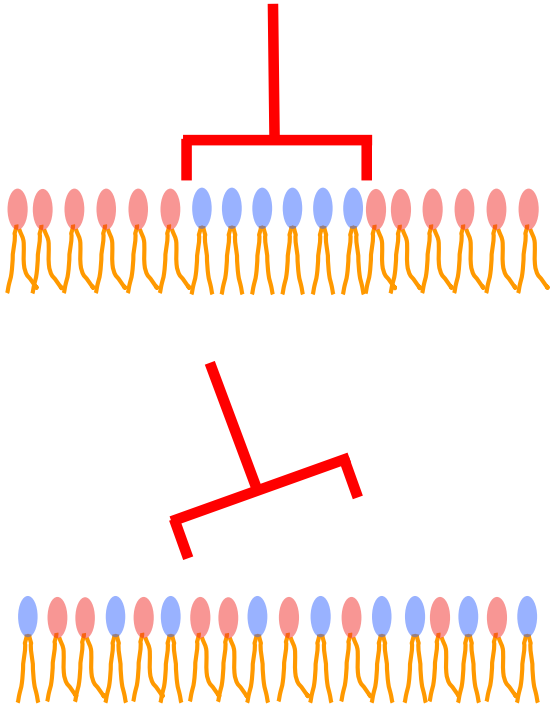


Kiyokawa 2005

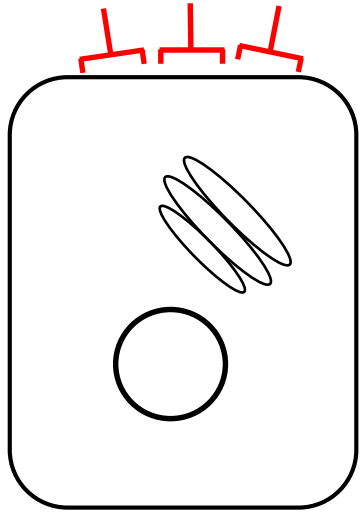
Detection of sphingomyelin clusters by lysenin

Model Membrane

Lysenin specifically binds sphingomyelin clusters



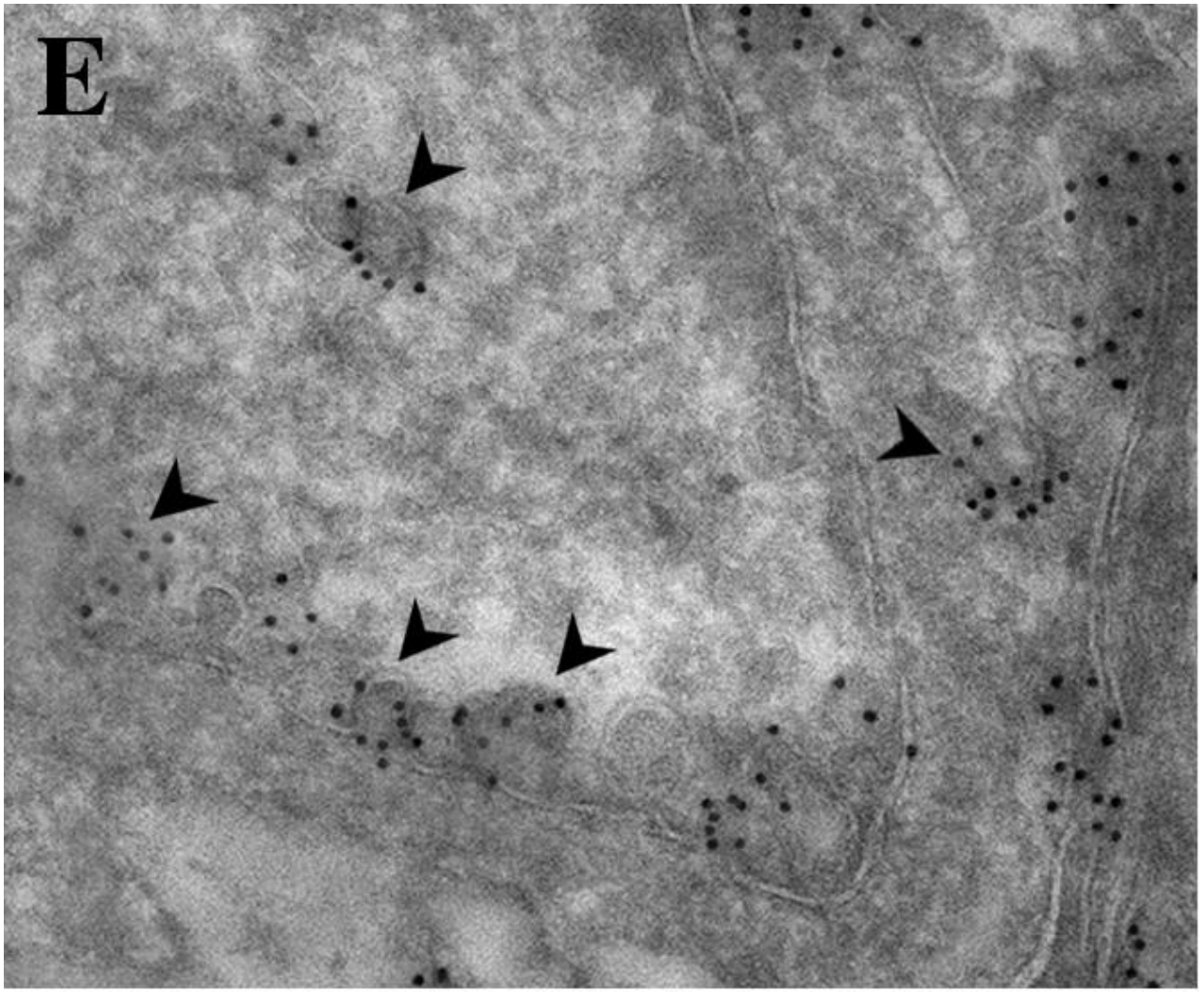
Cell Membrane



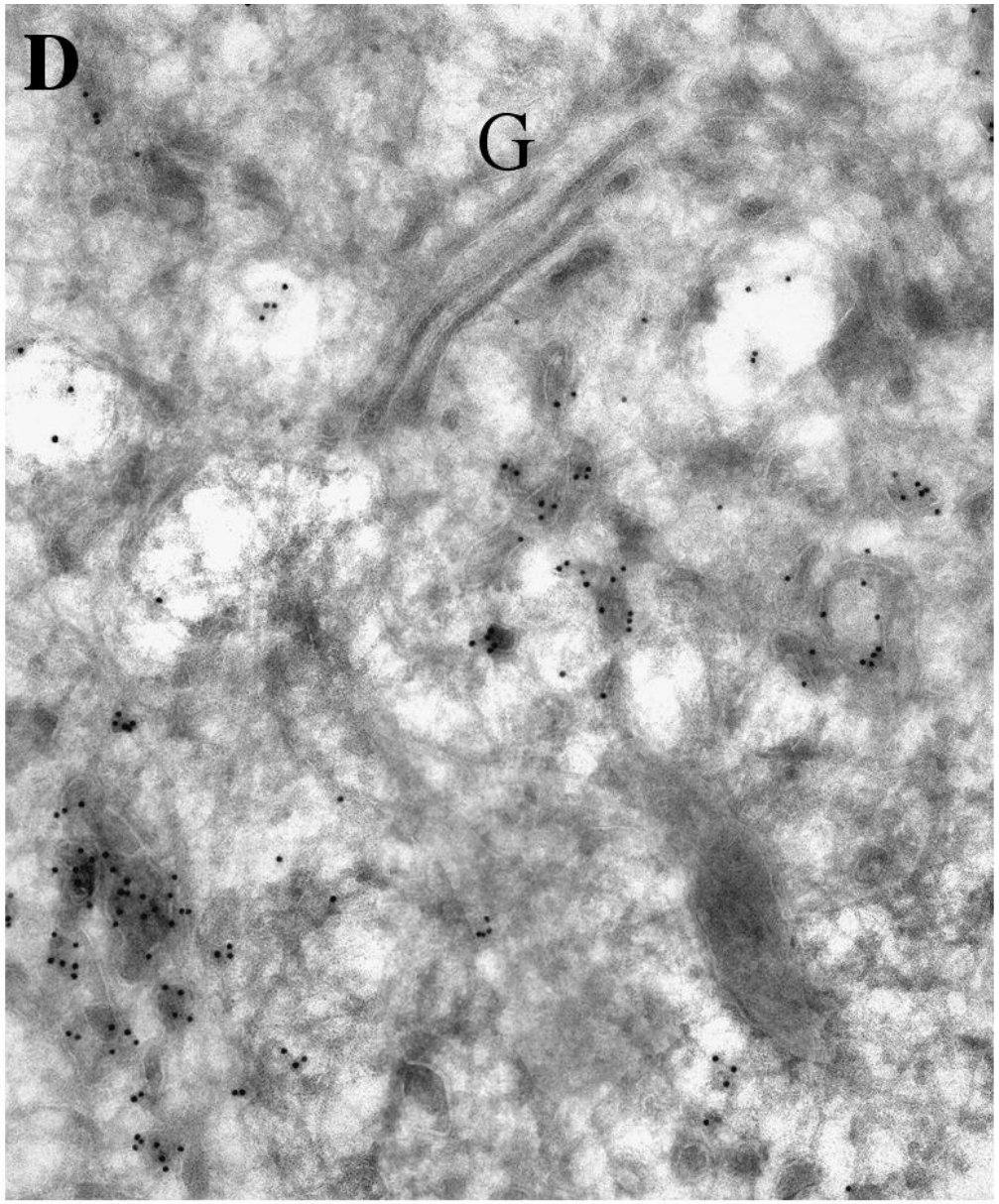
Binding means

Existence of the domain

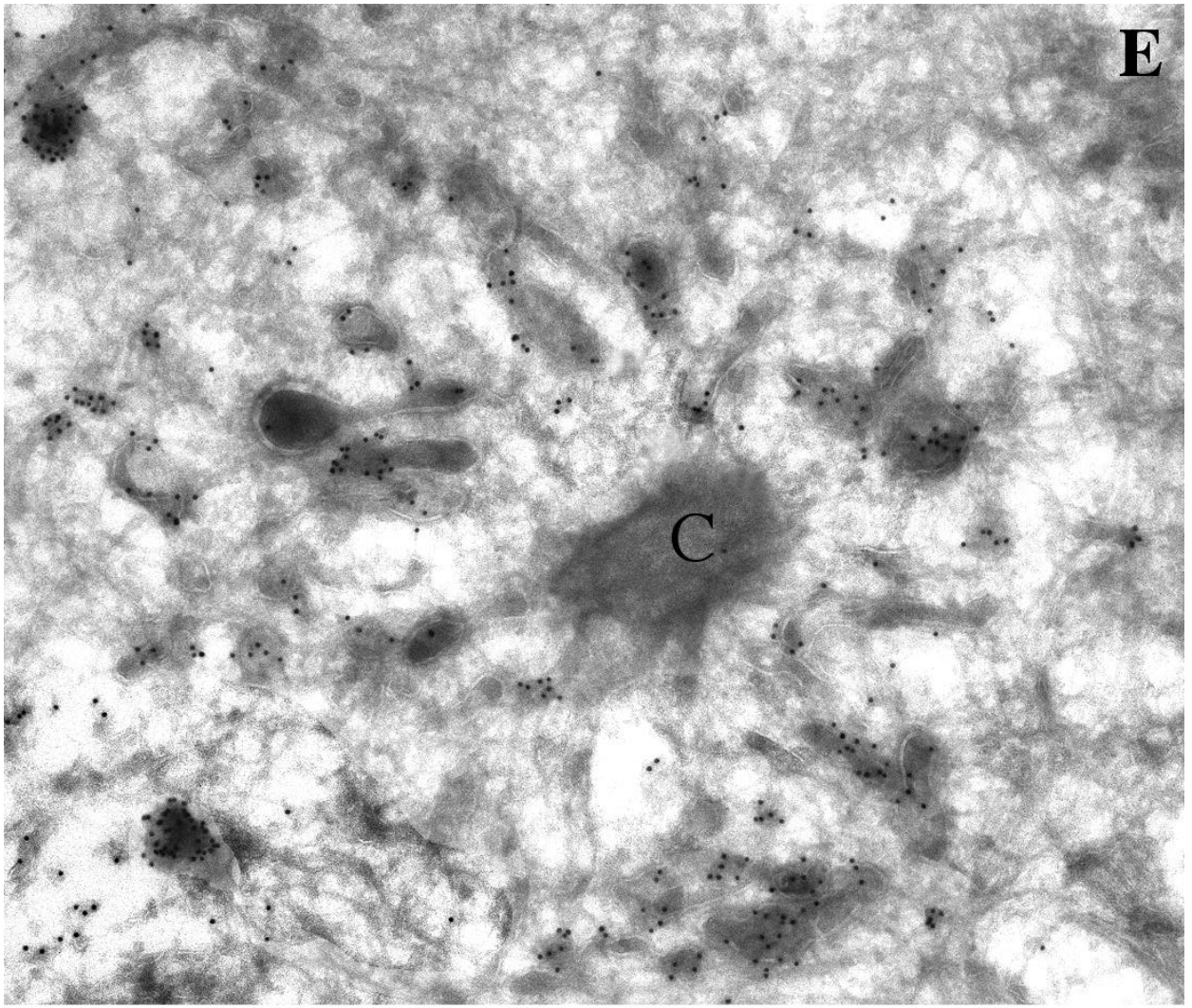
Sphingomyelin-rich domain localizes in caveolae

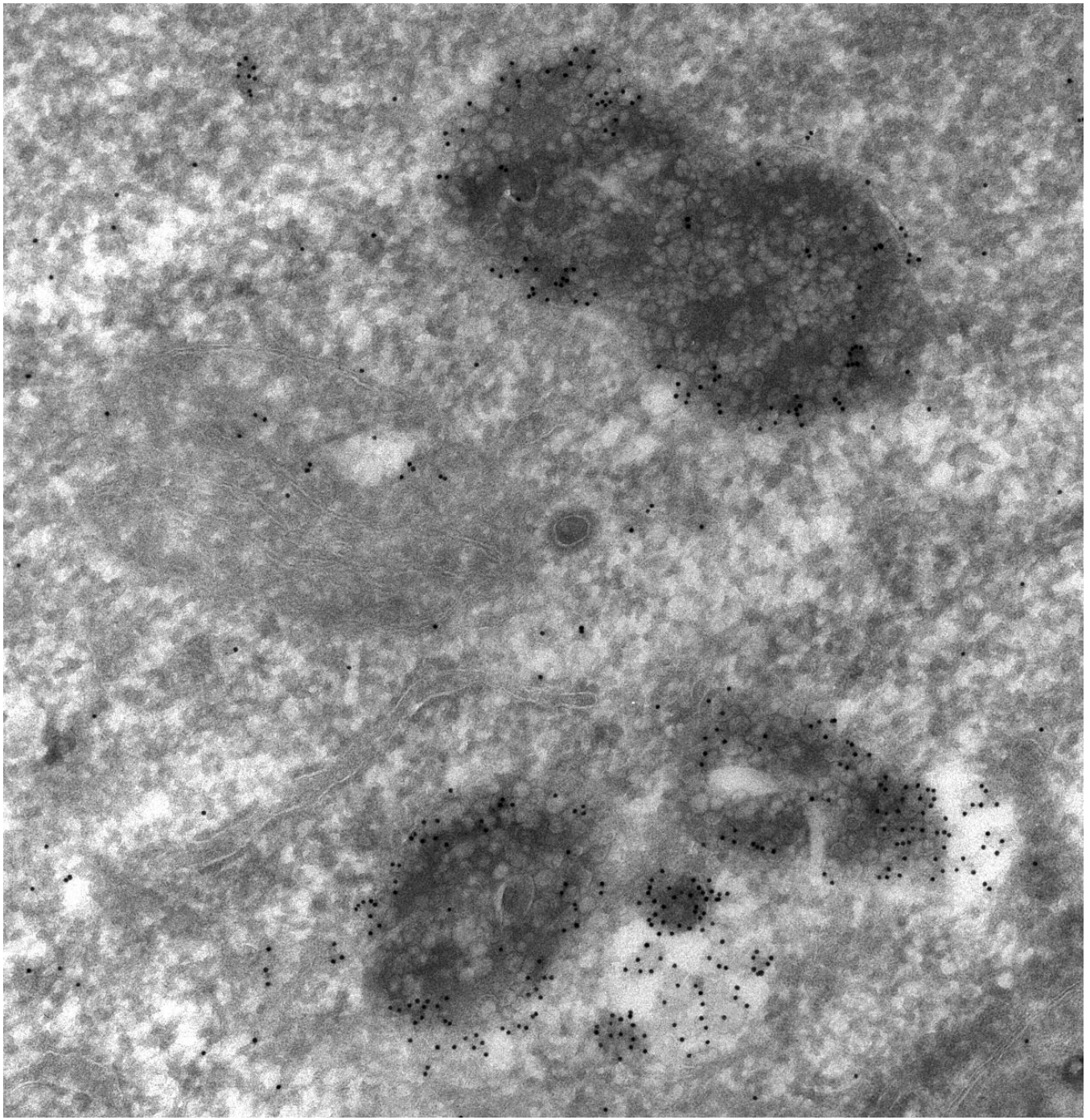


Sphingomyelin-rich domain does not localize in the Golgi apparatus



Sphingomyelin-rich domain localizes in pericentriolar endosomes





List of membrane lipid probes used

Sphingomyelin (SM) - Lysenin, SM-specific toxin
(Kiyokawa E. et al., J Biol Chem. 280, 24072 (2005))

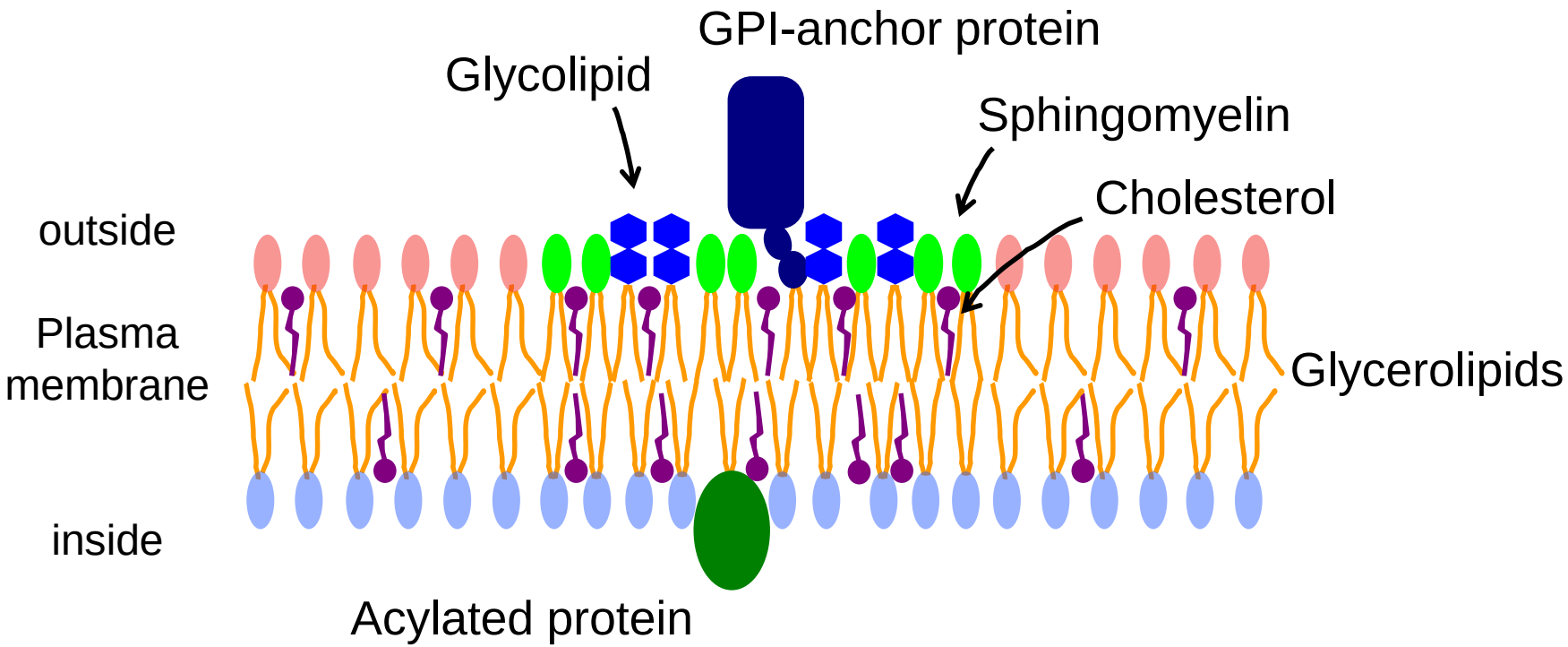
Phosphatidylethanolamine (PE) – Duramycin, PE-specific toxin
(Iwamoto K. et al., Biophys J. 93, 1608 (2007))

Phosphatidylcholine (PC) – anti-PC antibody
(Nam K. S. et al., Biochim Biophys Acta. 1046, 89 (1990))

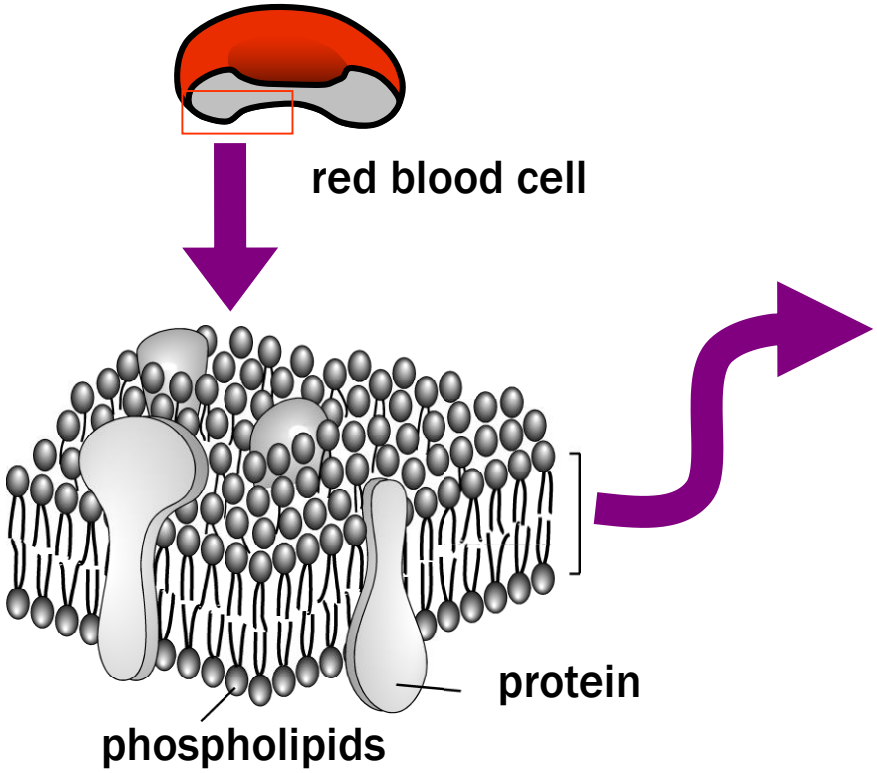
Phosphatidylserine (PS) + Phosphatidylinositol (PI) – anti-PS antibody
(Commercially available; Upstate Biotechnology, Lake Placid, NY.)

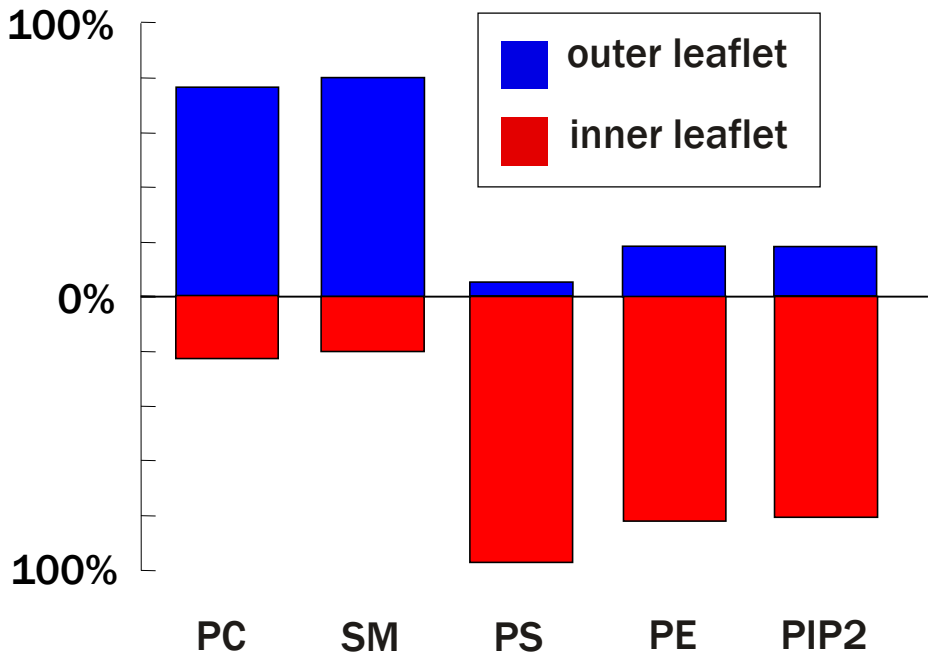
Phosphatidylinositol-4, 5-diphosphate (PIP2)– anti-PIP2 antibody
(Miyazawa A. et al., Mol. Immunol. 25, 1025 (1988))

Imaging Lipid Asymmetry

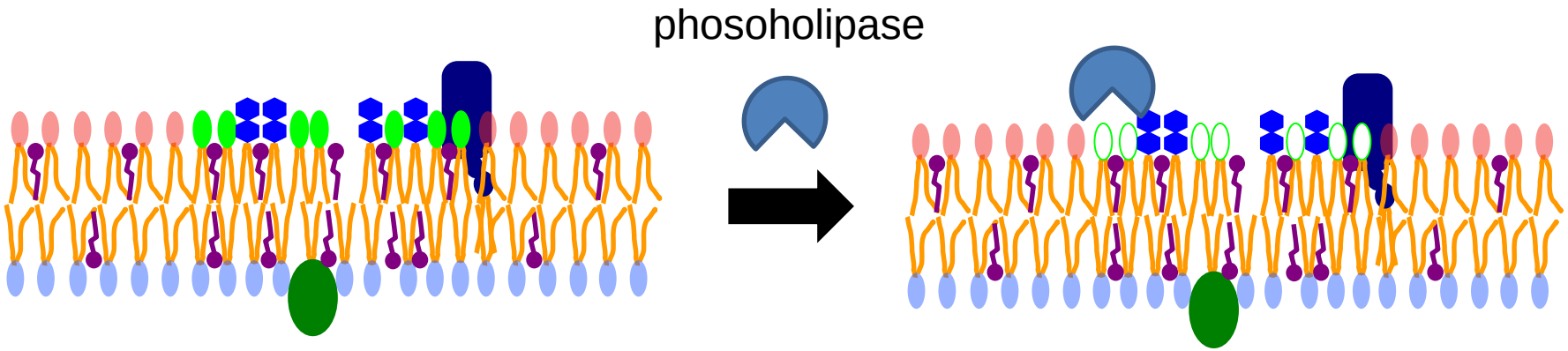


Asymmetric distribution of lipids in plasma membrane





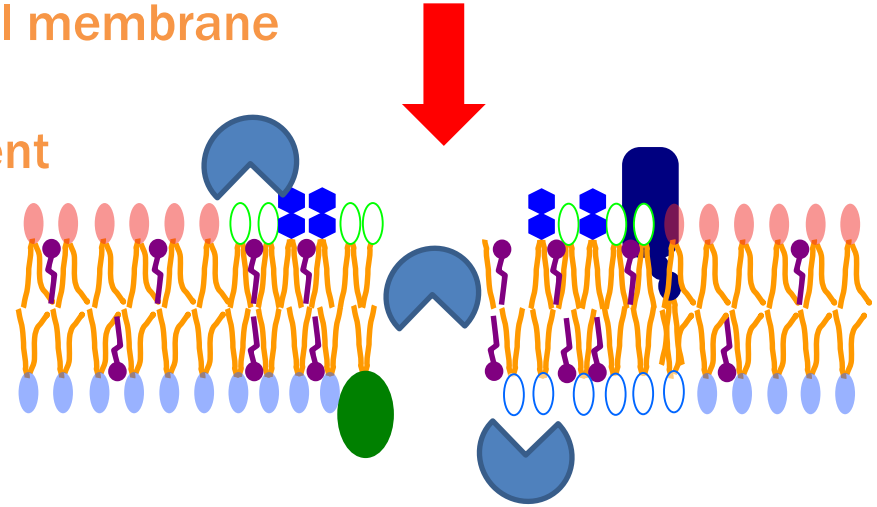
Zachowski A. Biochem. J. 294: 1-14. '93.
Gascard P. et al. Biochim. Biophys. Acta. 1069: 27-36. '91.



Only outer leaflet lipids are hydrolysed when the membrane is intact

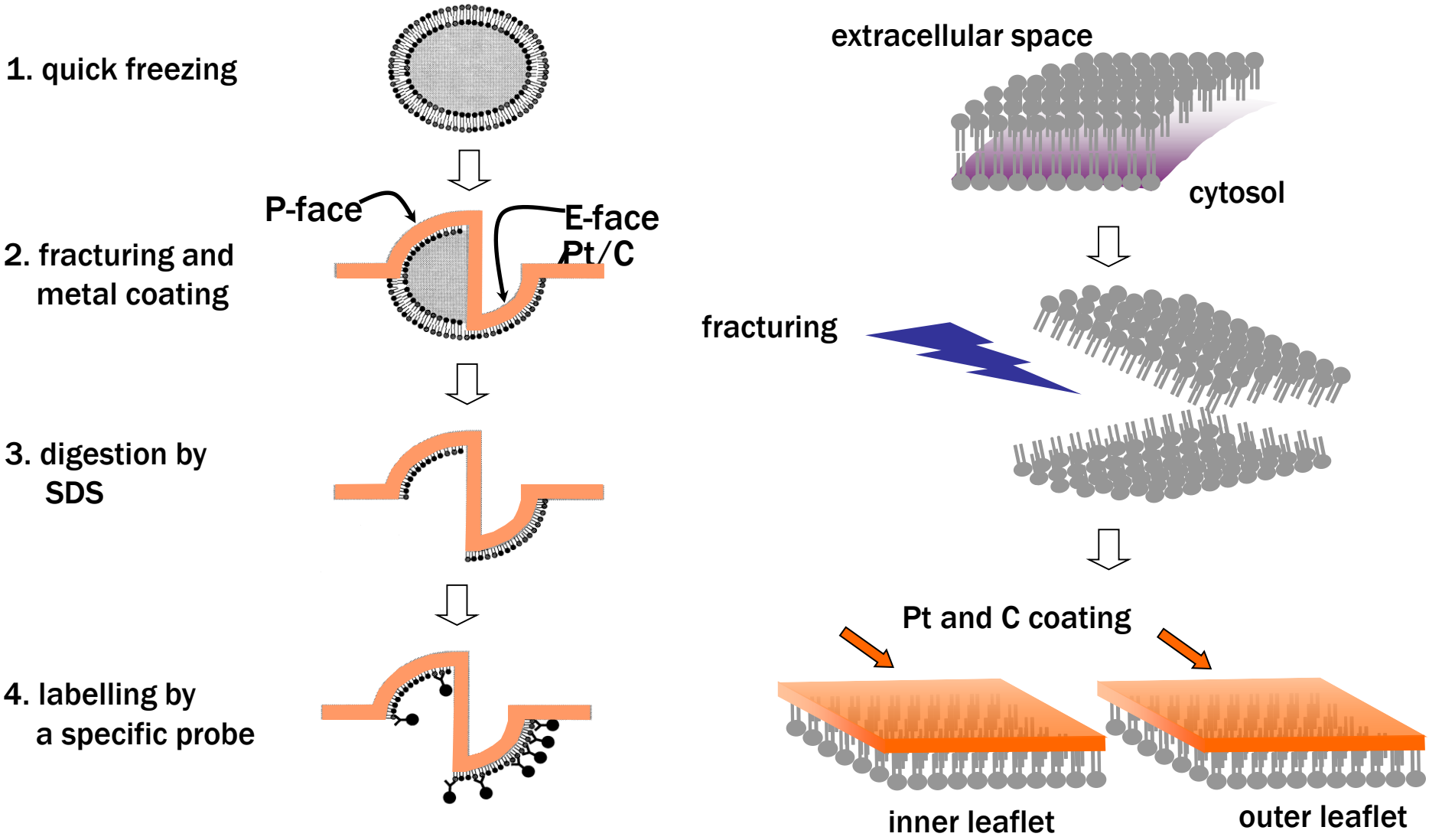
Problems of the biochemical method

1. cannot be used when there is additional membrane Inside the membrane
1. re-organization of lipids during treatment
2. only average value is obtained



Inner leaflet lipids are hydrolysed when the membrane is broken

SDS-digested Freeze-fracture Replica Labeling (SDS-FRL) method



Fujimoto K. J. Cell Sci. 108: 3443-3449. 1995.

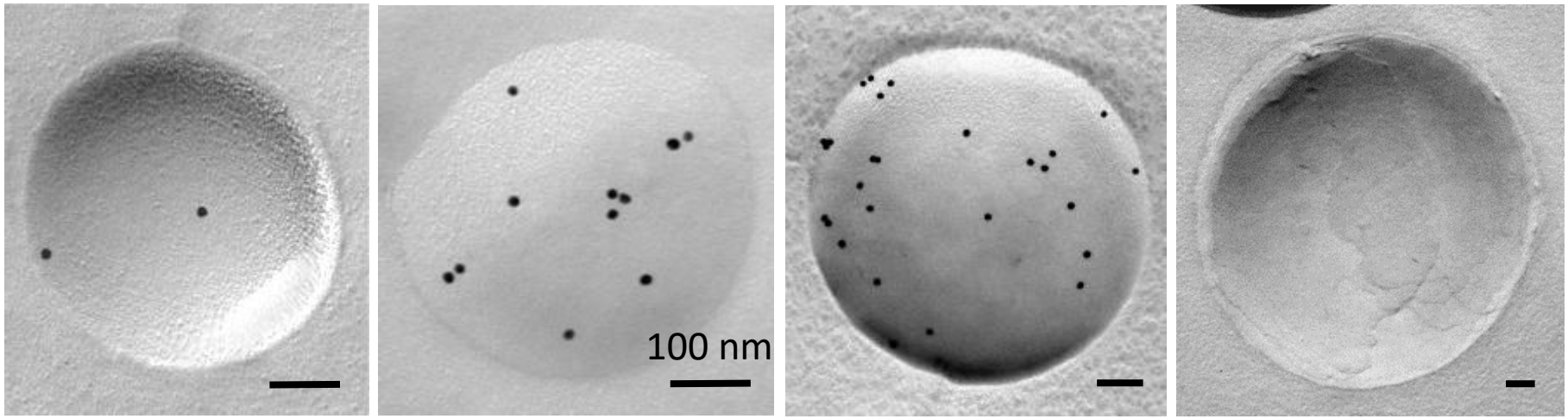
Fujimoto K, Umeda M, and Fujimoto T. J. Cell Sci. 109: 2453-2460. 1996.

Characteristics of SDS-FRL method in lipid biology

1. Minimal re-organization of lipids during sample preparation.

2. Information from individual cell is obtained.

3. Lateral distribution is demonstrated, in addition to transmembrane localization.

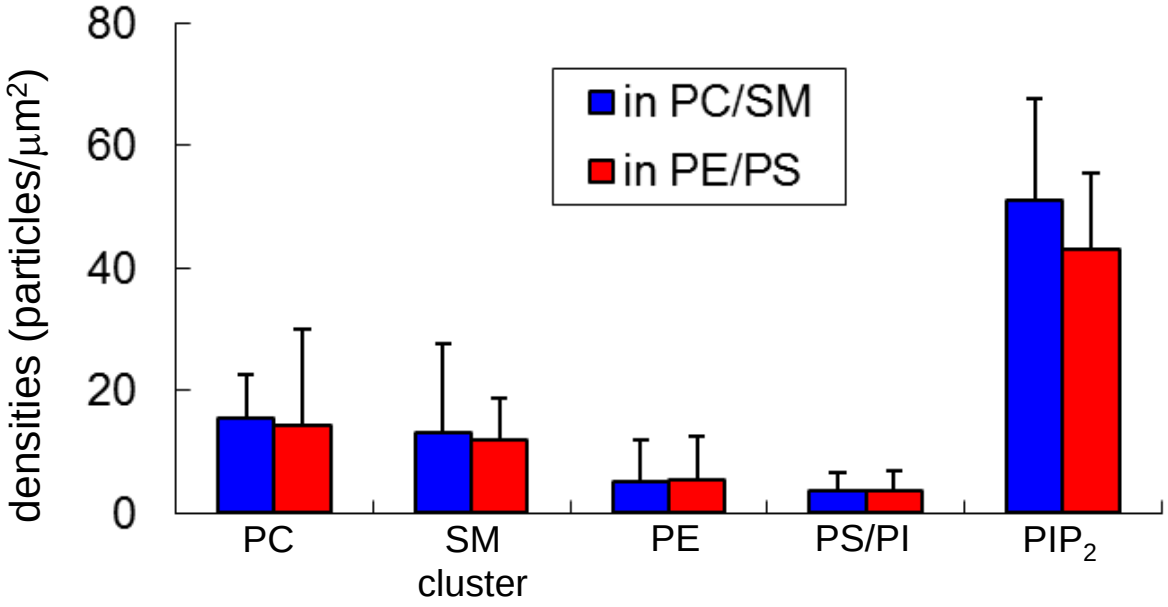


PIP_2 /PC (0.1:99.9)

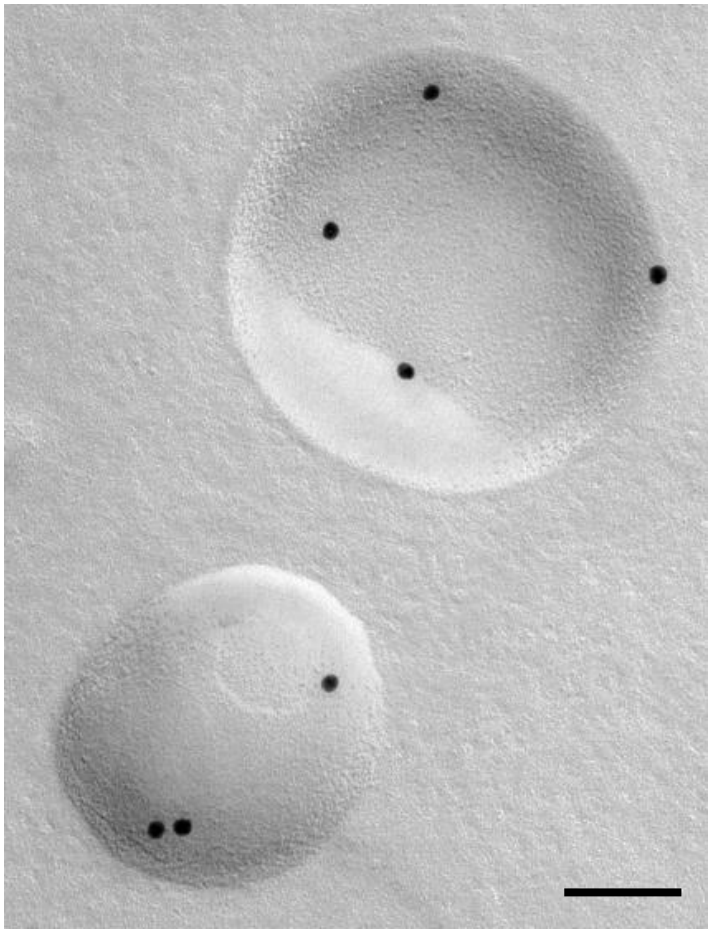
PIP_2 /PC (1:99)

PIP_2 /PE/PS (1:49.5/49.5)

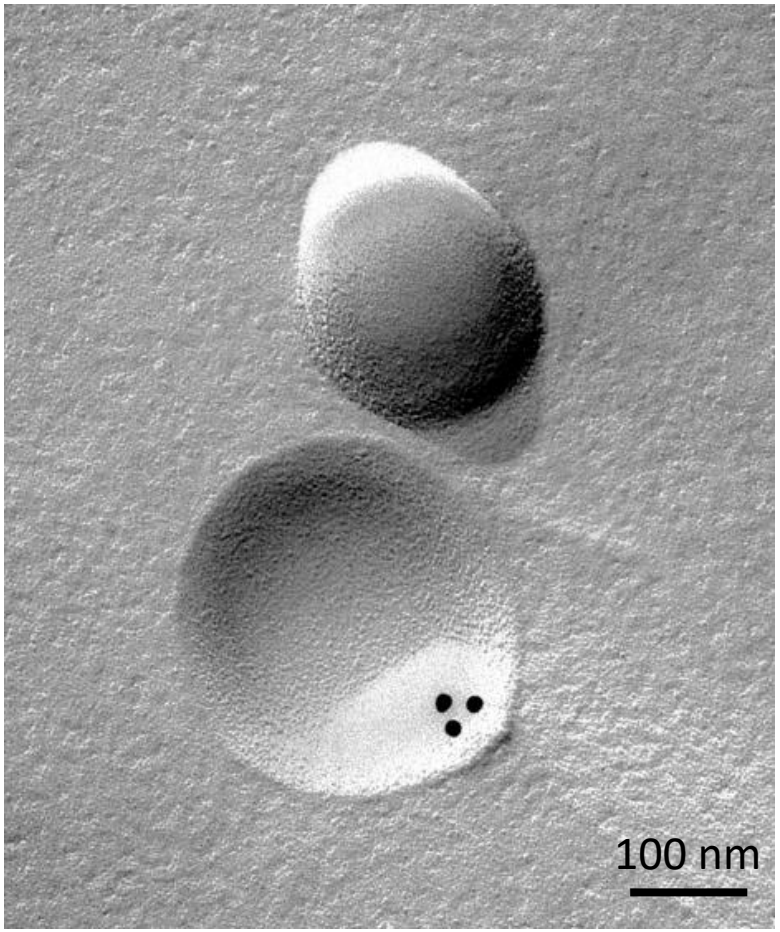
PS /PC (1:99)



Lipids are not scrambled during sample preparation



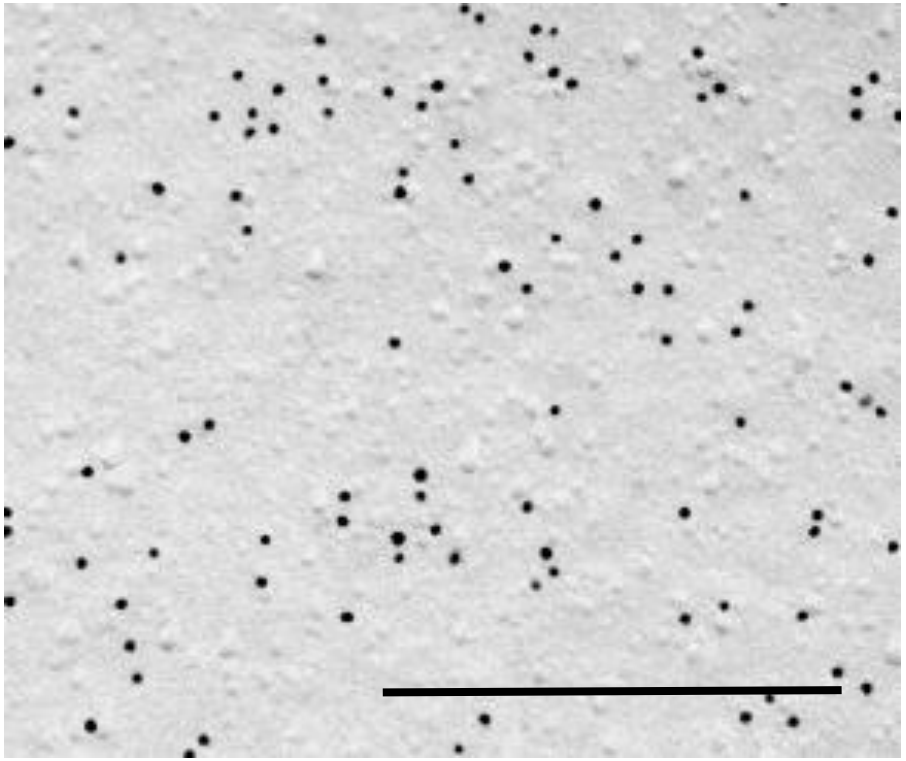
Lyso PE in outer and inner leaflet



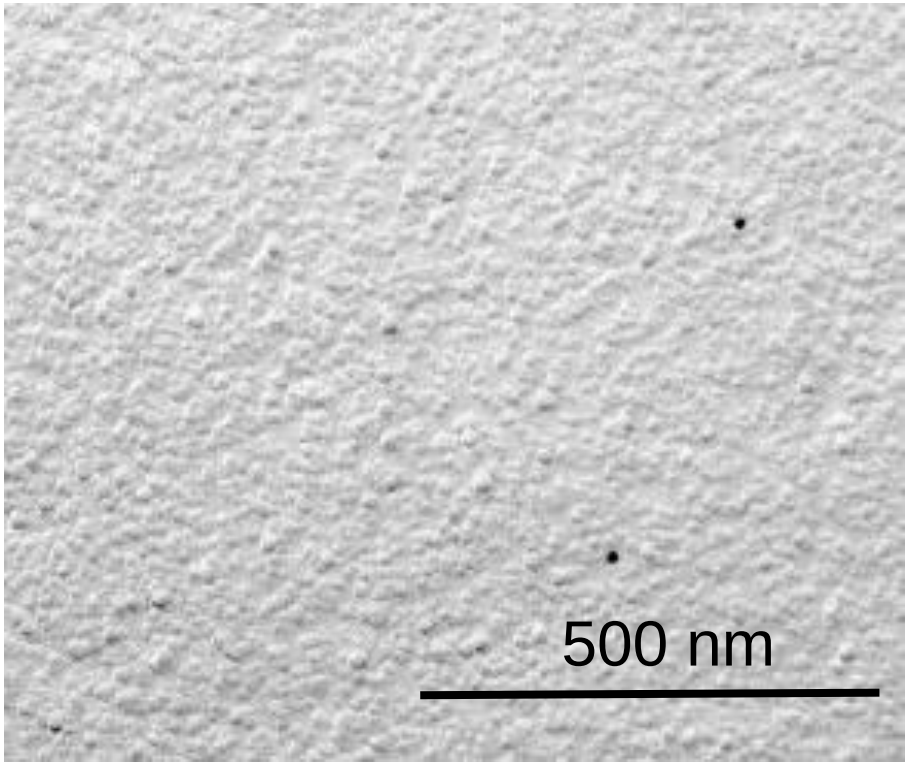
Lyso PE only in outer leaflet

Sphingomyelin (SM) labeling in human erythrocyte membrane

E-face（outer leaflet）

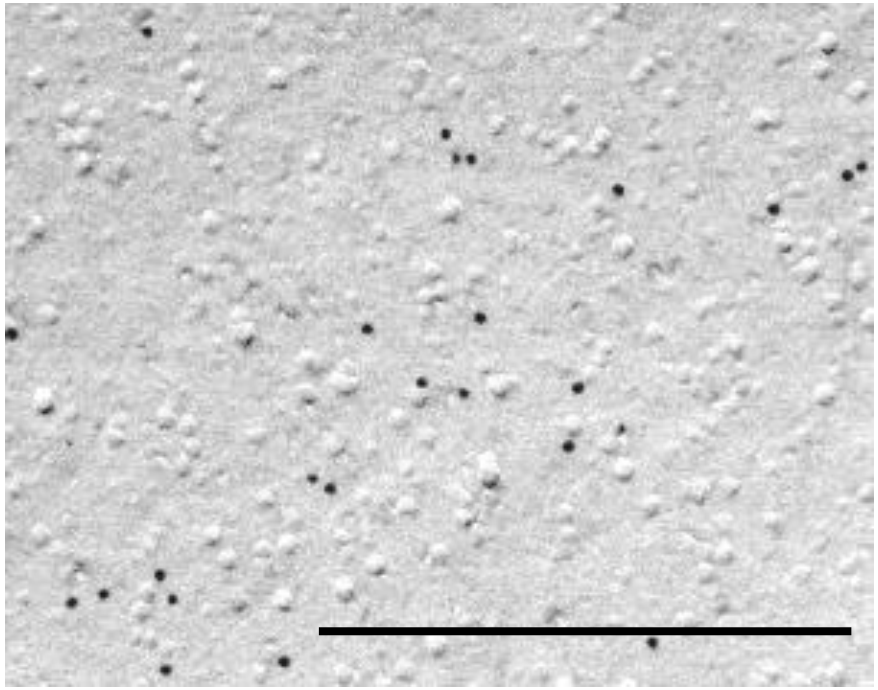


P-face（inner leaflet）

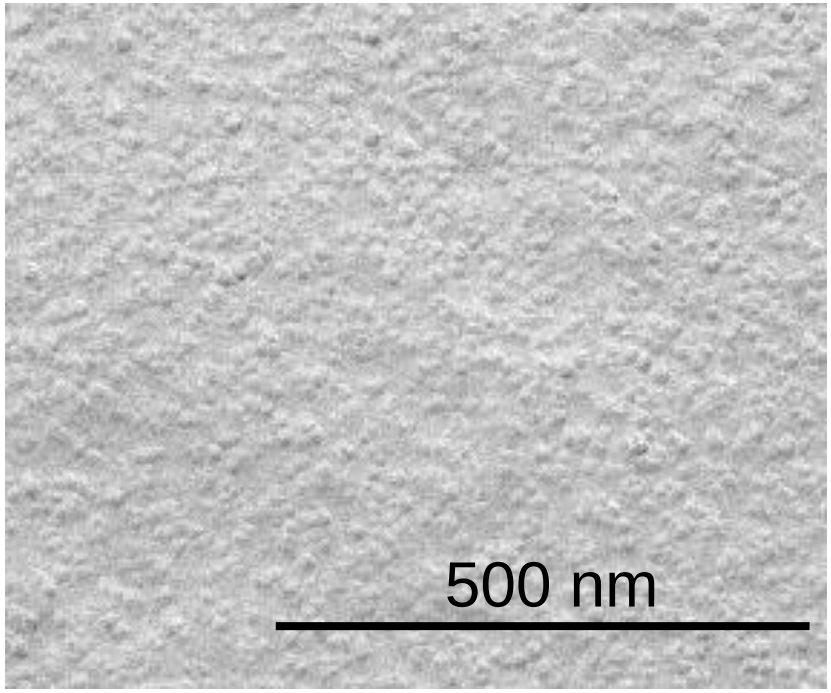


Phosphatidylcholine (PC) labeling in human erythrocyte membrane

E-face（outer leaflet）

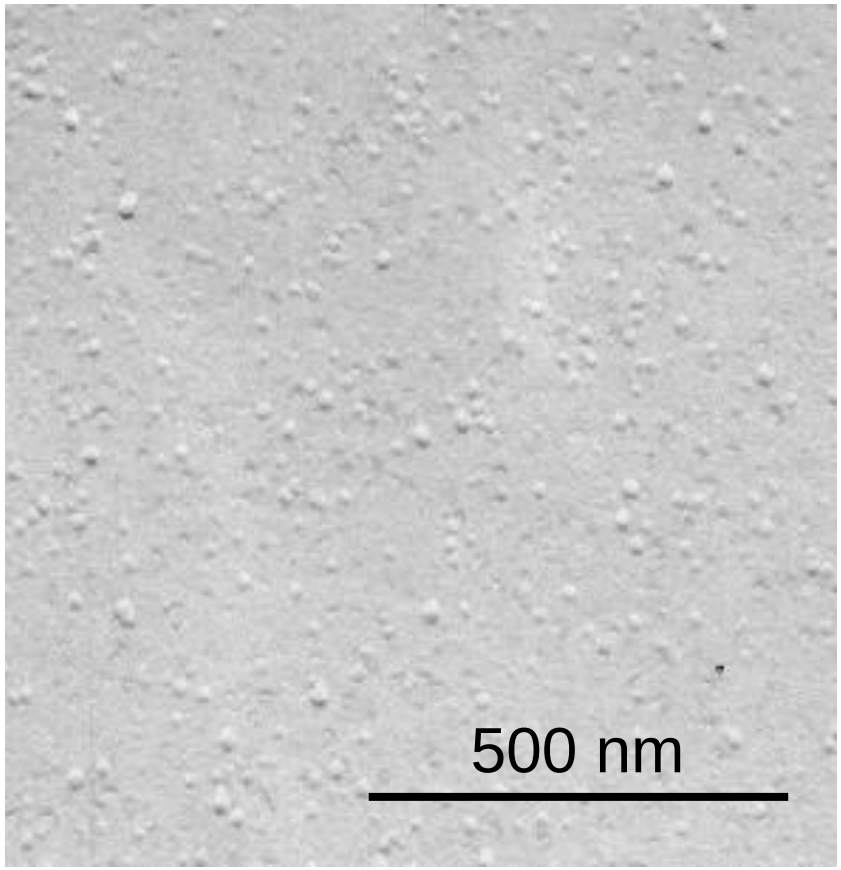


P-face（inner leaflet）

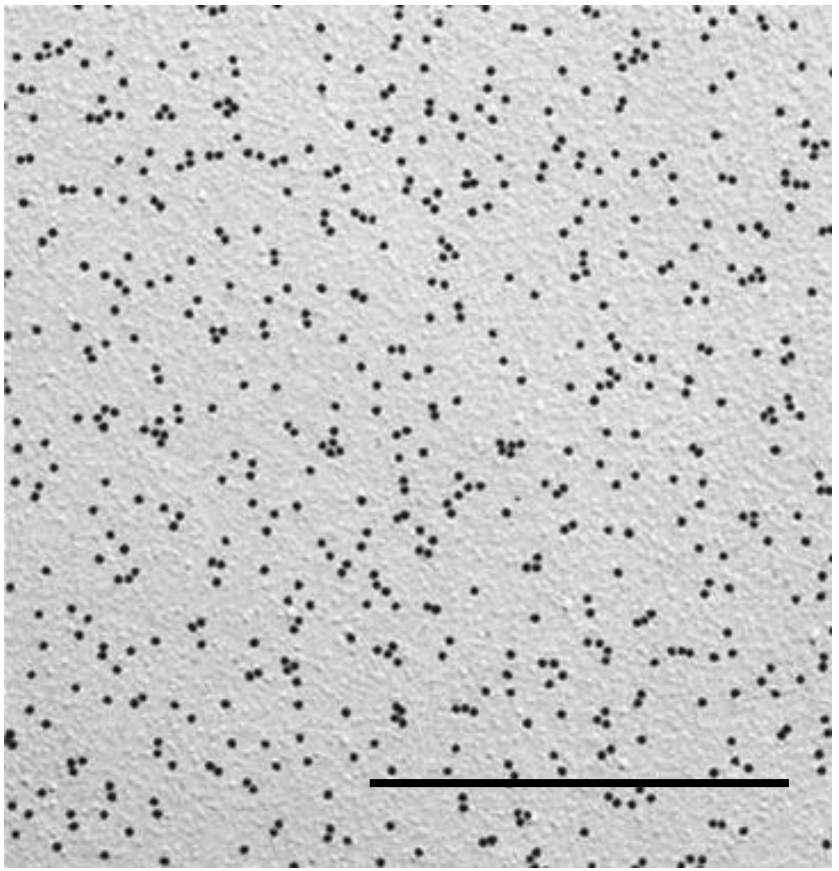


Phosphatidylethanolamine (PE) labeling in human erythrocyte membrane

E-face（outer leaflet）

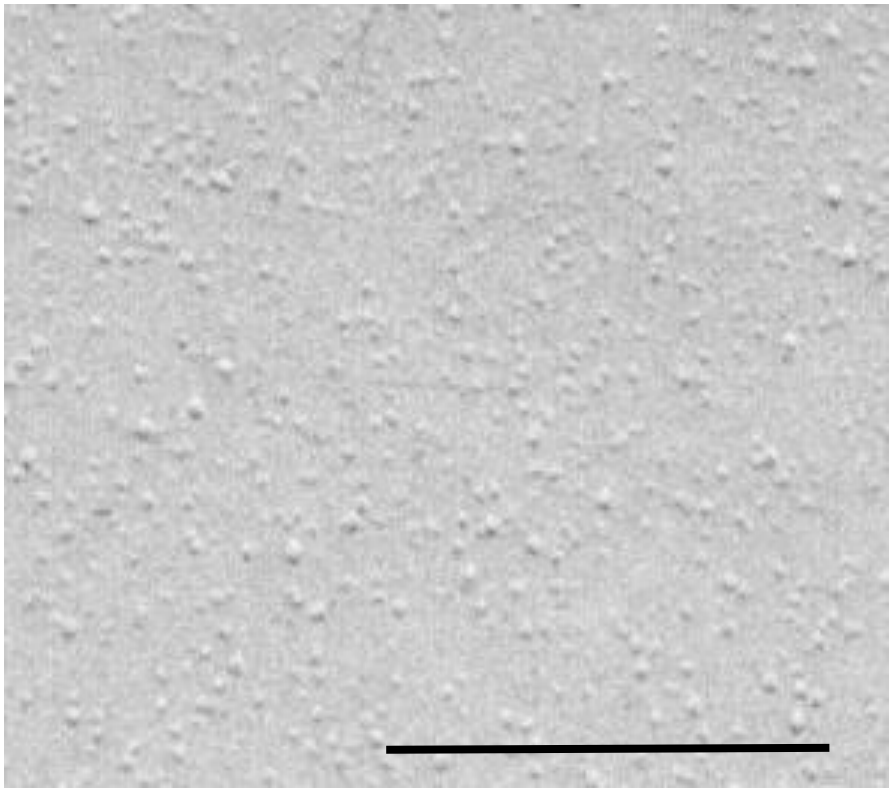


P-face（inner leaflet）

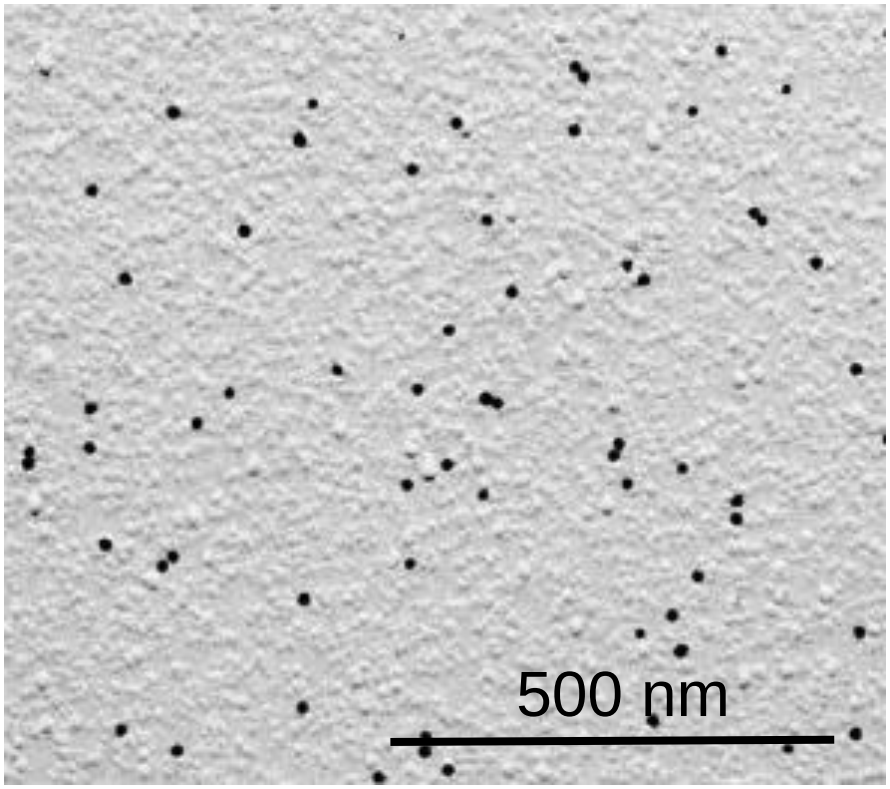


Phosphatidylserine (PS)/ phosphatidylinositol (PI) labeling in human erythrocyte membrane

E-face（outer leaflet）



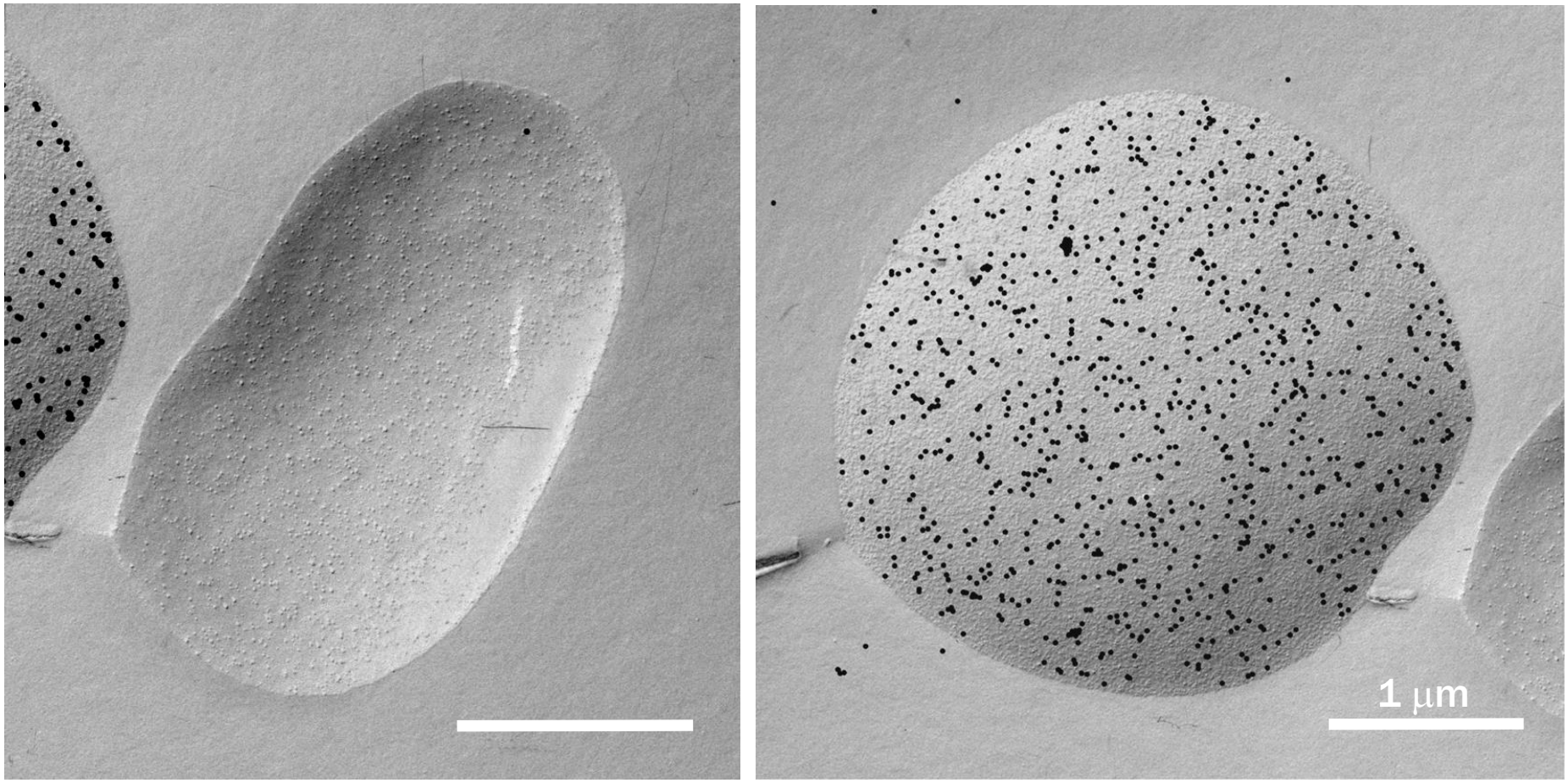
P-face（inner leaflet）



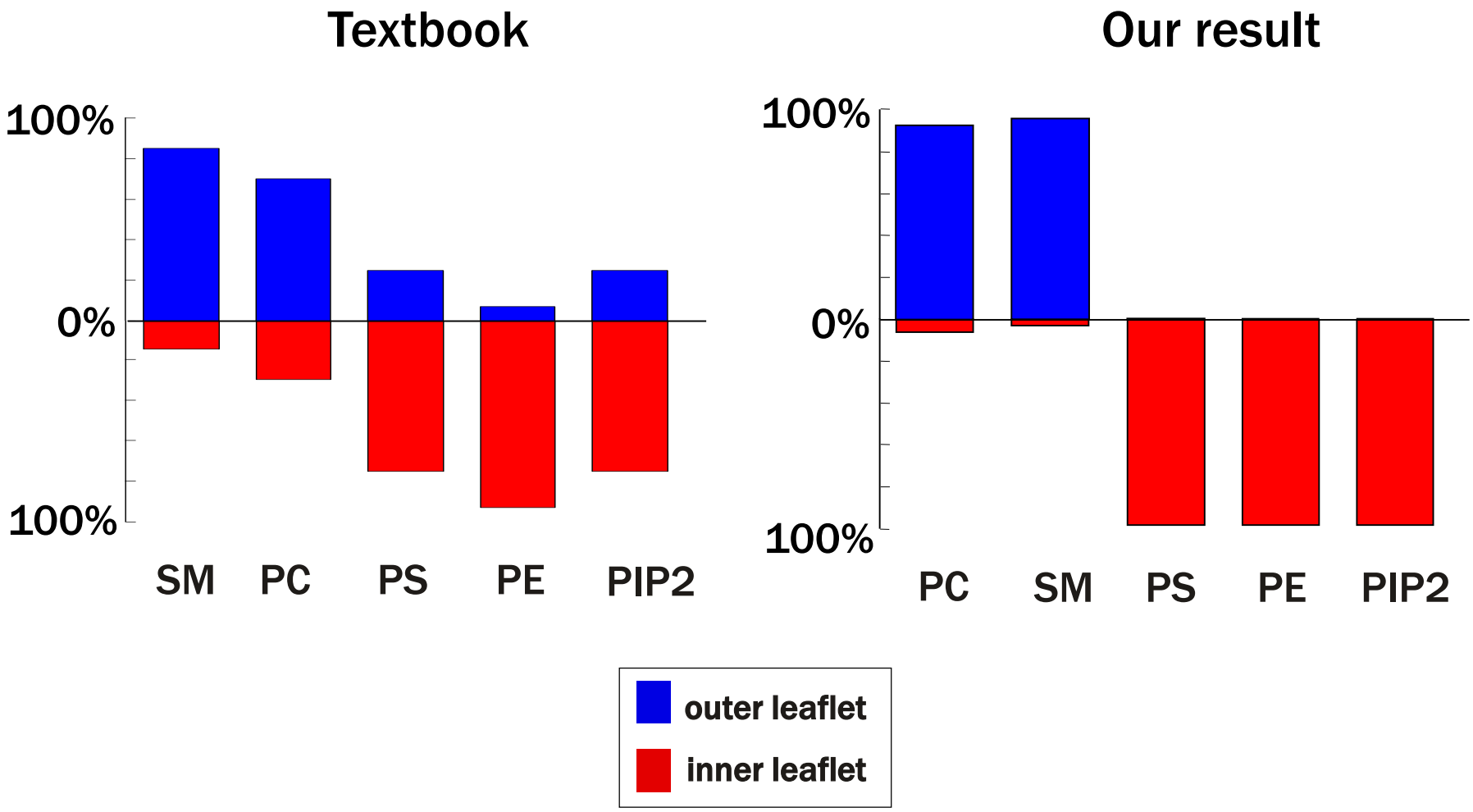
Phosphatidylinositol- 4,5-bisphosphate (PIP_2) labelling in human erythrocyte membrane

E-face（outer leaflet）

P-face（inner leaflet）

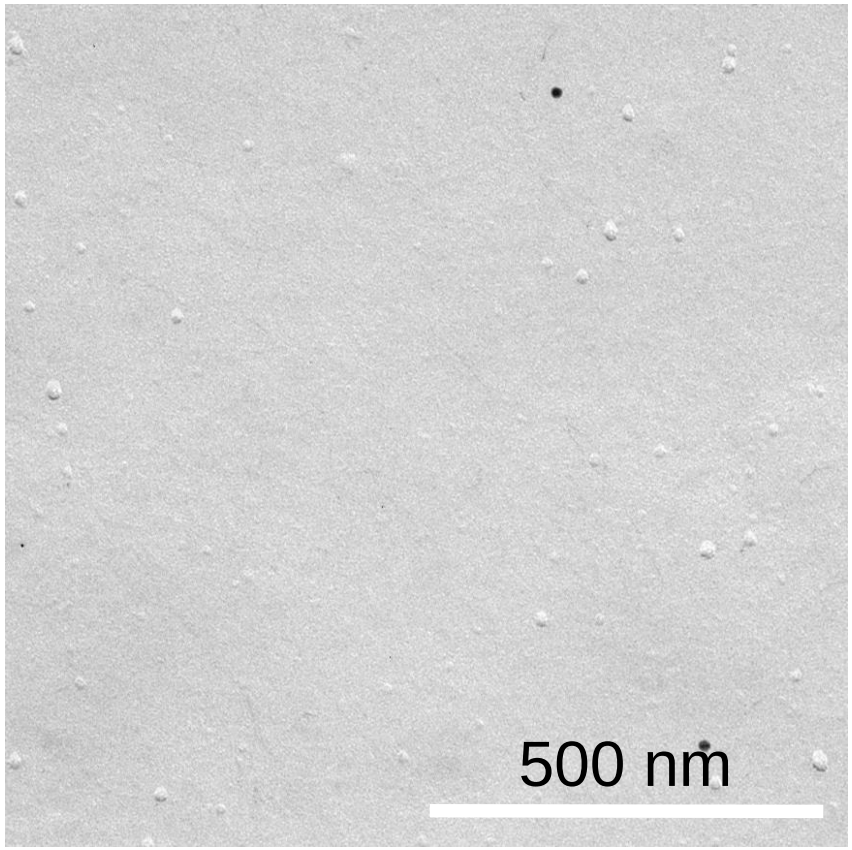


Revisiting asymmetrical distribution of phospholipids in the human erythrocyte membrane

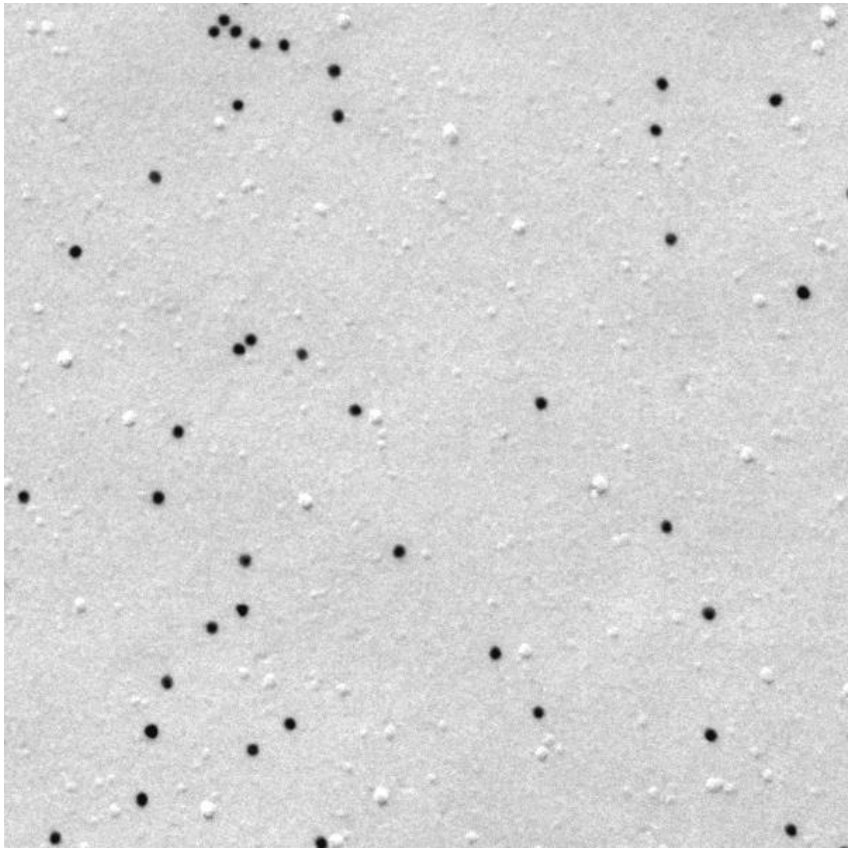


Distribution of phosphatidylethanolamine in the plasma membrane of fibroblast

E-face（outer leaflet）

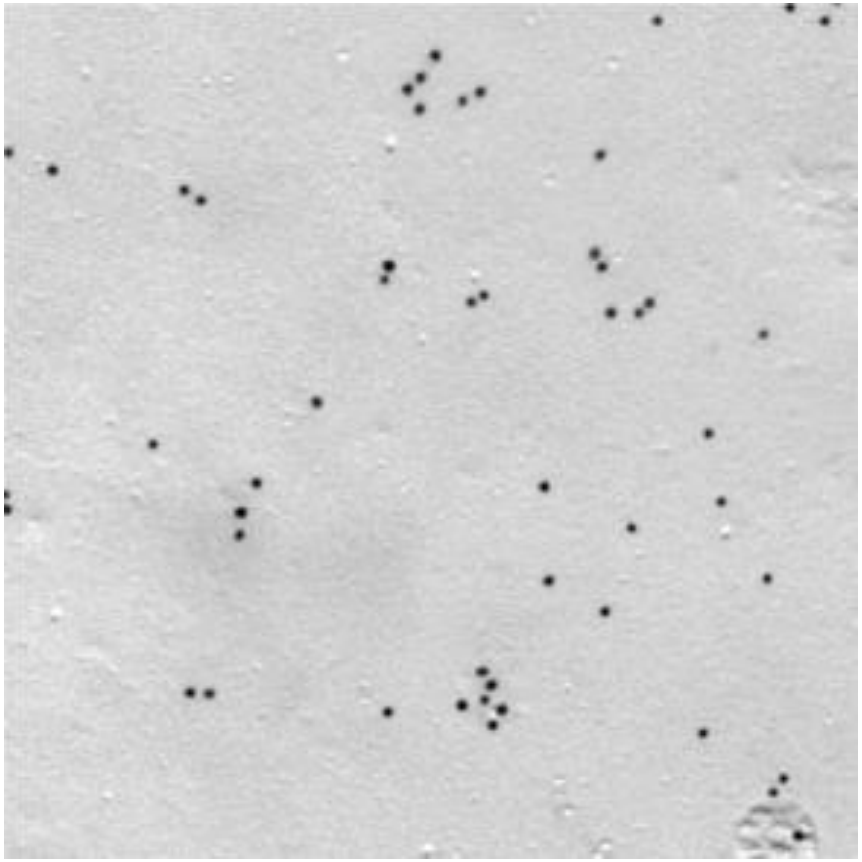


P-face（inner leaflet）

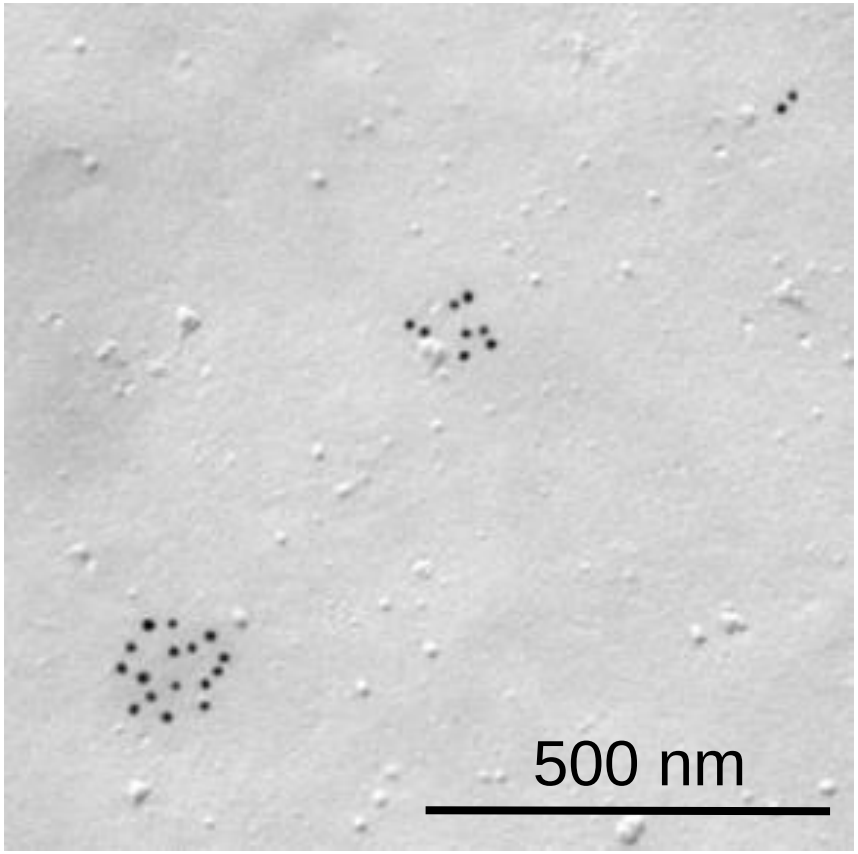


Distribution of sphingomyelin in the plasma membrane of fibroblast

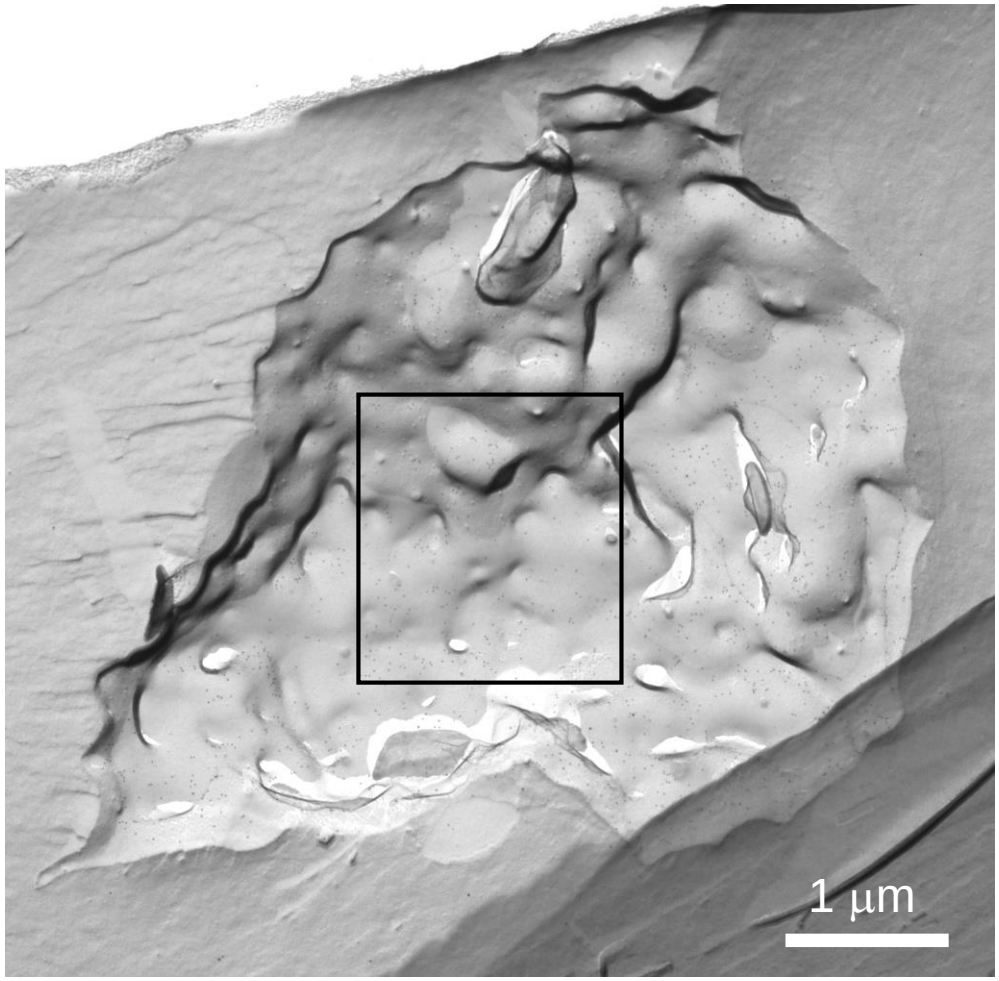
E-face (outer leaflet)

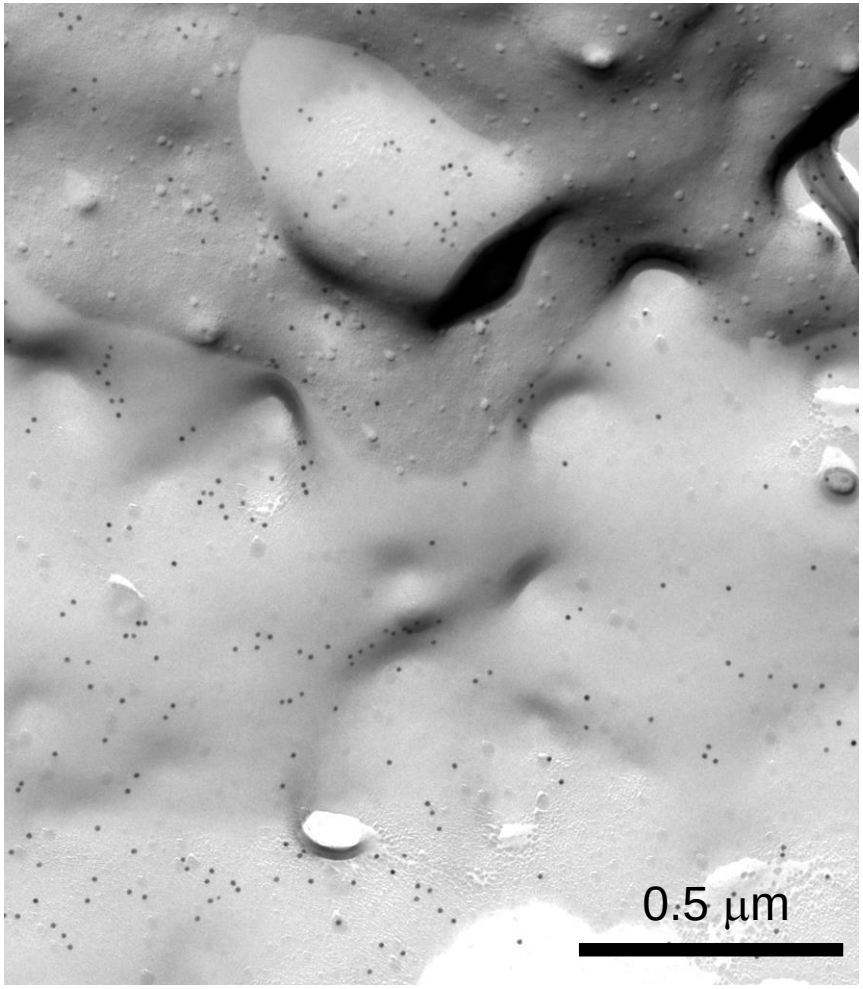


P-face (inner leaflet)

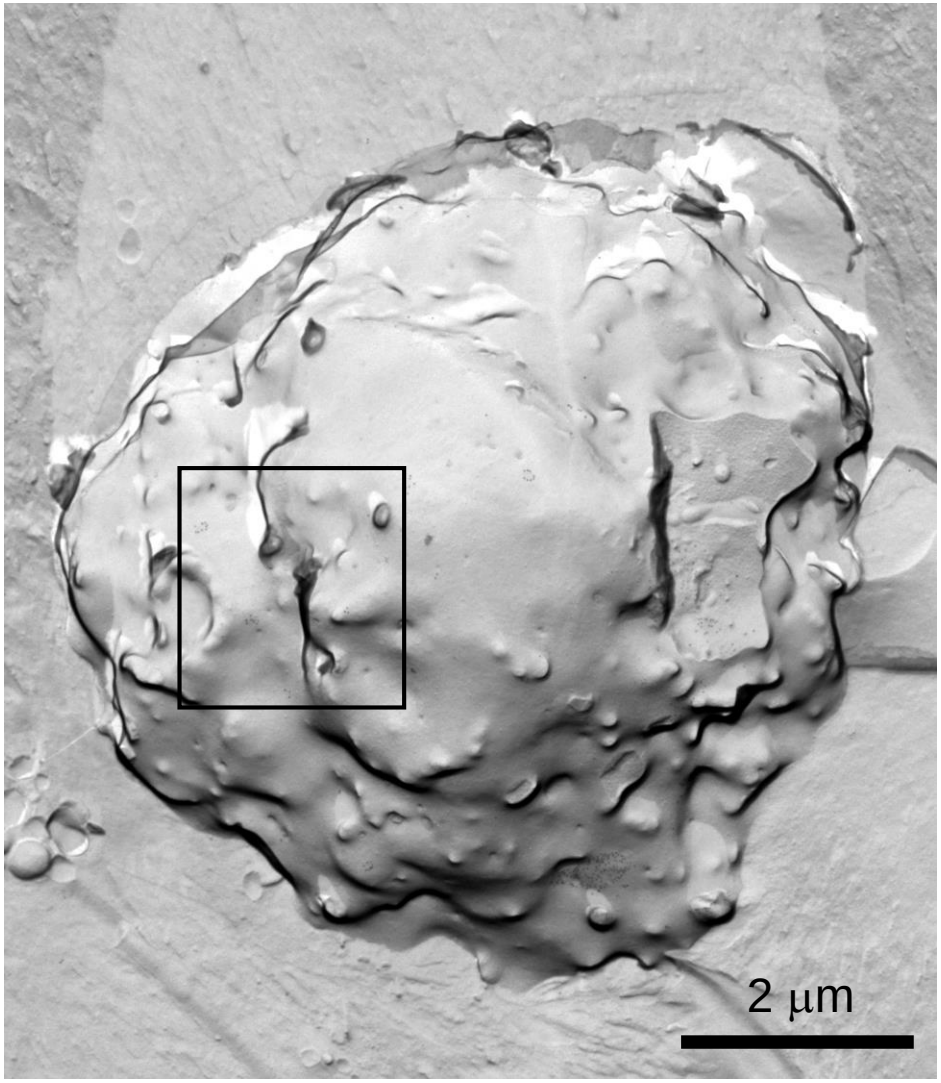


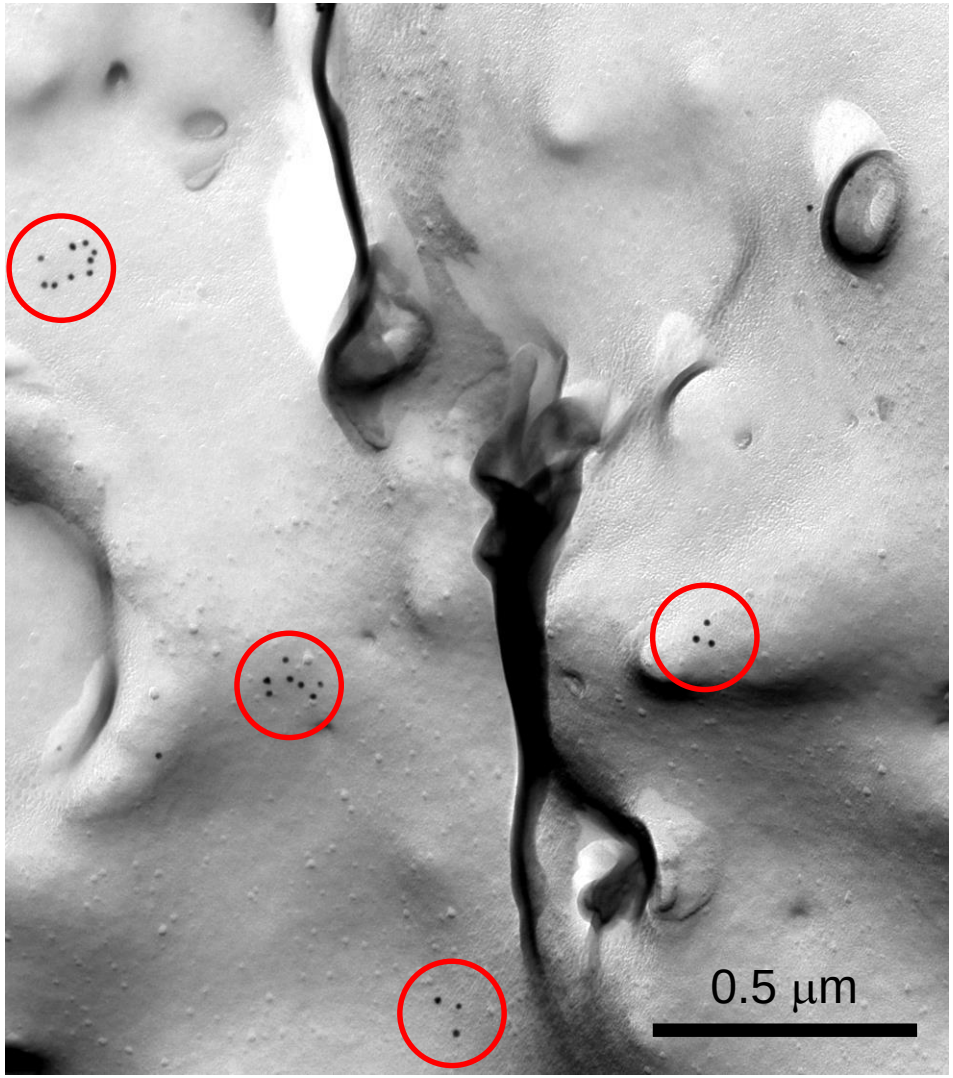
Distribution of sphingomyelin in the plasma membrane of neutrophils (E-face (outer leaflet))



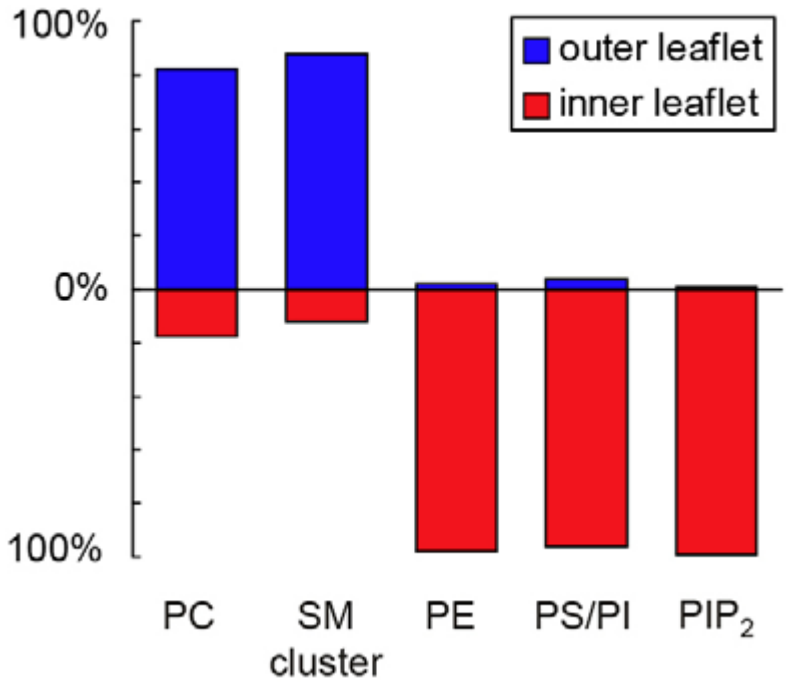


Distribution of sphingomyelin in the plasma membrane of neutrophils (P-face (inner leaflet))





Distribution of phospholipids in the plasma membrane from human skin fibroblasts



Summary 1

-In red blood cells, most lipids are distributed exclusively in outer or inner leaflet.

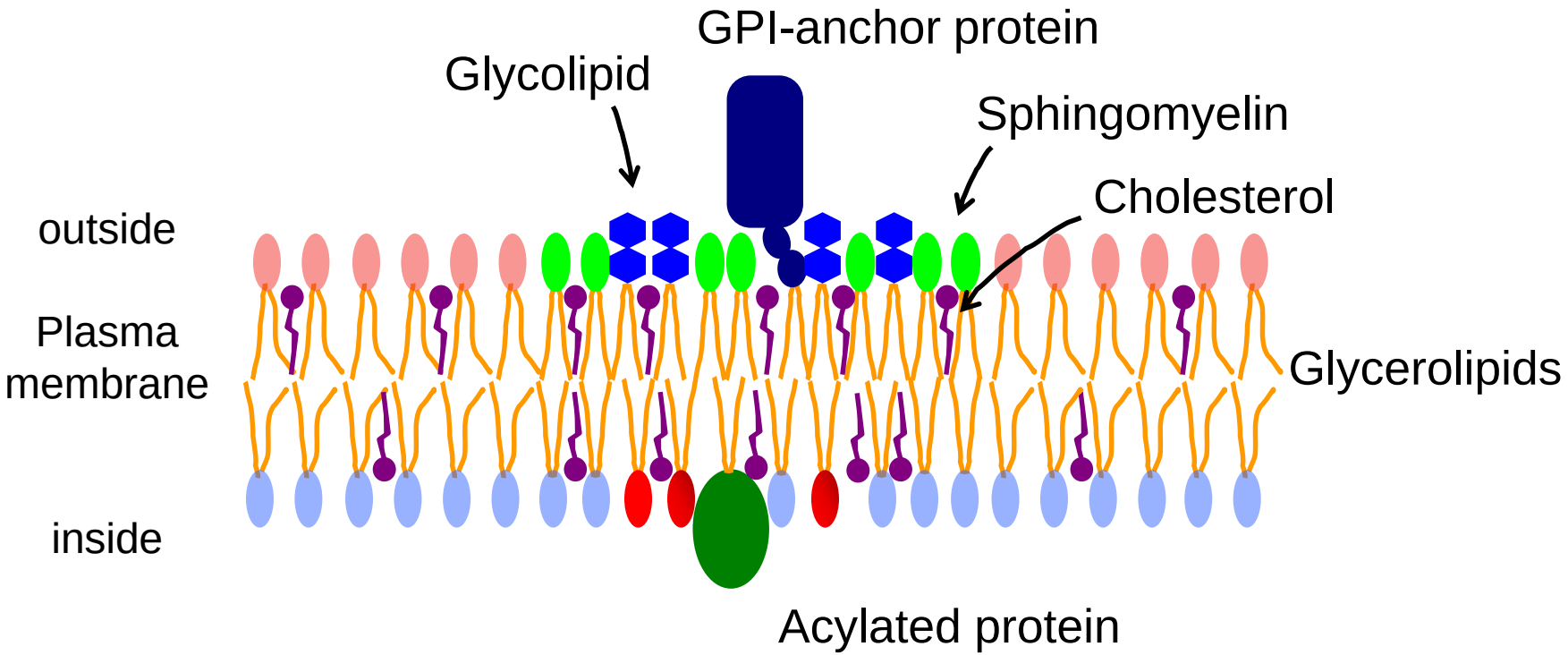
-In nucleated cells, sphingomyelin is distributed both in outer and inner leaflet.

-Sphingomyelin forms clusters in the inner leaflet of the plasma membrane of nucleated cells.

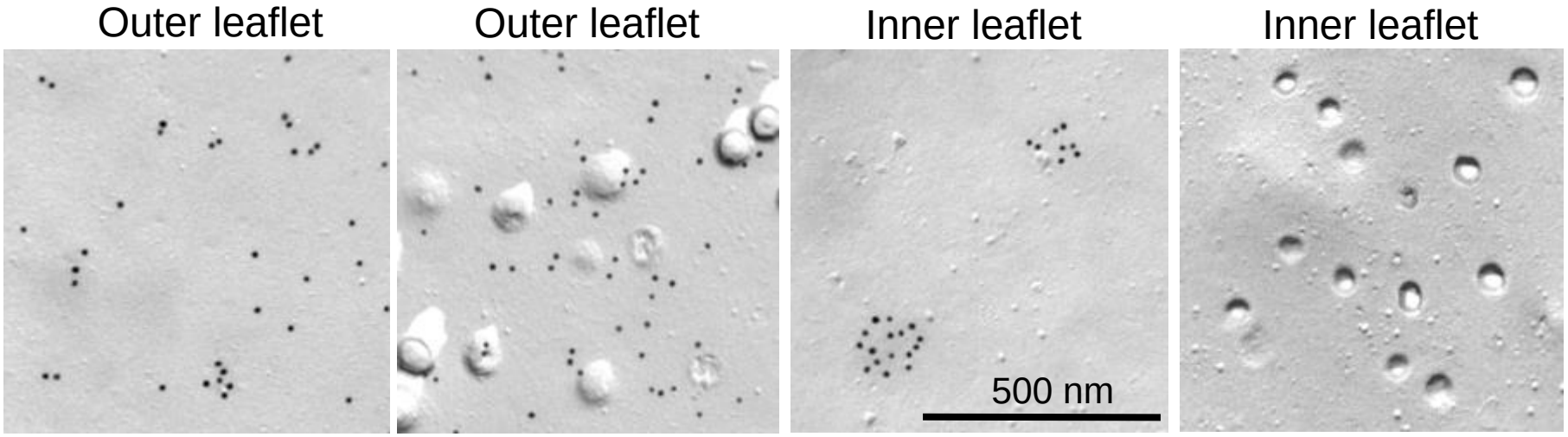
-Platelet microparticles are released from the membrane domain where lipid asymmetry is abolished.

Murate 2015

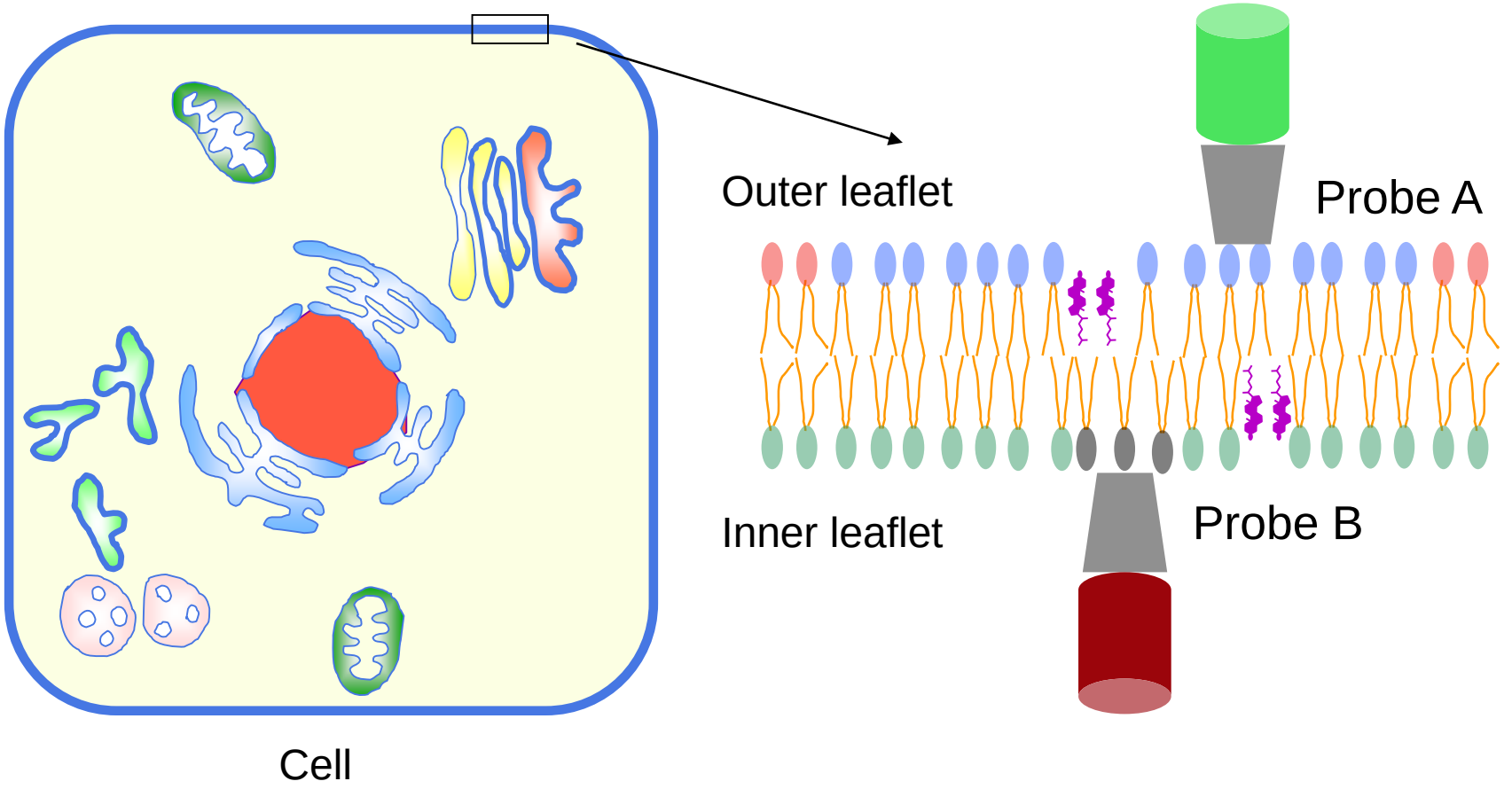
Communication between outer and inner leaflet



Outer leaflet sphingomyelin and inner leaflet sphingomyelin do not co-localize

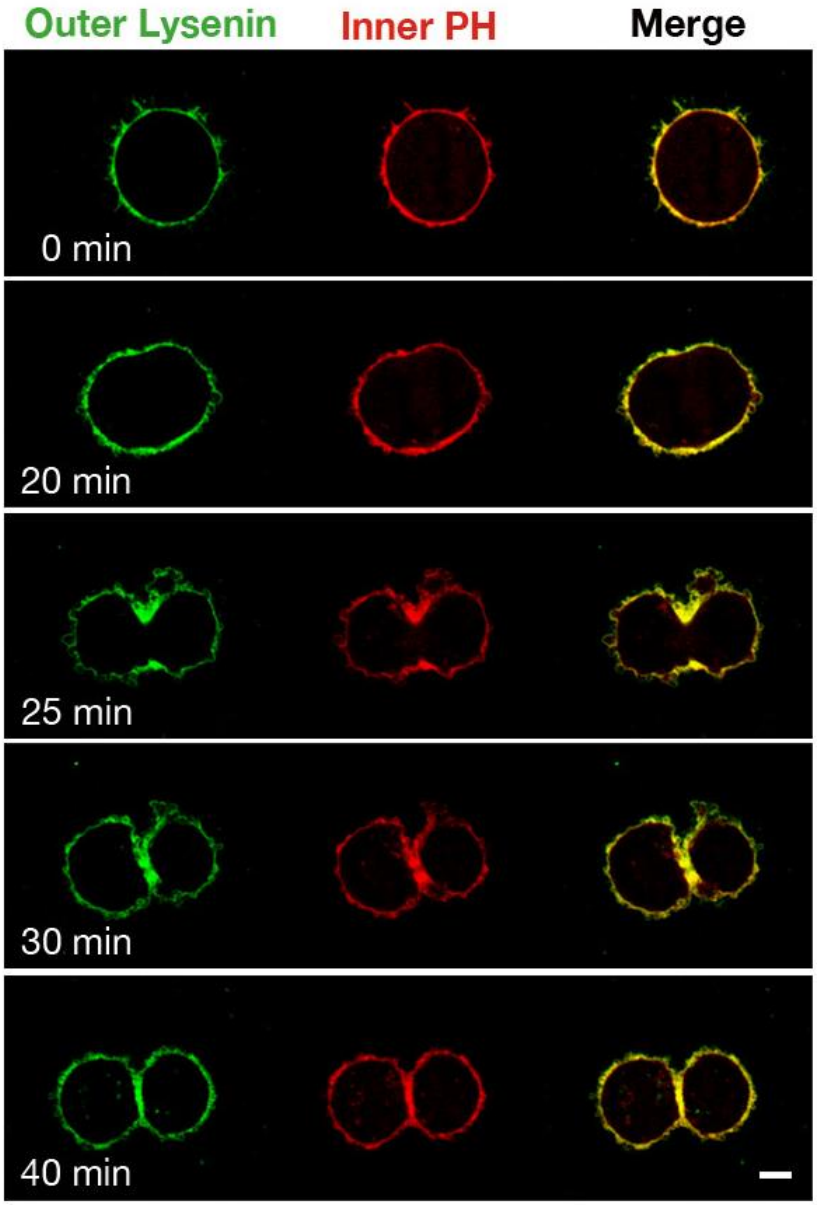


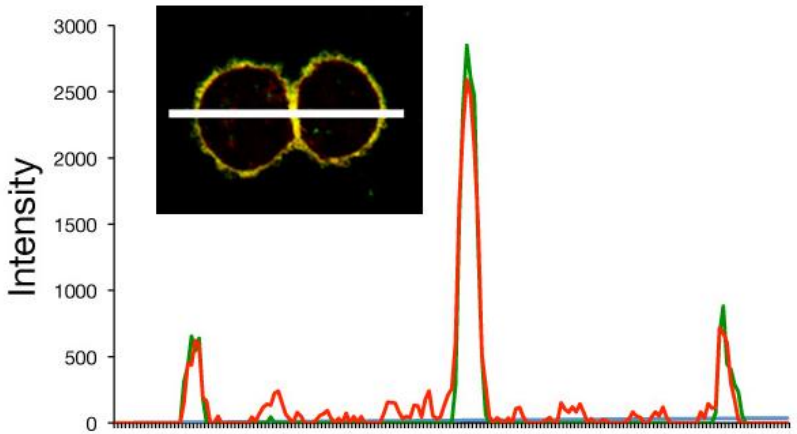
Simultaneous observation of inner leaflet and outer leaflet lipids



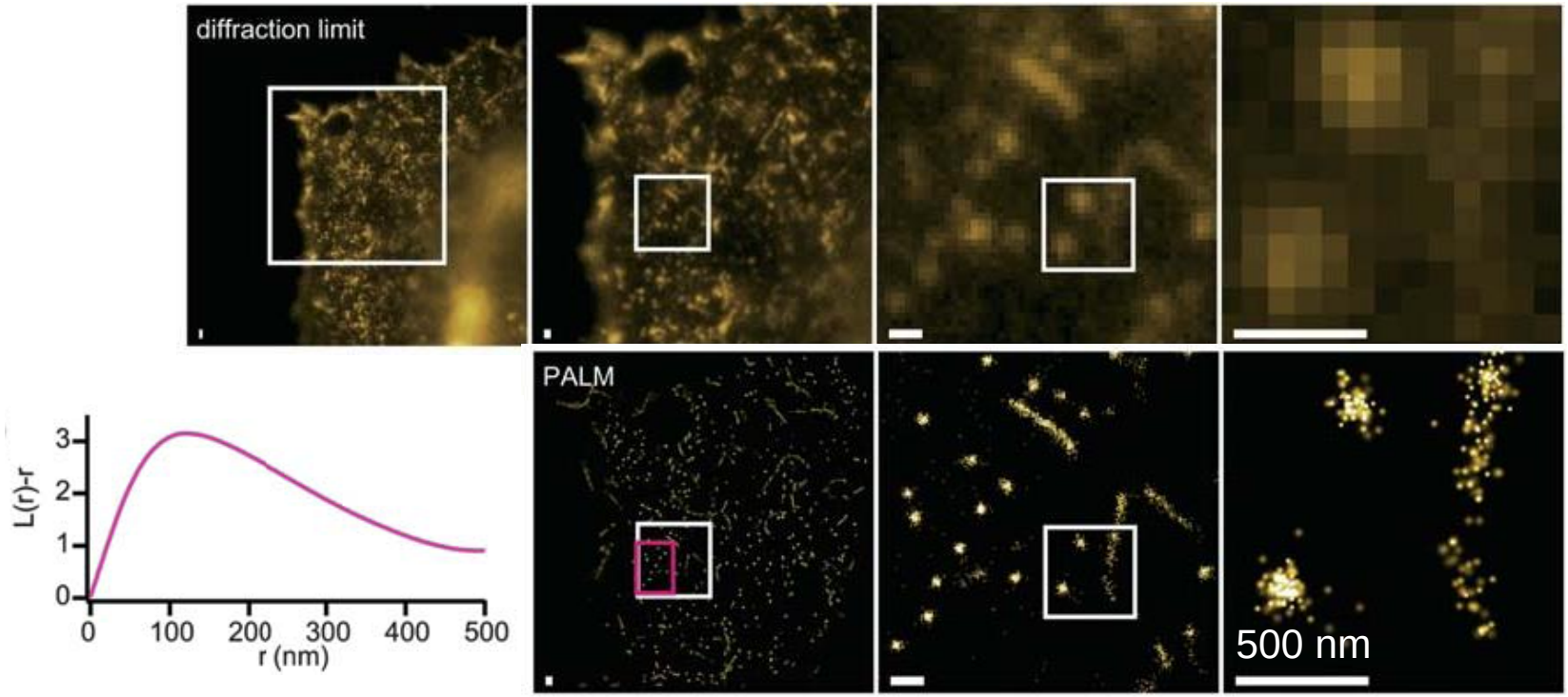
Probe A expressed and purified from *E.coli*
Probe B expressed in cells

Colocalization of sphingomyelin and PIP_2



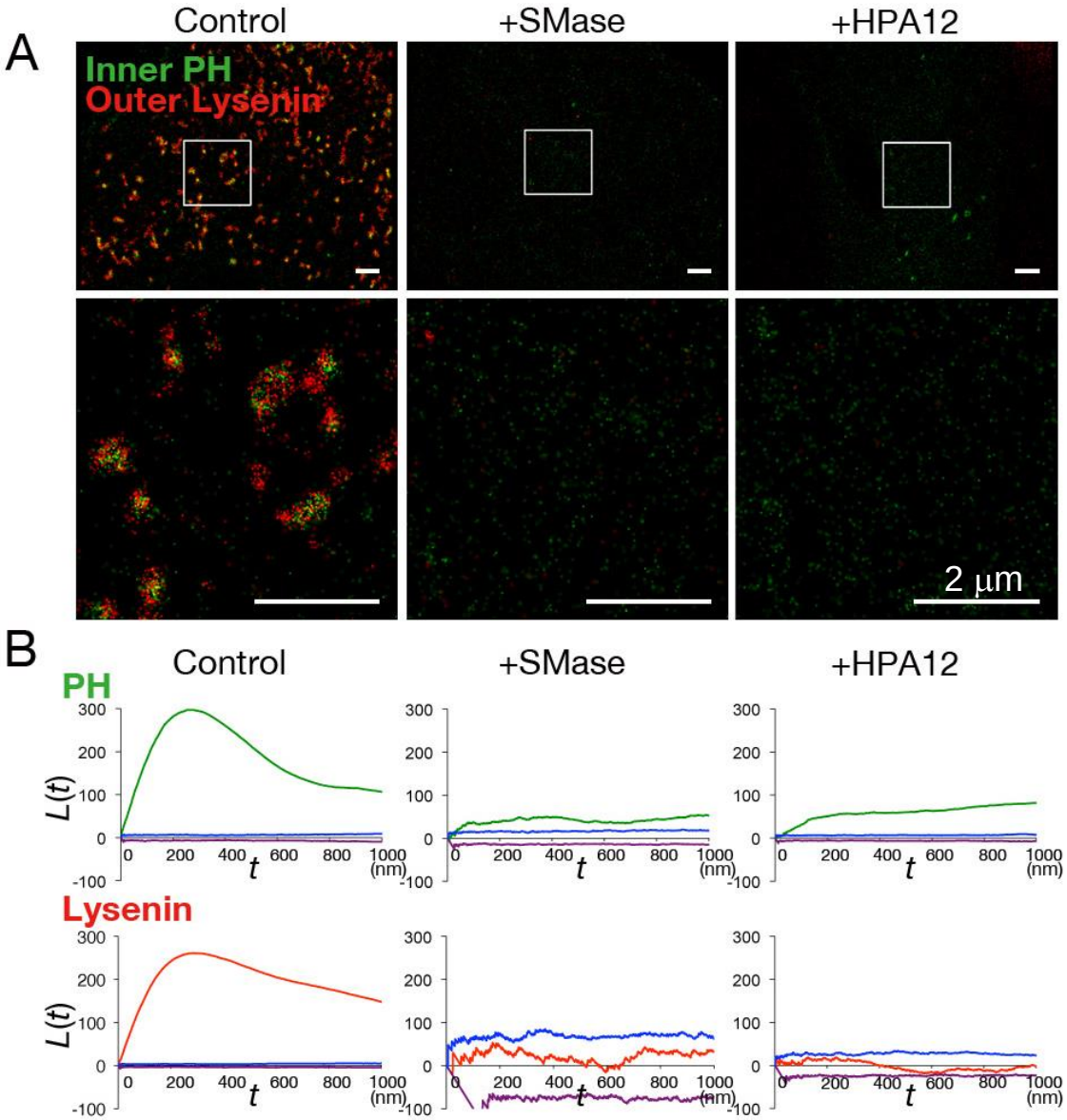


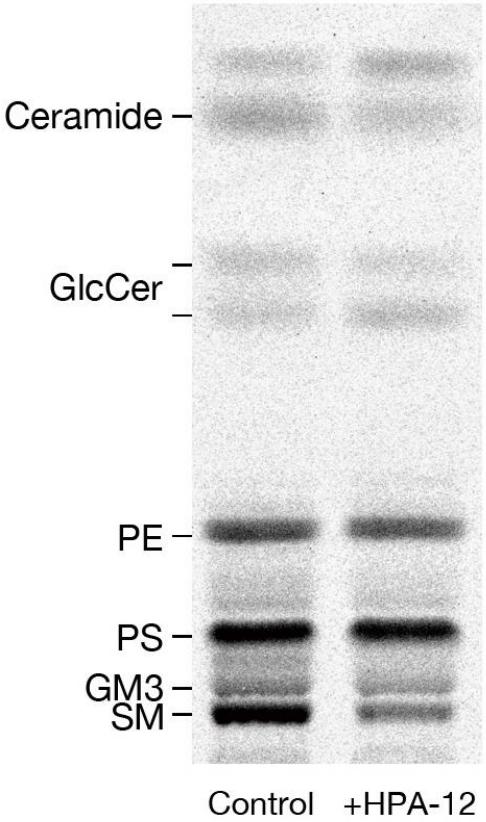
Photoactivation localization microscopy (PALM) image of cholesterol-rich membrane domains



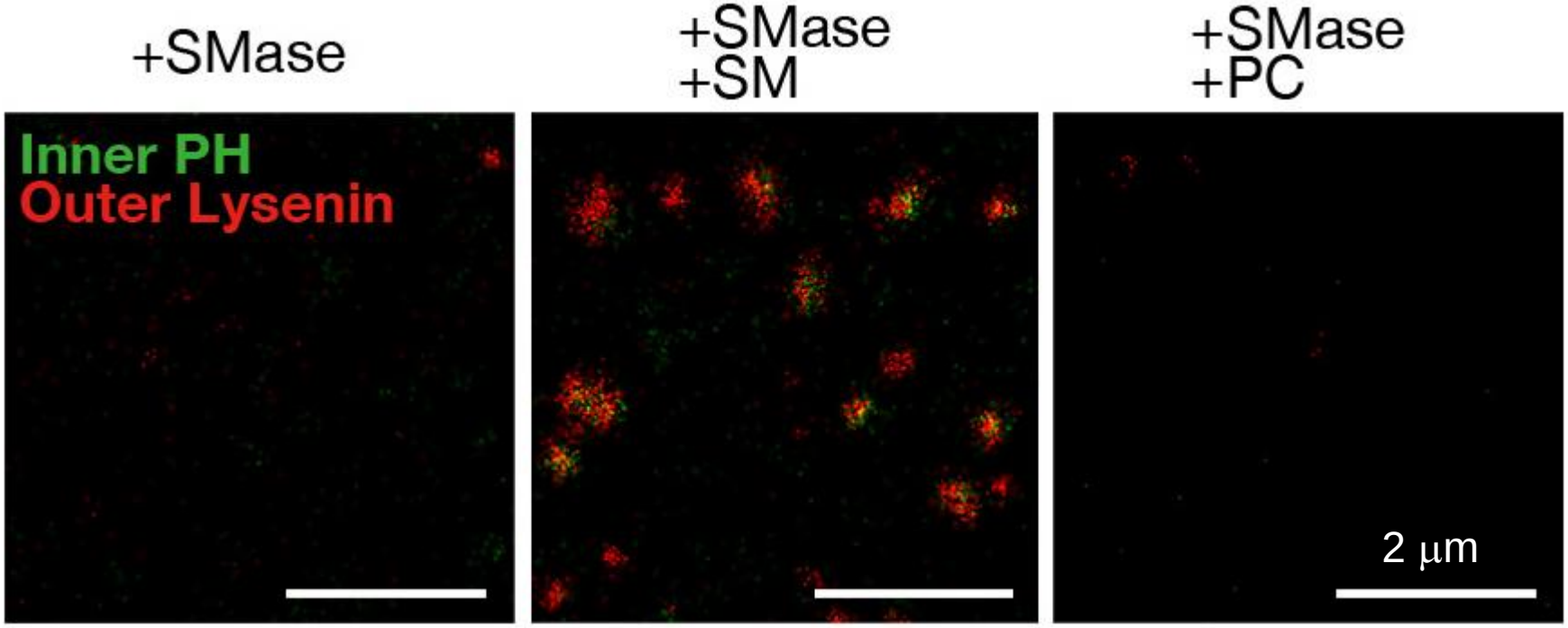
Mizuno 2011

Sphingomyelin-rich domains colocalize with PIP_2 and are required to maintain PIP_2 domains

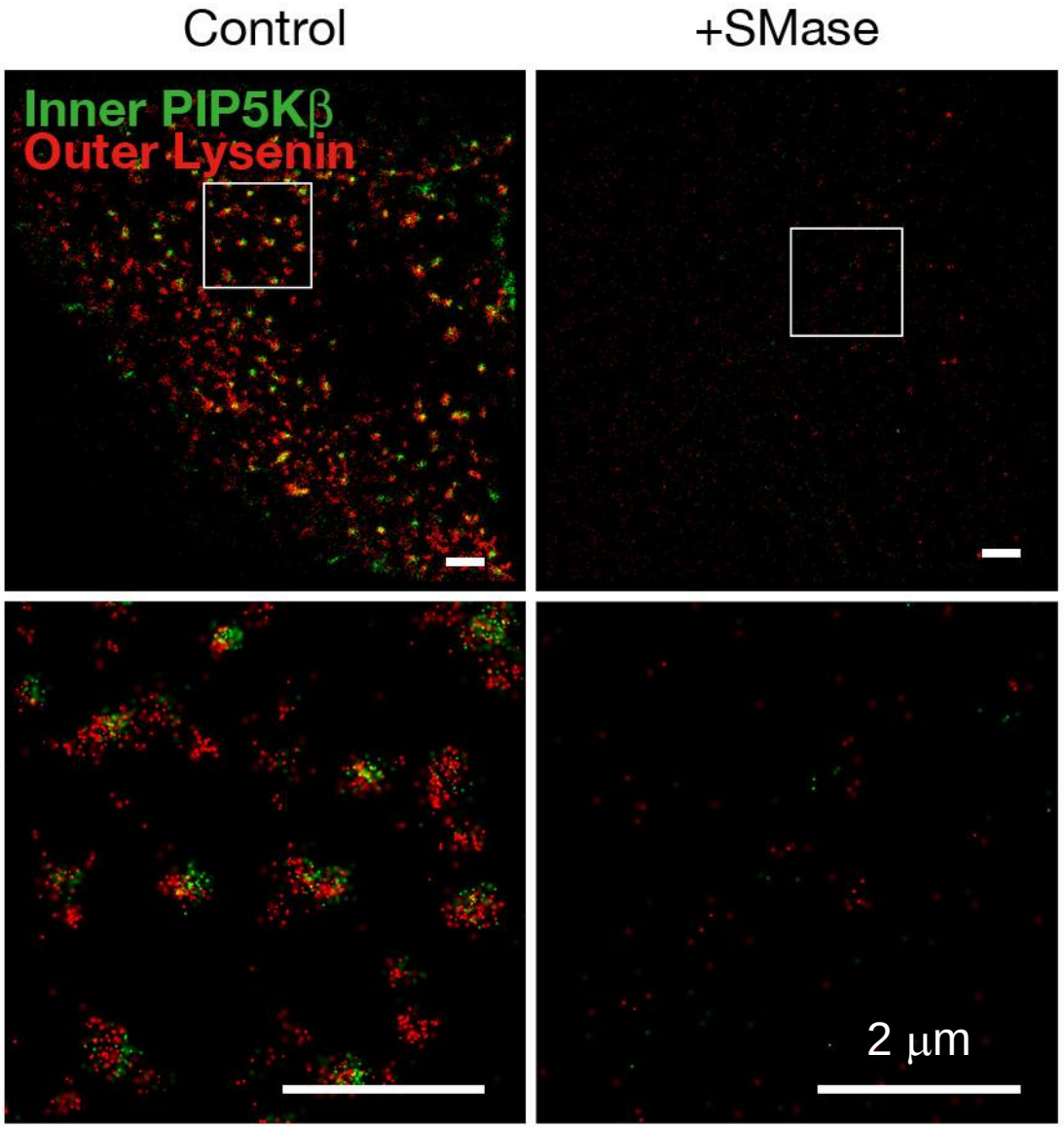




Addition of exogenous sphingomyelin restores the PIP_2 domain in sphingomyelinase-treated cells



Transbilayer colocalization of sphingomyelin-rich domain and PIP5Kβ



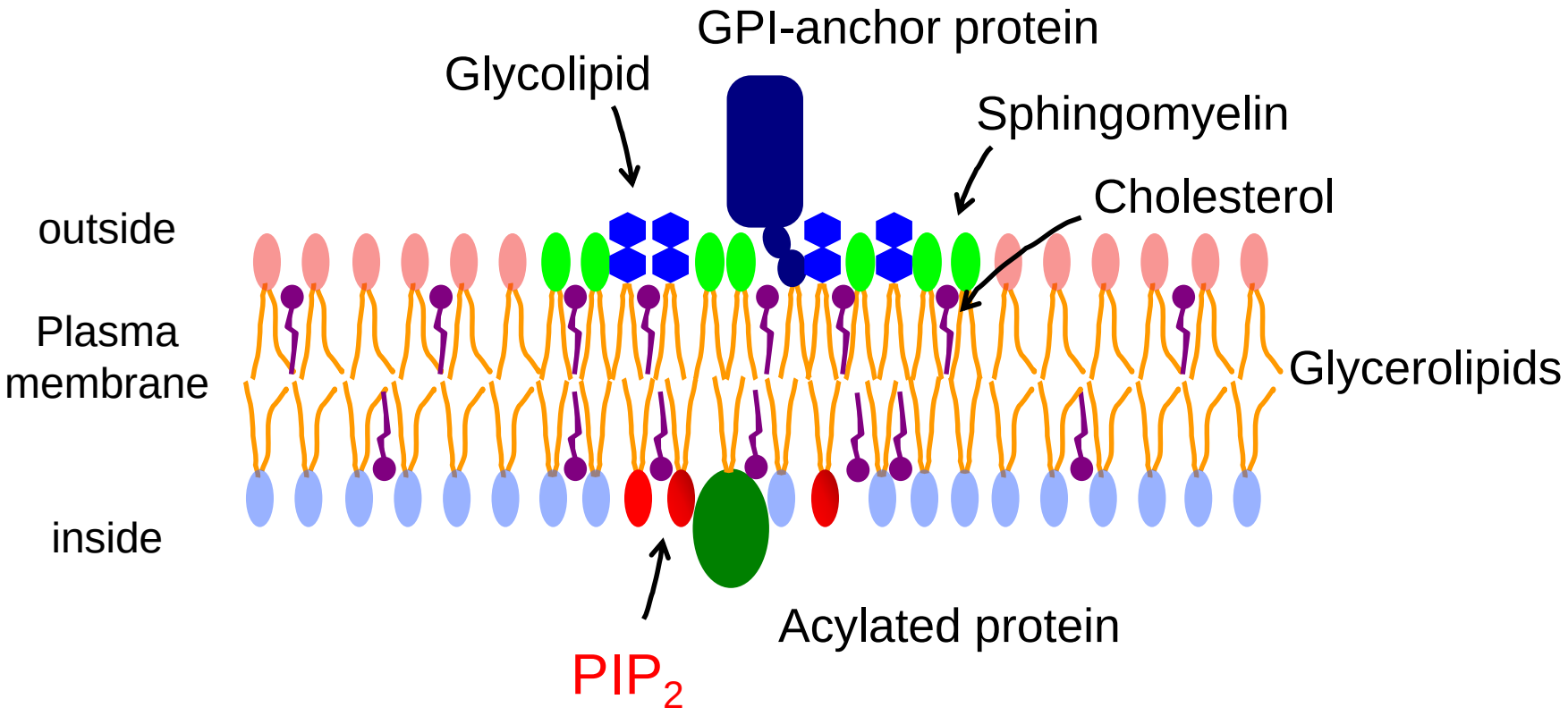
Summary 2

-PIP_2 domains are located to the opposite side of sphingomyelin-rich domains.

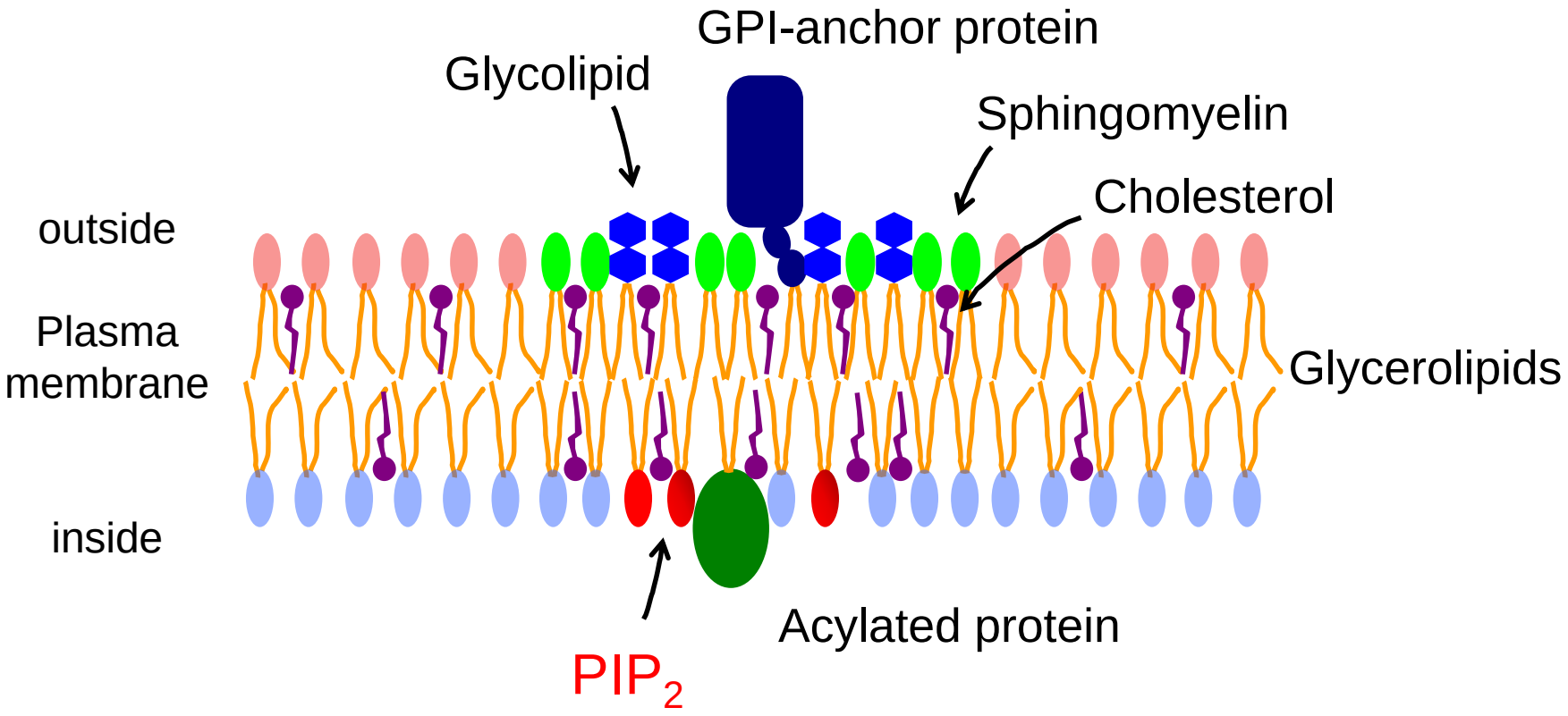
-Sphingomyelin domains are required for the formation of PIP_2 domains.

Abe 2012

Interbilayer co-localization of sphingomyelin and PIP_2



Interbilayer co-localization of sphingomyelin and PIP_2

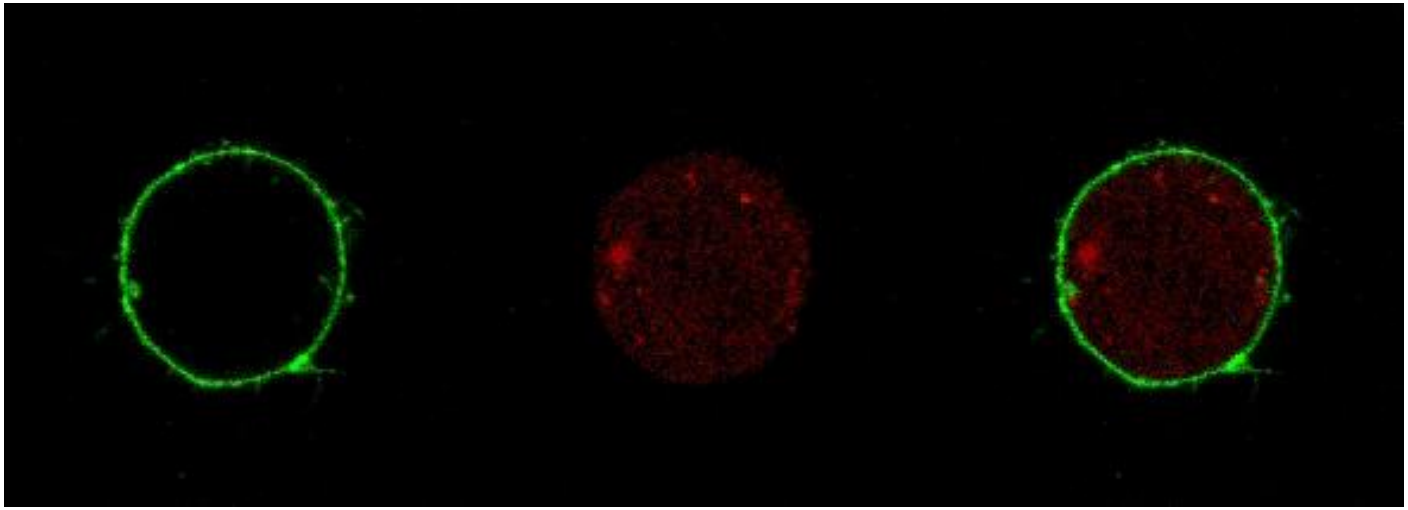


Physiological significance?

Role of sphingomyelin in cell division

Sphingomyelin is accumulated to the cleavage furrow during cytokinesis

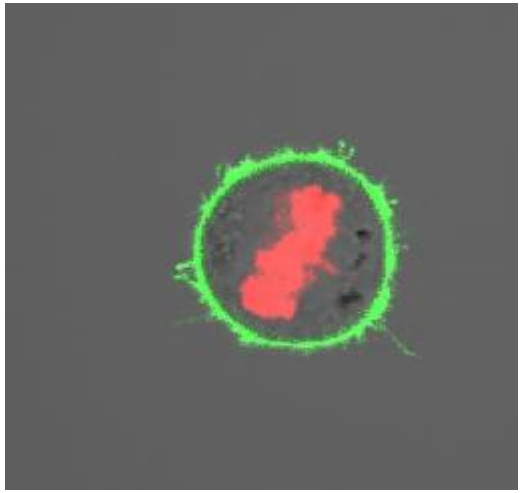
Outer leaflet Inner leaflet Merge



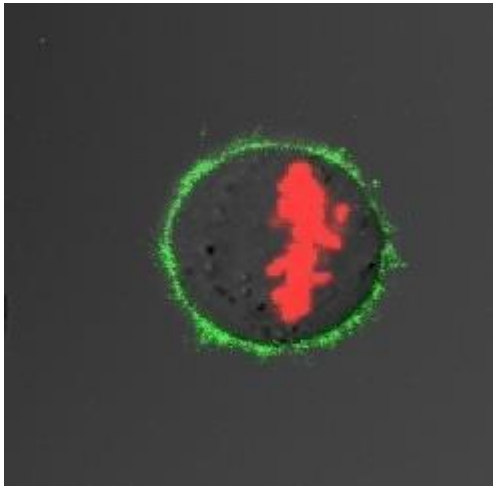
Sphingomyelin is accumulated to the outer leaflet of the cleavage furrow.

Sphingomyelinase treatment inhibits the completion of cytokinesis

Control



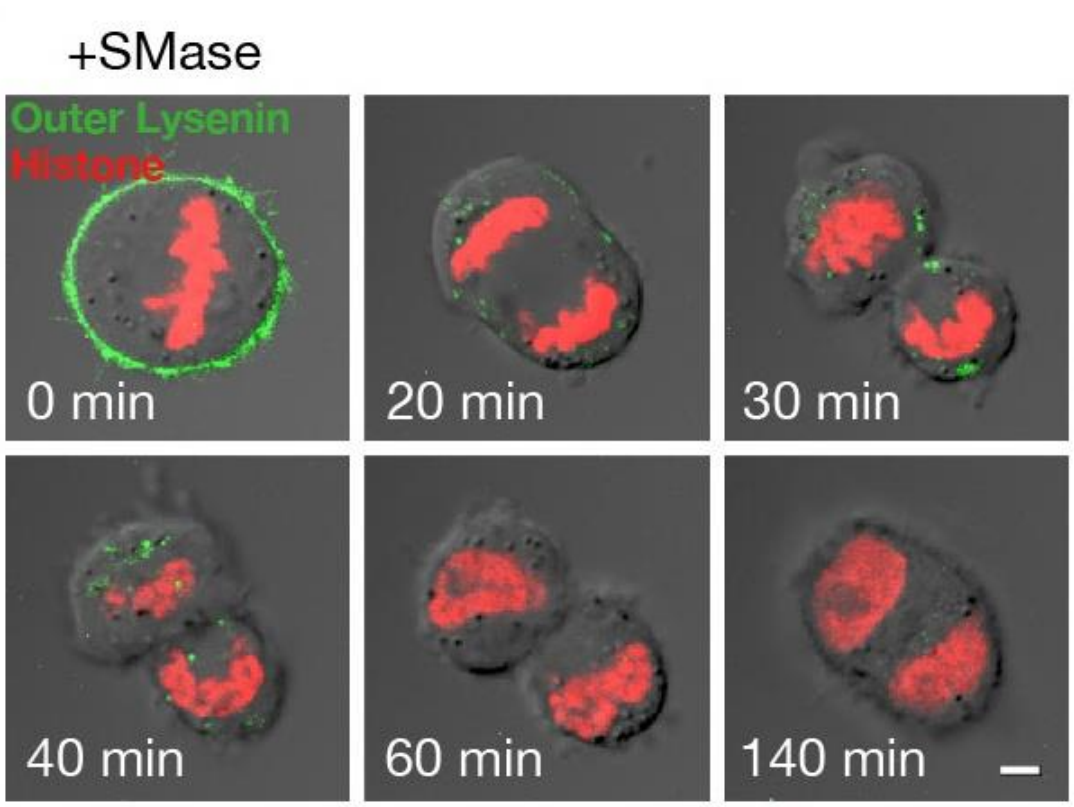
Treatment with sphingomyelinase, which degrades sphingomyelin

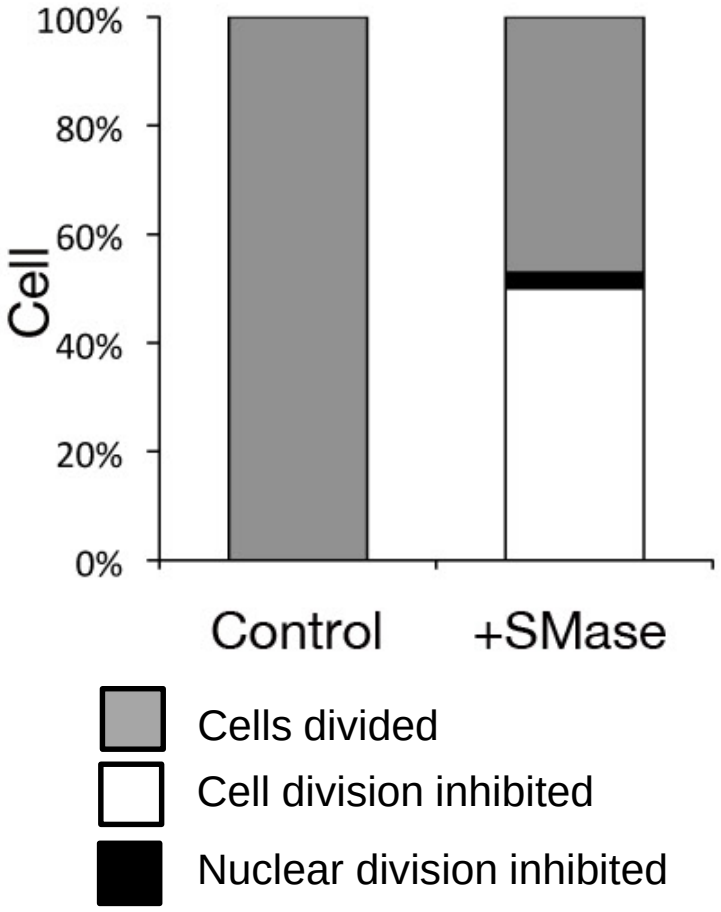


Sphingomyelin, DNA

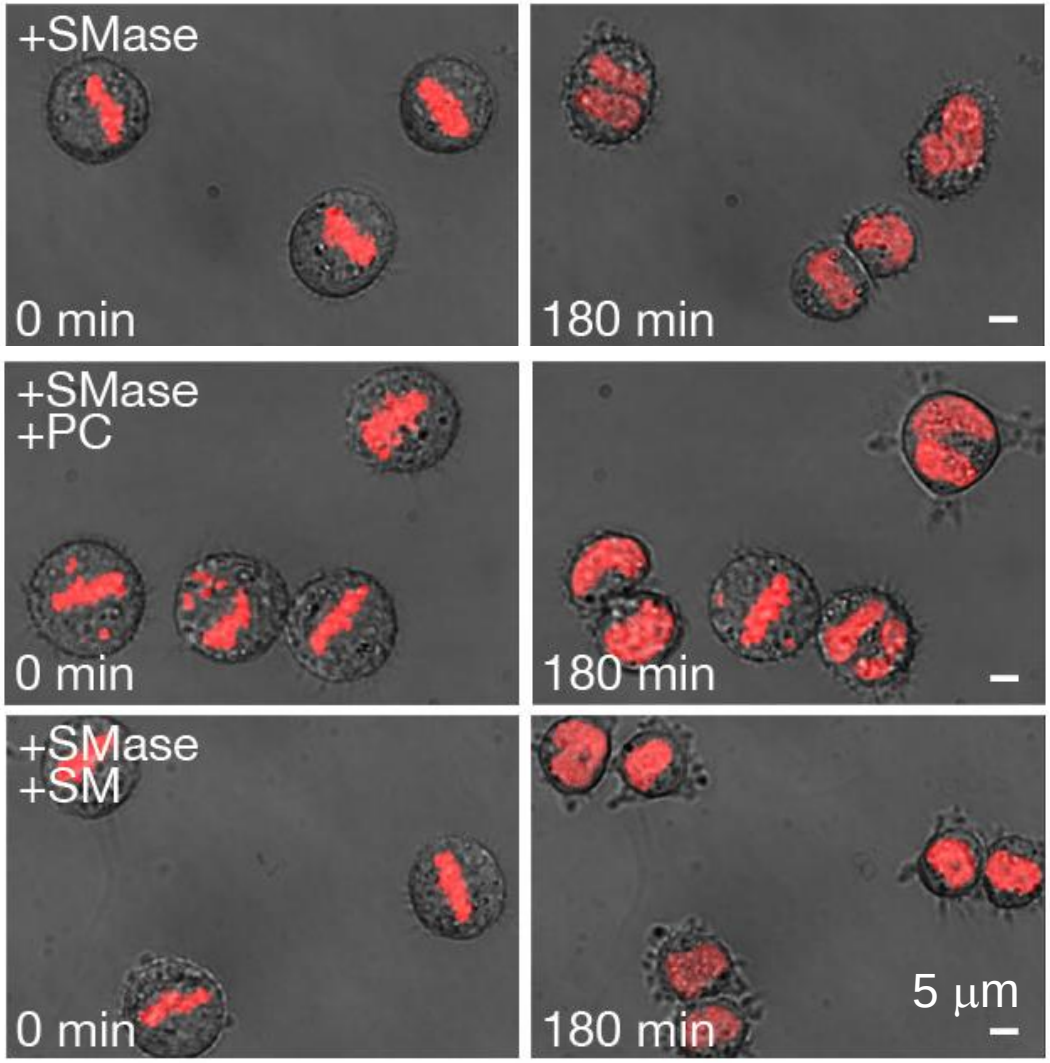
Cleavage furrow is formed but regressed.

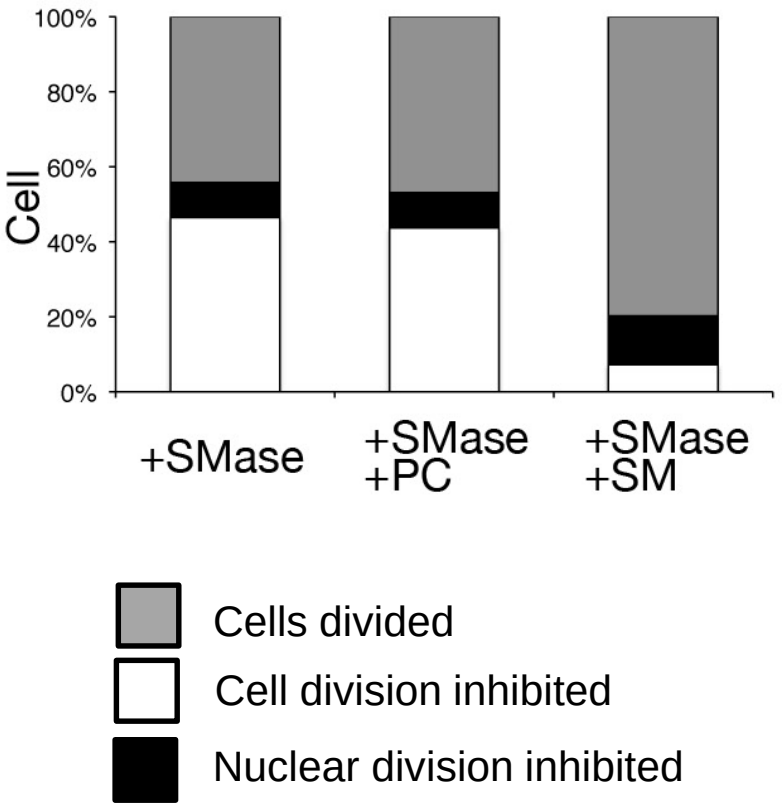
Sphingomyelinase treatment results in the inhibition of the completion of cytokinesis





Addition of exogenous sphingomyelin restores cell division in sphingomyelinase-treated cells





Sphingomyelin is accumulated to the cleavage furrow during cell division.

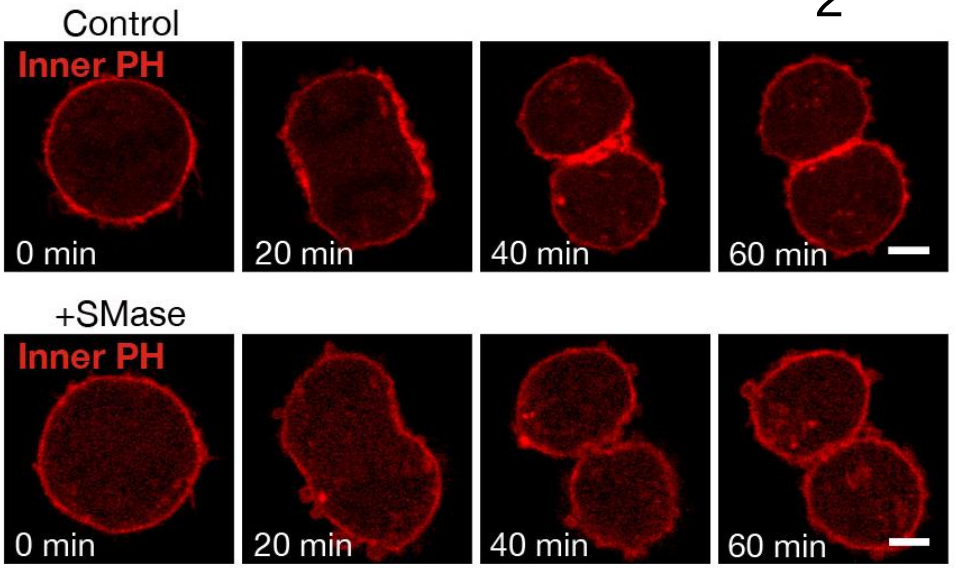
Cell surface sphingomyelin is required for cell division.

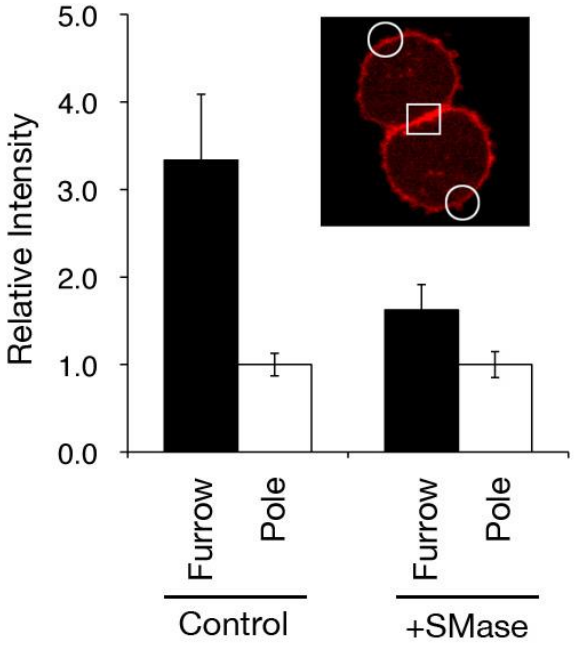
Accumulation of phosphatidylinositol 4,5-bisphosphate to the cleavage furrow is crucial for cytokinesis

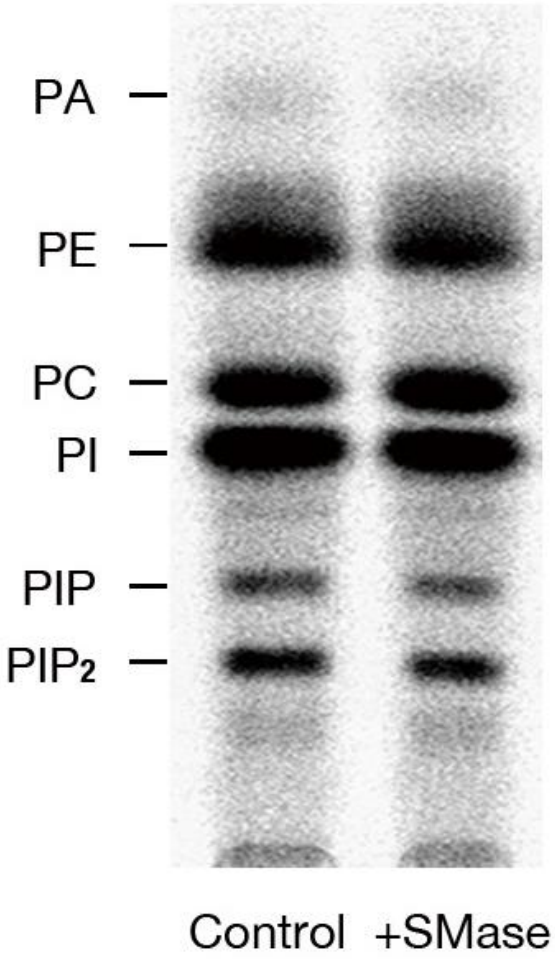
Yoshida S, Bartolini S, Pellman D
Mechanisms for concentrating Rho1 during cytokinesis
Genes Dev, 23, 810-23 (2009)

Field SJ, Madson N, Kerr ML, Galbraith KA, Kennedy CE, Tahiliani M, Wilkins A, Cantley LC
PtdIns(4,5)P_2 functions at the cleavage furrow during cytokinesis
Curr Biol, 15, 1407-12 (2005)

Sphingomyelinase treatment abolishes the accumulation of PIP_2 to the cleavage furrow

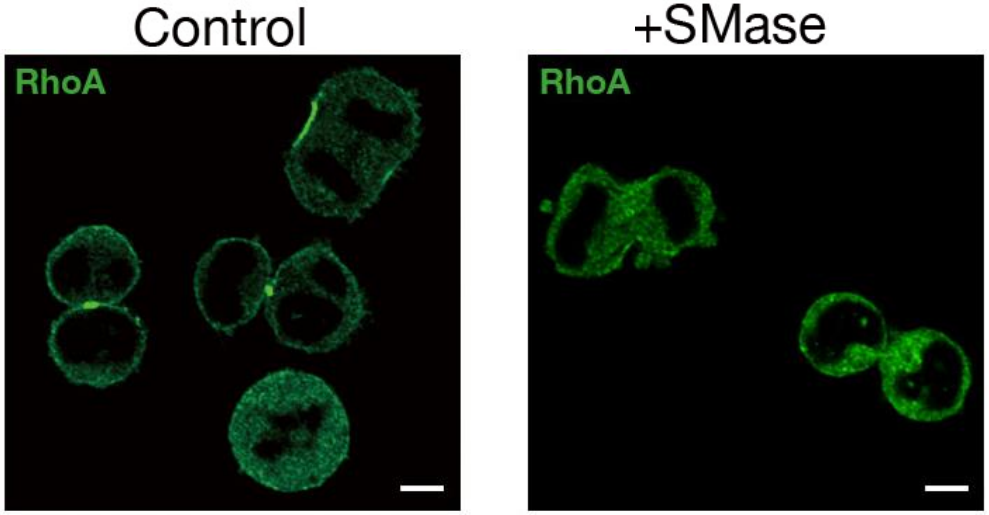


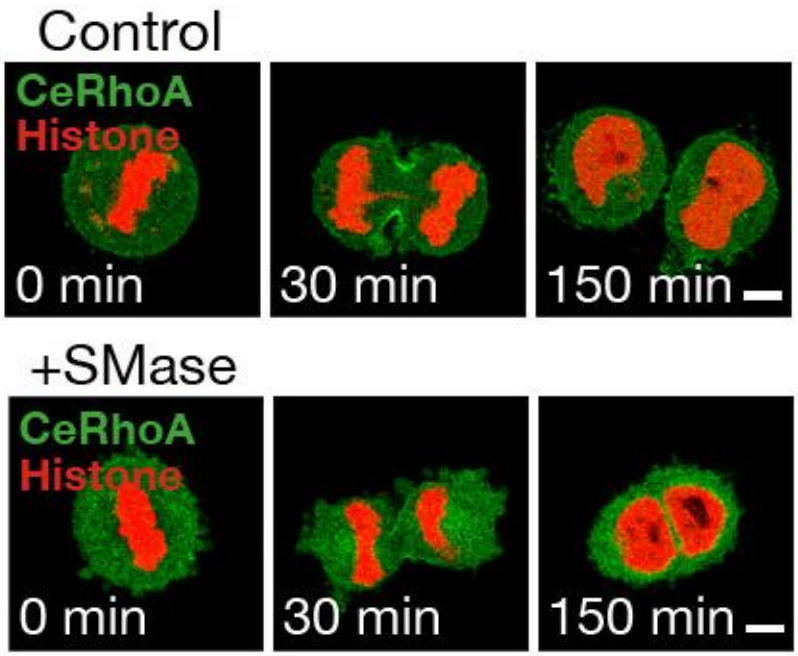


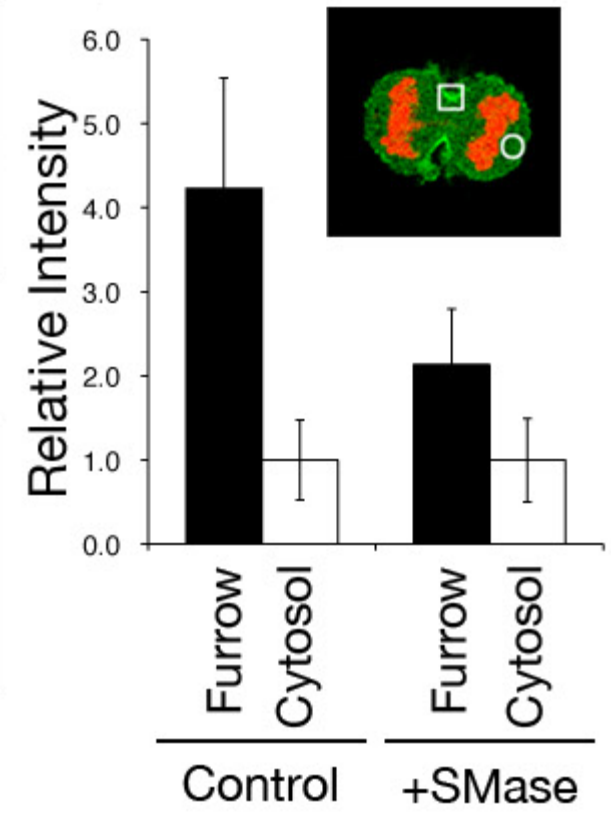


[^{33}P]phosphate labeling

Sphingomyelinase treatment abolishes the accumulation of RhoA to the cleavage furrow







Summary 3

-Sphingomyelin-rich membrane domains are accumulated to the cleavage furrow during cytokinesis.

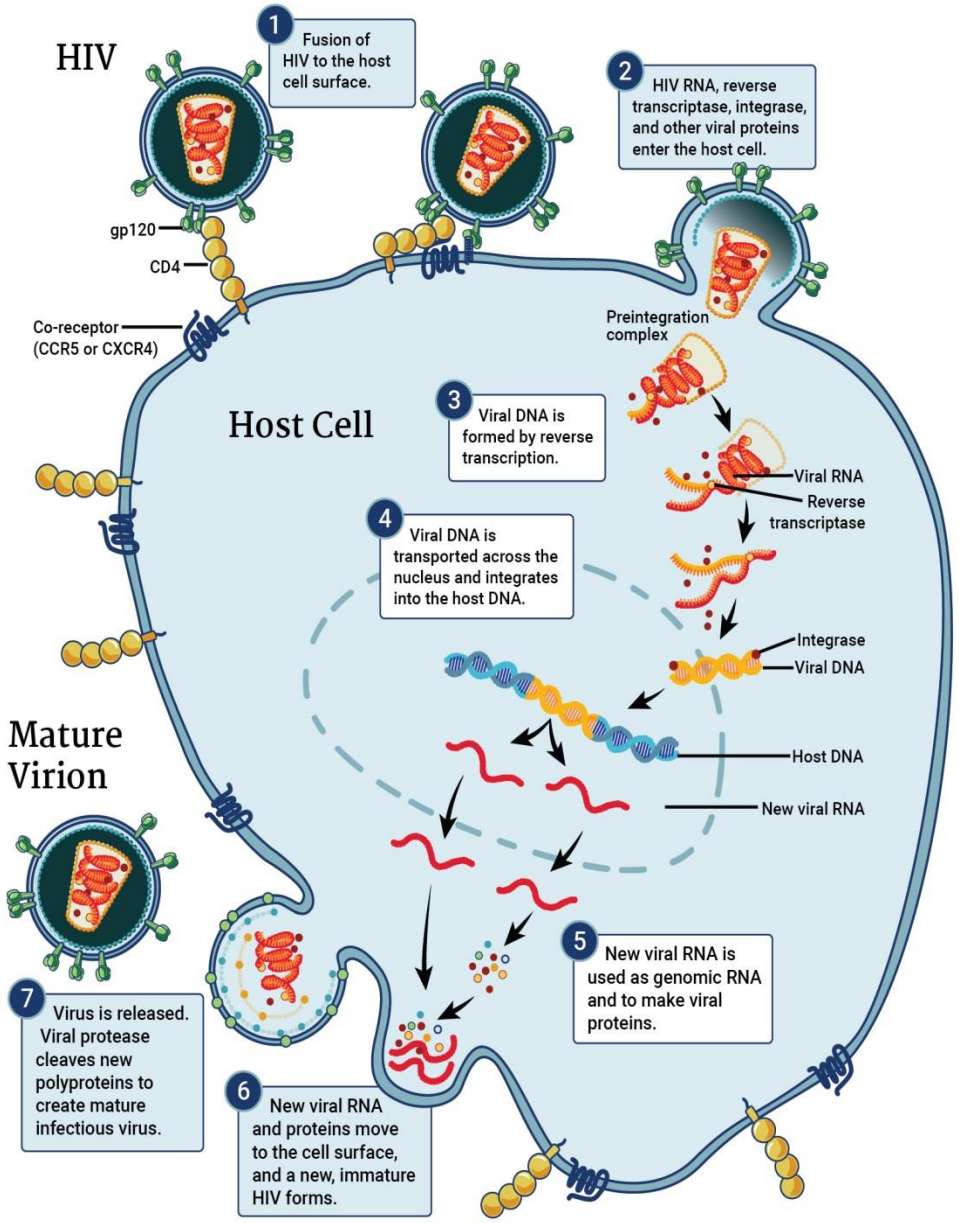
-Cholesterol is required for the formation of sphingomyelin-rich membrane domains in mitotic cells.

-Sphingomyelinase treatment inhibits cytokinesis by inhibiting the formation of PIP_2 domains.

Abe 2012

Role of sphingomyelin-PIP_2 interaction in virus budding

HIV-1 (human immunodeficiency virus type 1)



HIV-1 is a retrovirus that causes HIV infection. AIDS is the most advanced stage of HIV infectio

- Number of people living with HIV is 37 million in the world.
- 1 million people died from AIDS-related illness in 2016.
- People newly infected with HIV is 1.8 million.

UNAIDS 2017 report

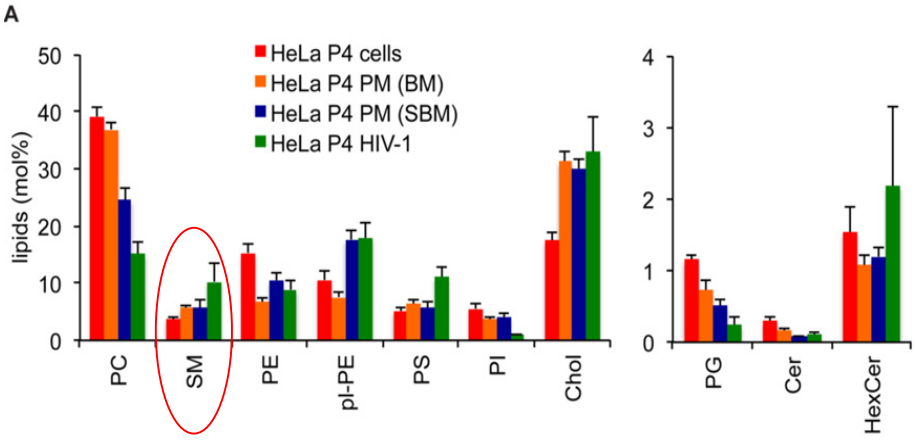
HIV still remains a threat for human.

Lipids incorporated in HIV-1 particles

Table 1. Phospholipid composition of MT-4 cells and HIV-1

	MT-4 cells (mol % ± SD)	HIV-1 (mol % ± SD)
PC	43.0 ± 2.9	16.0 ± 1.0
SM + DHSM	10.4 ± 1.6	33.1 ± 1.2
PE	17.0 ± 1.5	8.2 ± 1.3
pl-PE	15.9 ± 0.5	27.0 ± 3.3
PS	7.4 ± 0.8	15.5 ± 2.2

Brugger et al (2006) PNAS



Lorizate et al (2013) Cellular Microbiol

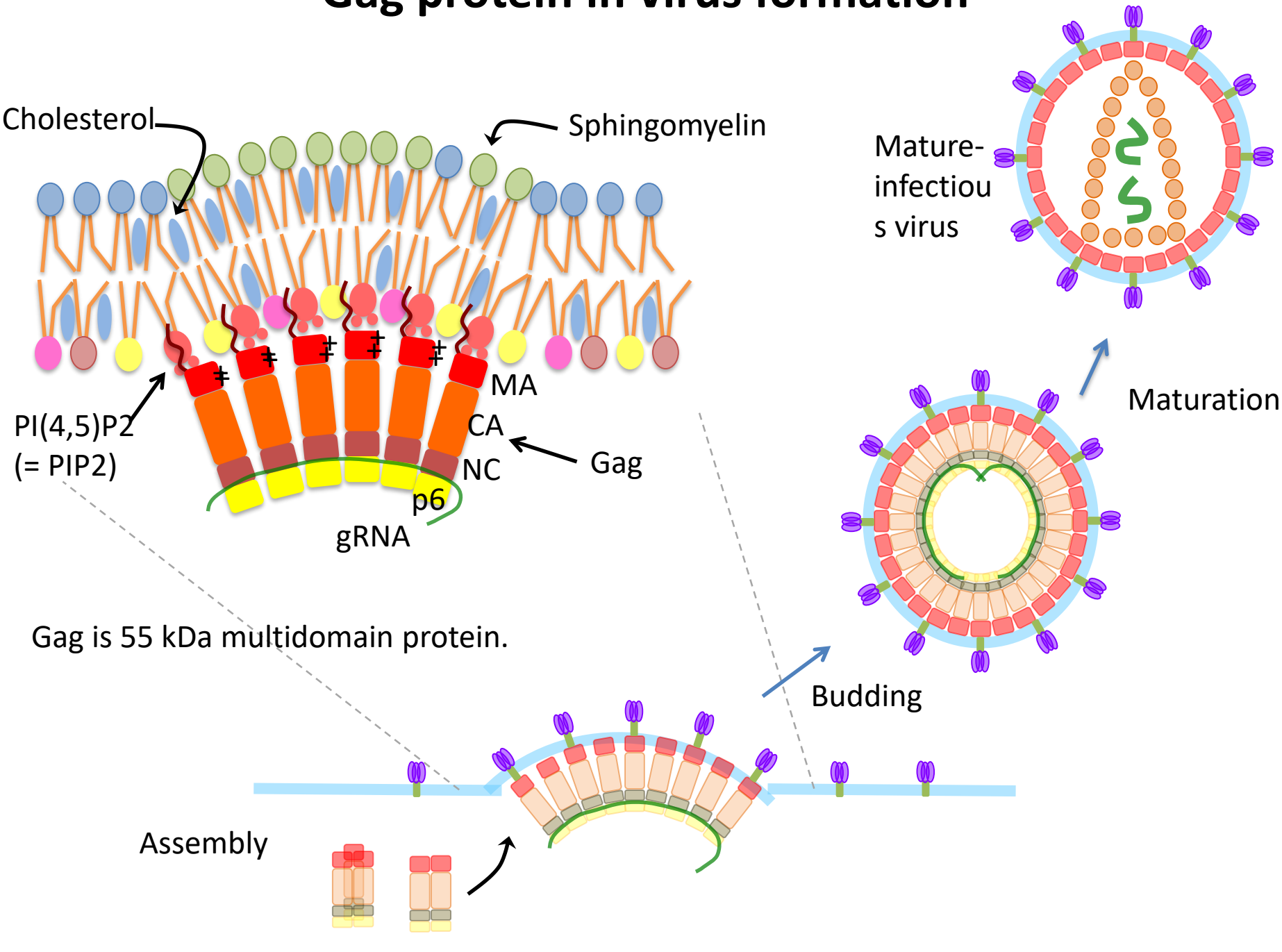
B

	This study		Brugger et al, 2006
Virus	HIV	MLV	HIV
Cell Line	H9	REF	MT4
PS	2.8*	2.5*	2.1
PI	-0.4*	-0.1*	n/a
PIP	1.4*	1.2	n/a
PIP_2	2.4*	5.9*	n/a
PE	1.6	-0.9	-0.5
pl-PE	2.2*	1.3	1.7
PC	-0.7*	-0.6*	-0.4
ePC	-0.3*	-0.6	n/a
SM	2.1*	1.8*	3.2
dhSM	2.5*	2.3*	
Cer	-0.3*	-0.3*	-0.3
Glu-Cer	-0.6*	1.2	2.6

Chan et al (2008) J Virol

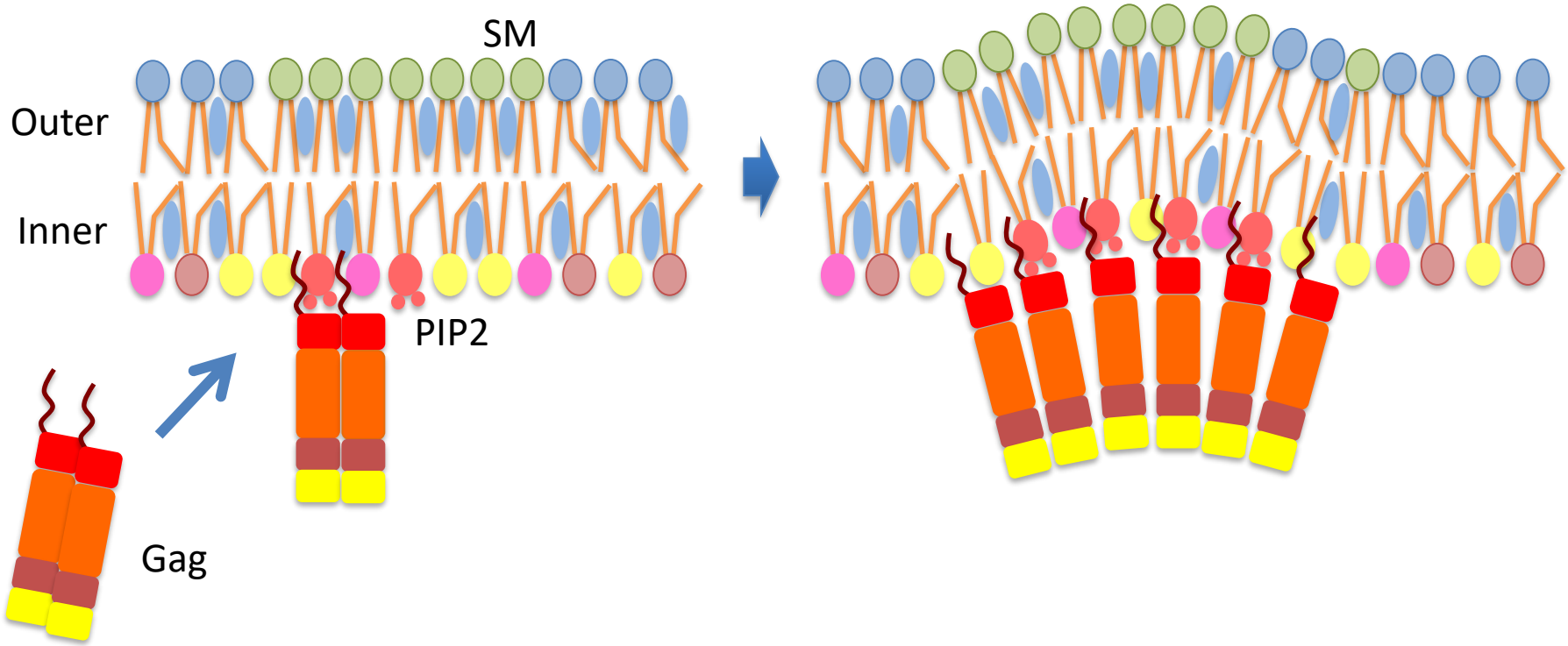
Lipidomic analyses revealed that HIV-1 viral particle are rich in SM and PIP_2

Gag protein in virus formation



Gag is 55 kDa multidomain protein.

Gag assembly in plasma membrane



Expression of Gag is sufficient to promote the formation of virus-like particles.

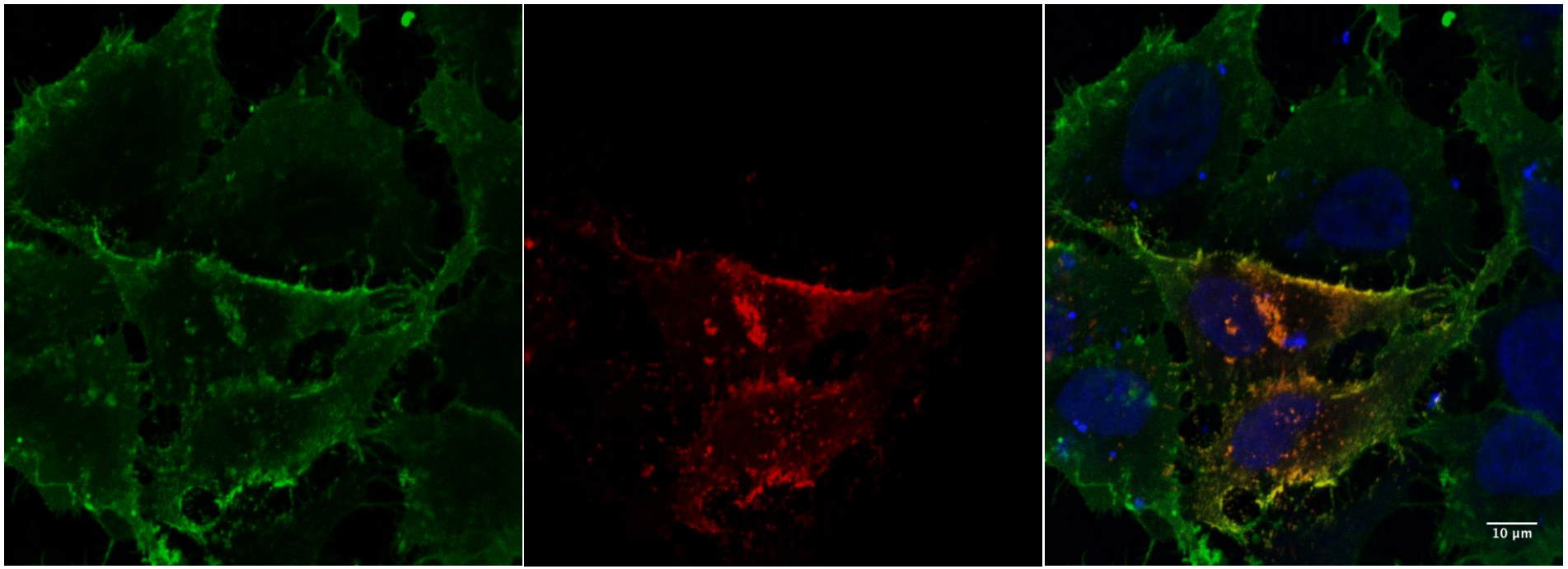
Recruitment of Gag to the plasma membrane requires negatively charged lipid, PIP2

Virus particle size: 100 – 150 nm

EGFP-Lys and Gag-mCherry observed by confocal microscop

EGFP-NT-Lys

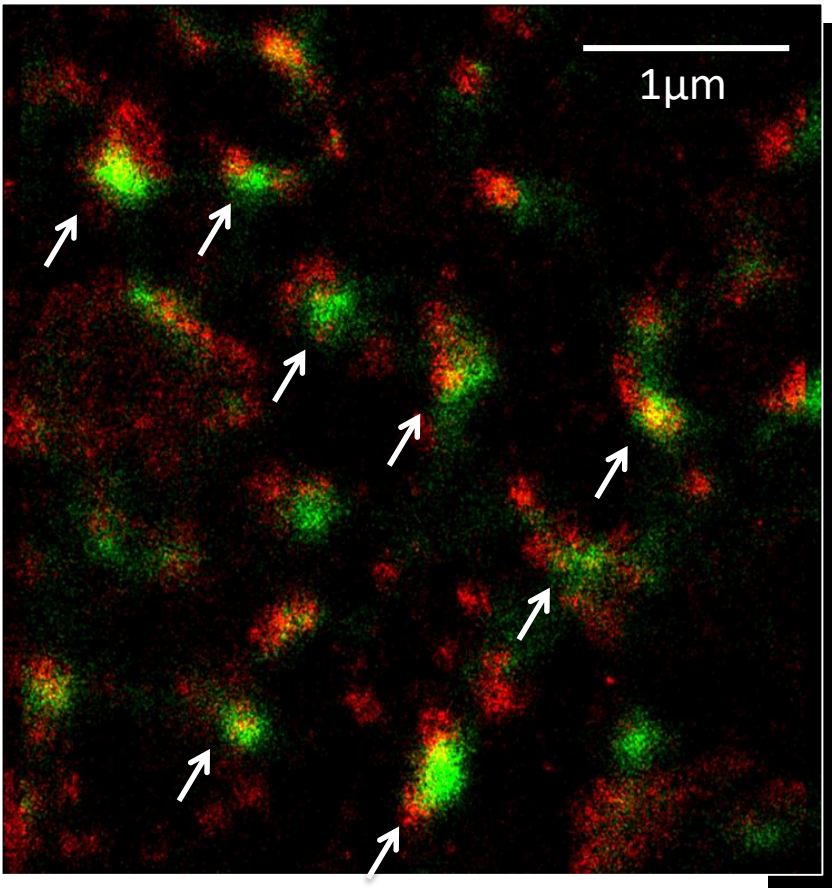
Gag-mCherry

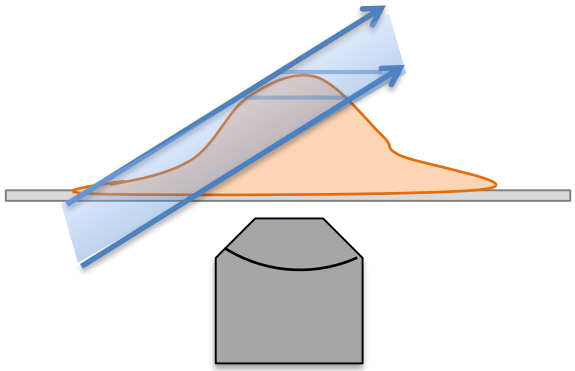


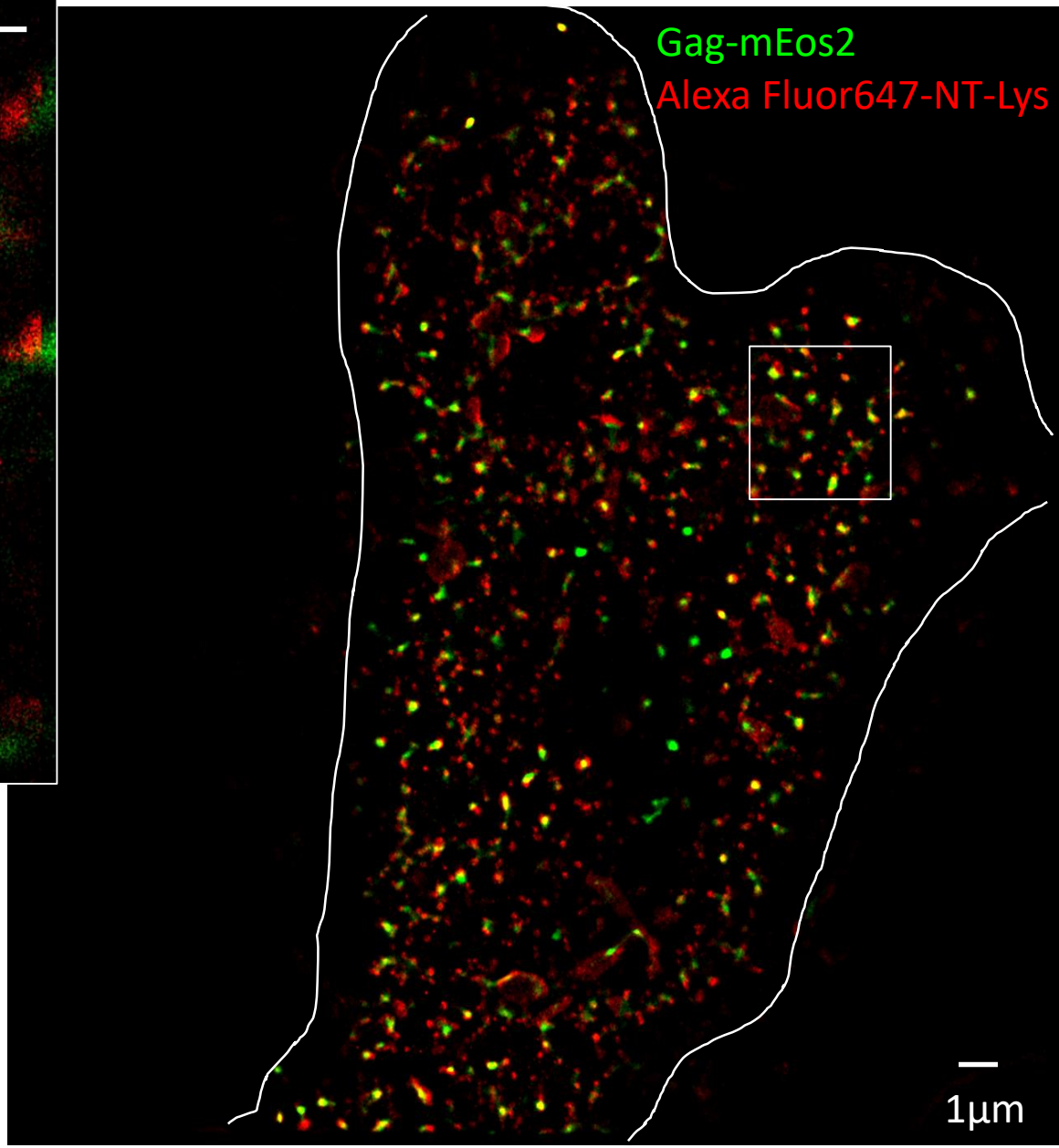
Bar, 10 µm

HeLa cells were labeled with EGFP-NT-Lys 24 h after transfection with Gag-mCherry and Gag-

PALM/STORM image of Gag-mEos2 and Alexa Fluor647-NT-Lys

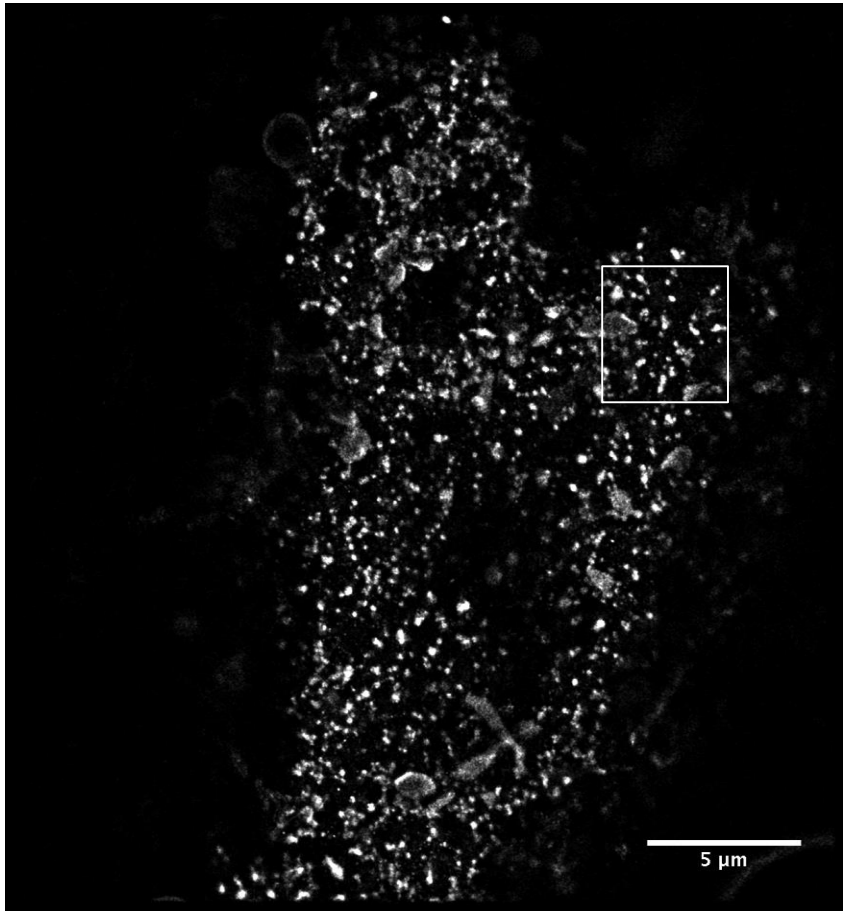




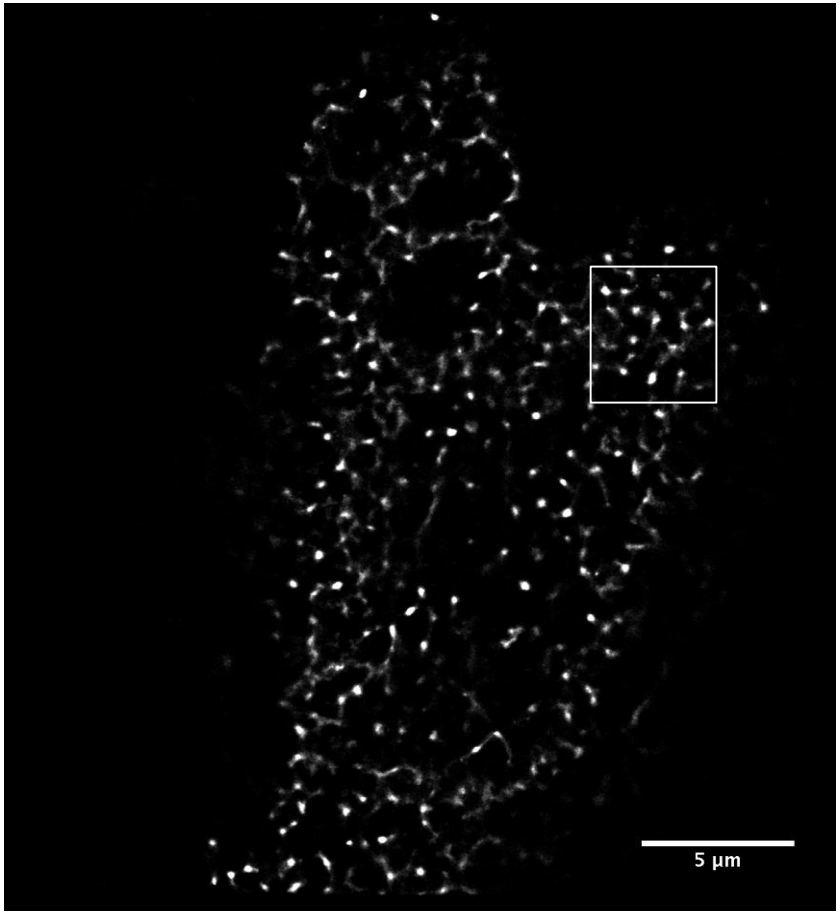


Does the size of SM cluster change in the presence of Gag cluster?

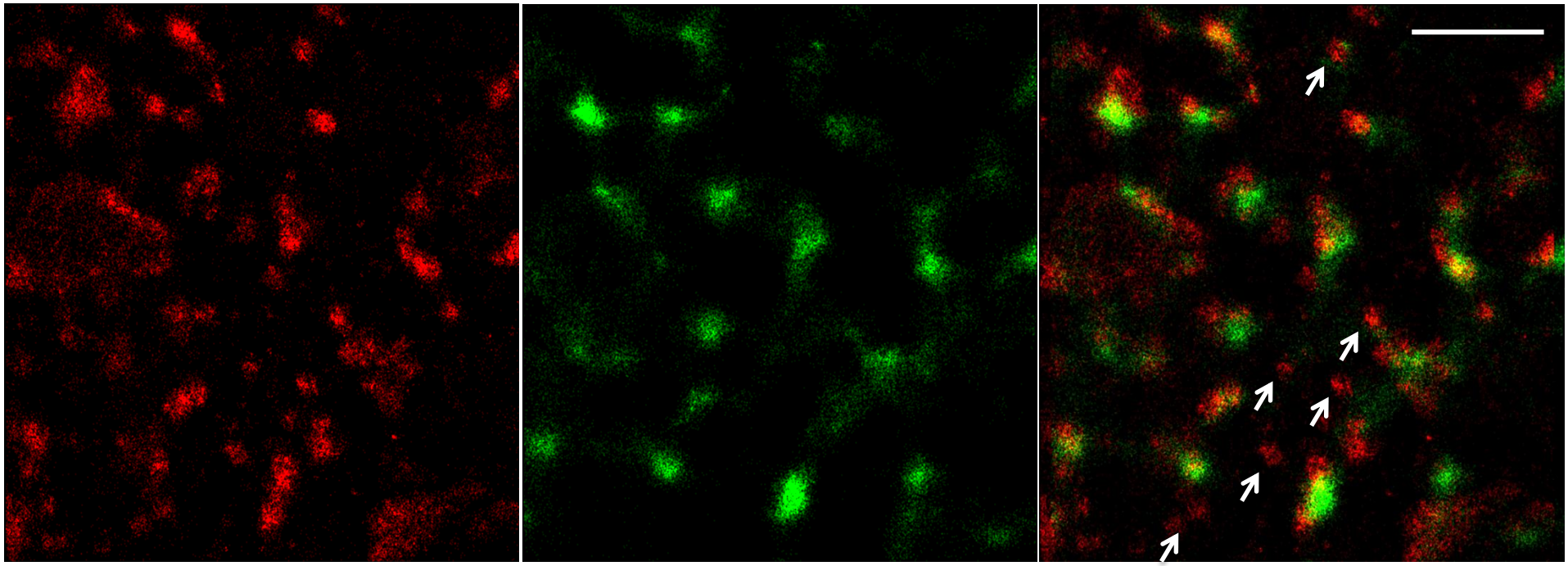
AF647-NT-Lys



Gag-mEos2

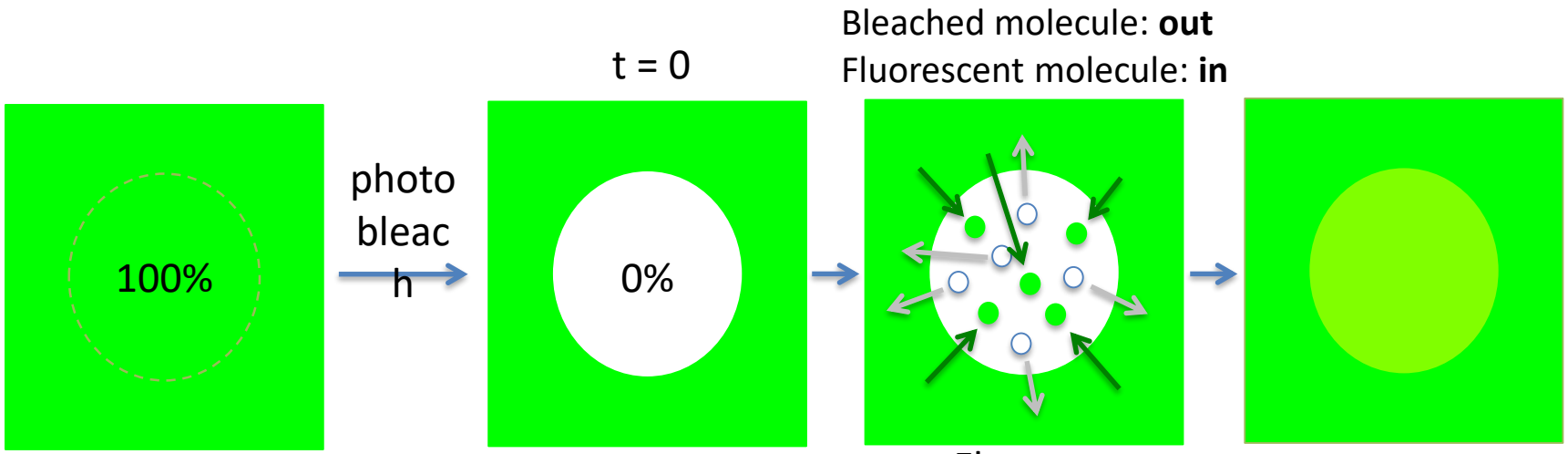


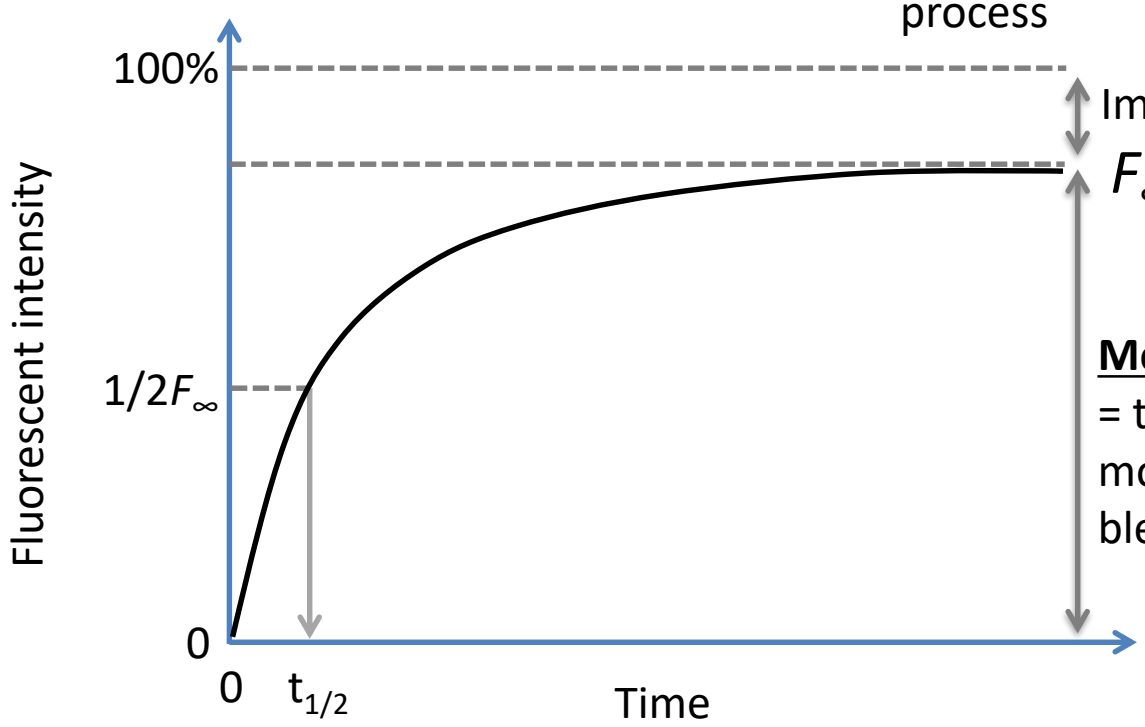
SM cluster in proximity of Gag cluster is larger than that without Gag cluster



Gag cluster gathers SM domains?

FRAP (fluorescent recovery after photobleaching)

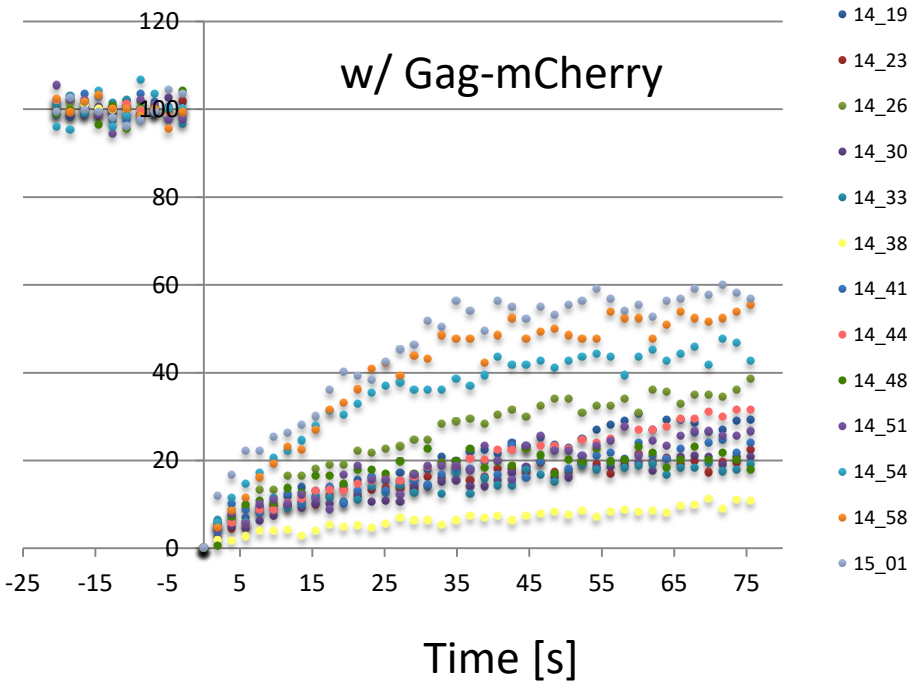


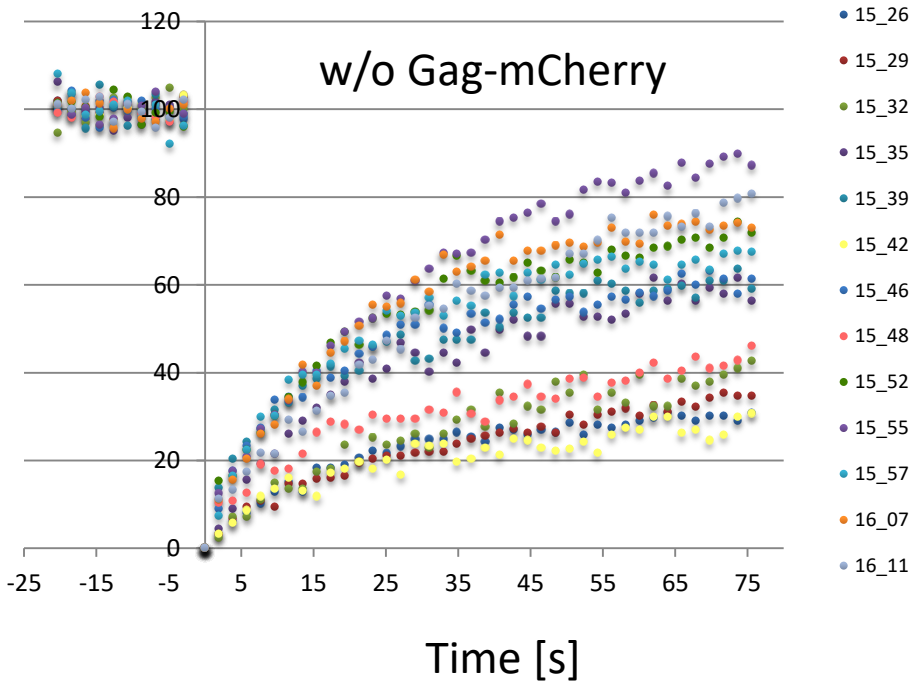


Mobile fraction
= the percentage of molecule moving into bleached area

Diffusion coefficient (*D*)
= status of environment, size of particle, etc

FRAP experiment of EGFP-NT-Lys

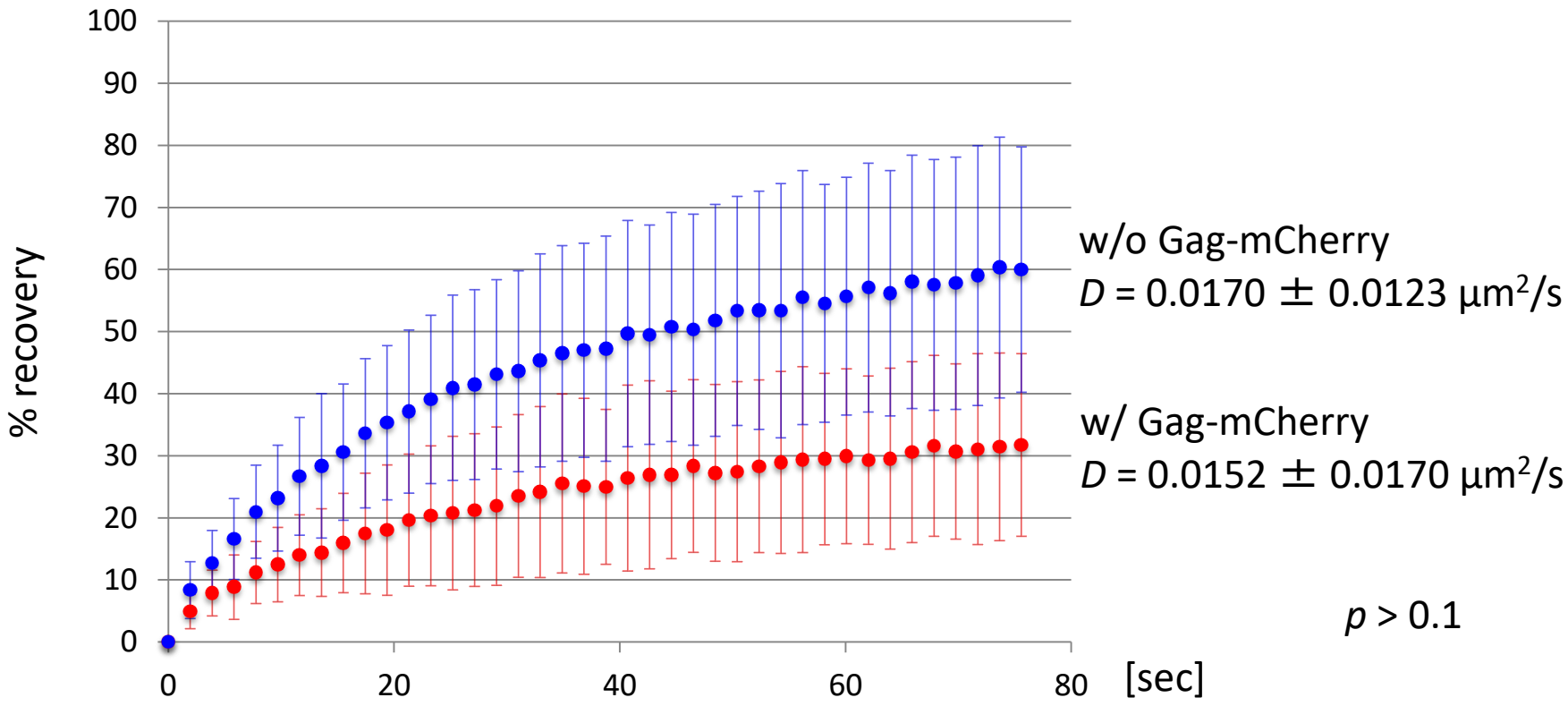




Experiment:

- Hela cells were labeled with EGFP-NT-Lys 20 hours after transfection w/ or w/o Gag-mCherry
- EGFP-NT-Lys was photobleached with 488 nm laser

Summary: FRAP experiment of EGFP-NT-Lys



1. **Diffusion coefficient of SM did not change in the presence of Gag**

2. **Presence of Gag increased immobile fraction of SM**

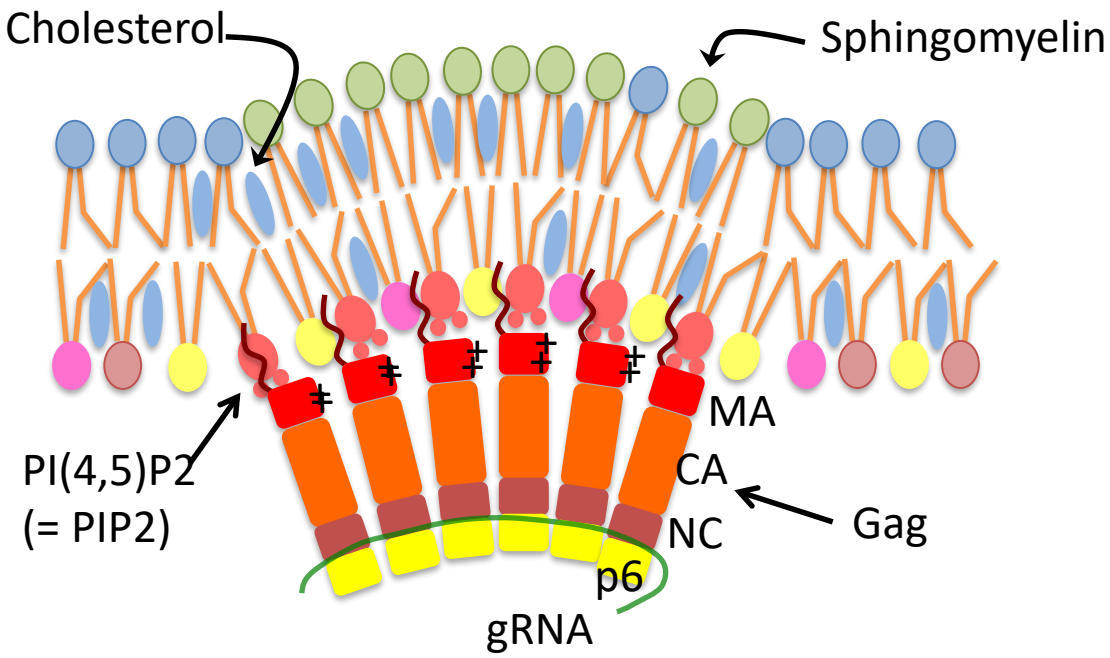
Summary 4

-Expressed Gag in the inner leaflet colocalizes with sphingomyelin-rich domains in the outer leaflet.

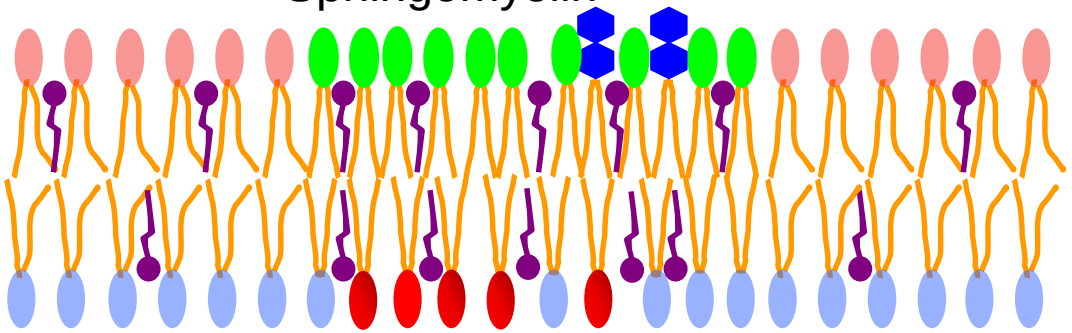
-Gag increases the size of sphingomyelin domains.

-Gag alters the dynamics of the sphingomyelin domains.

How does inner leaflet Gag affect outer leaflet sphingomyelin?

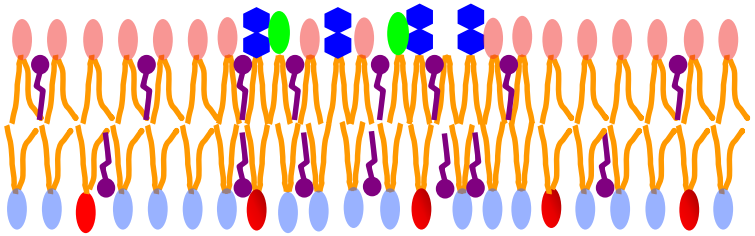


Sphingomyelin



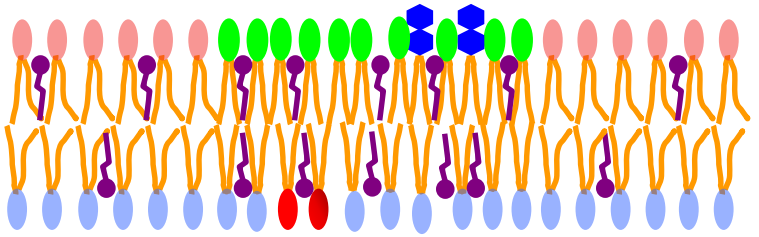
PIP_2

-Decrease of sphingomyelin



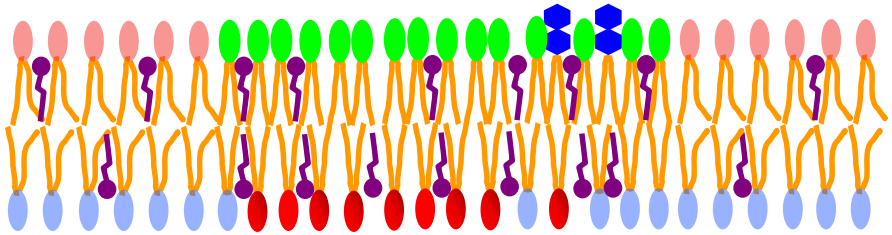
→ disperses PIP_2

→ no change in sphingomyelin



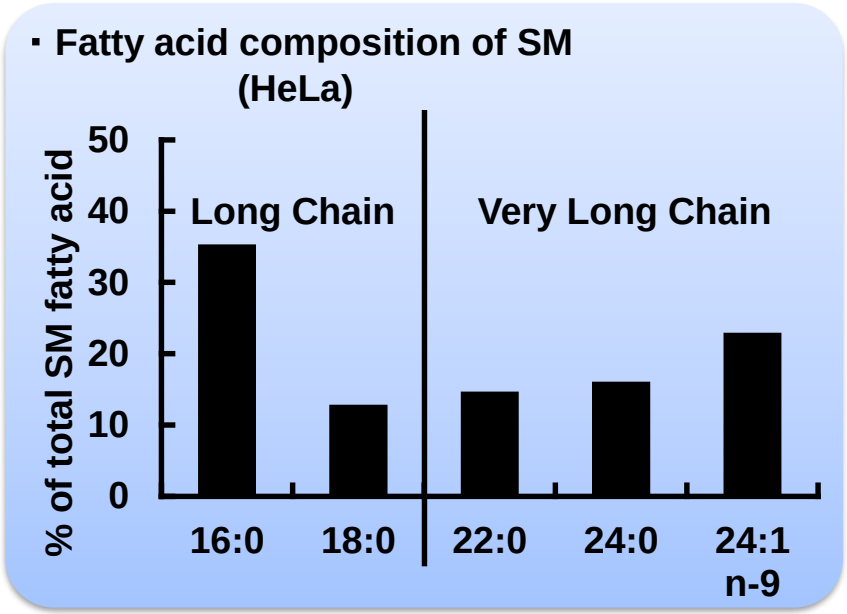
-Decrease of PIP_2

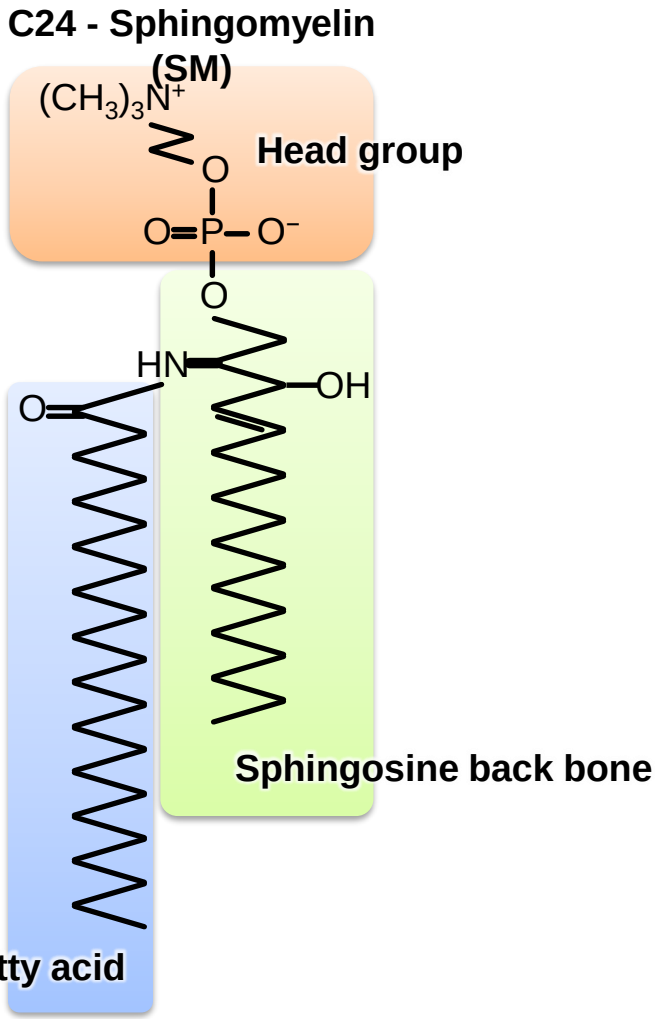
→ merges sphingomyelin domains

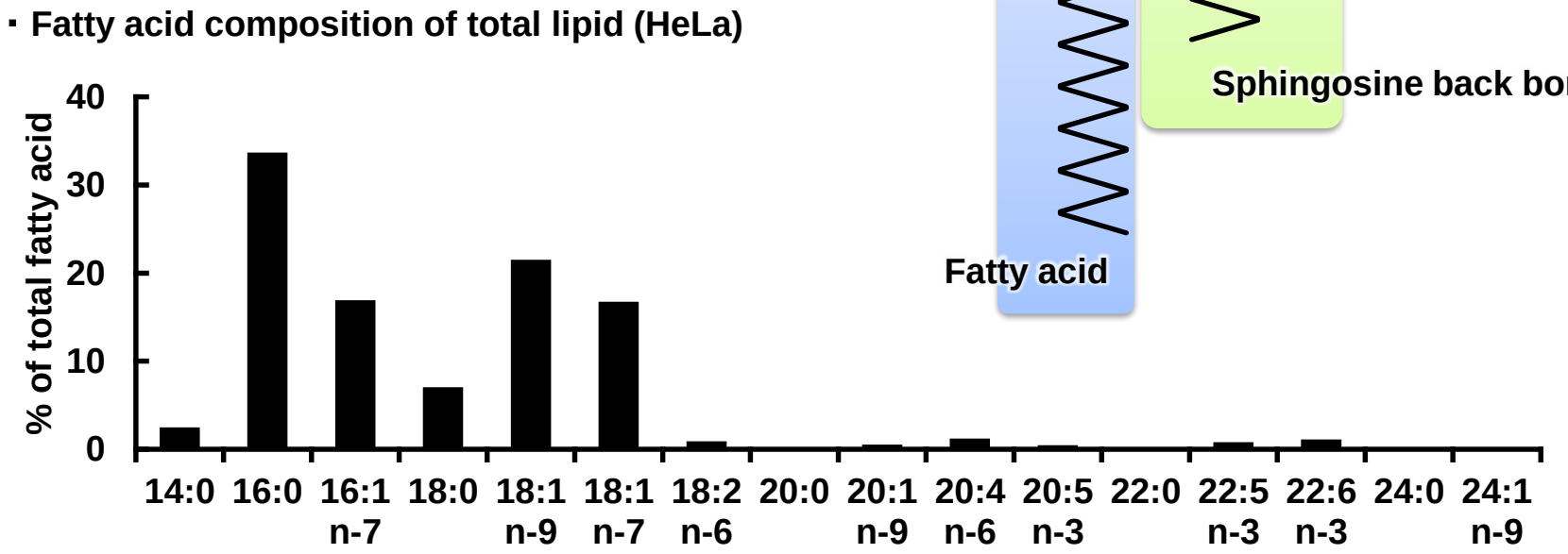


-Crosslinking PIP_2

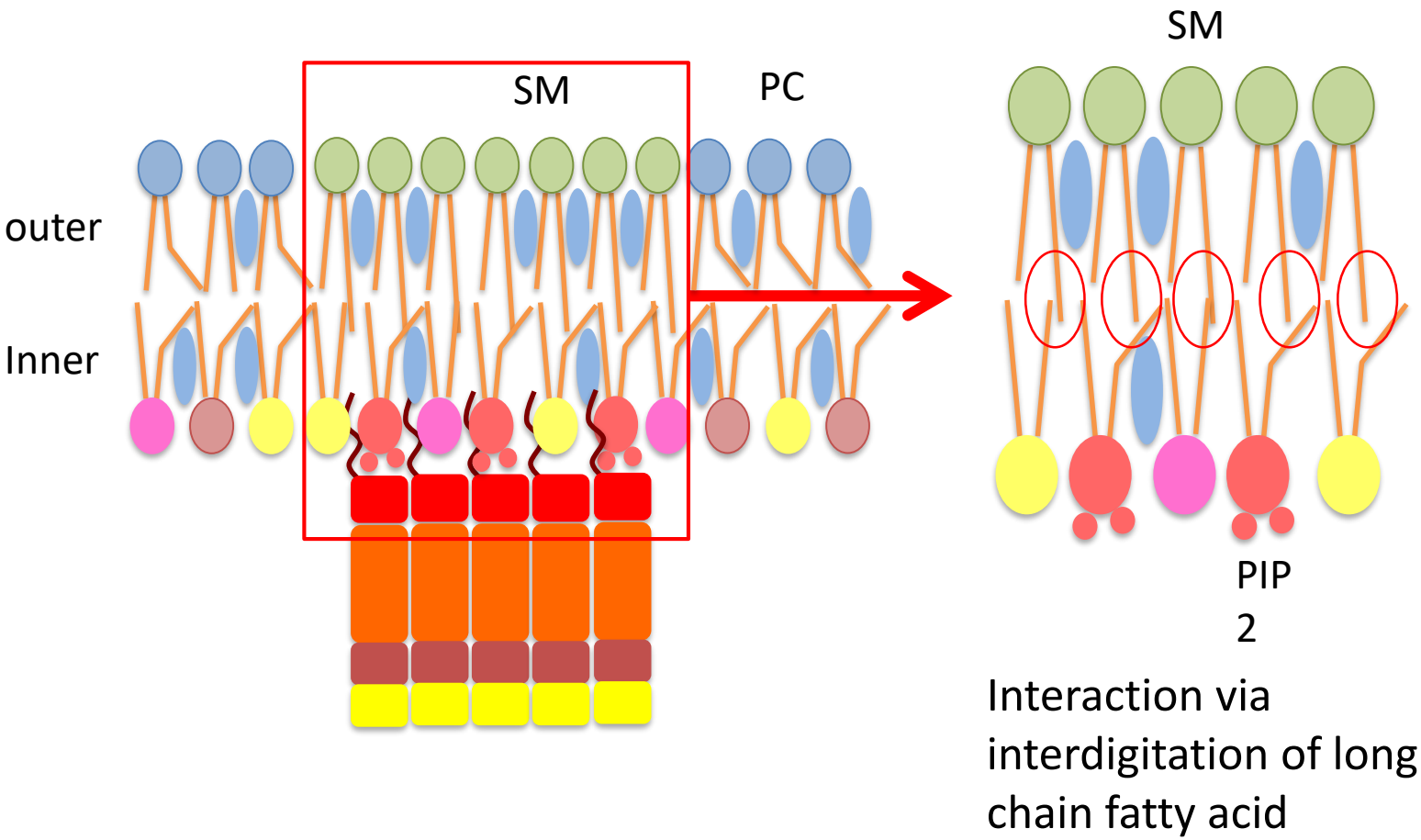
Sphingomyelin has unique fatty acid composition





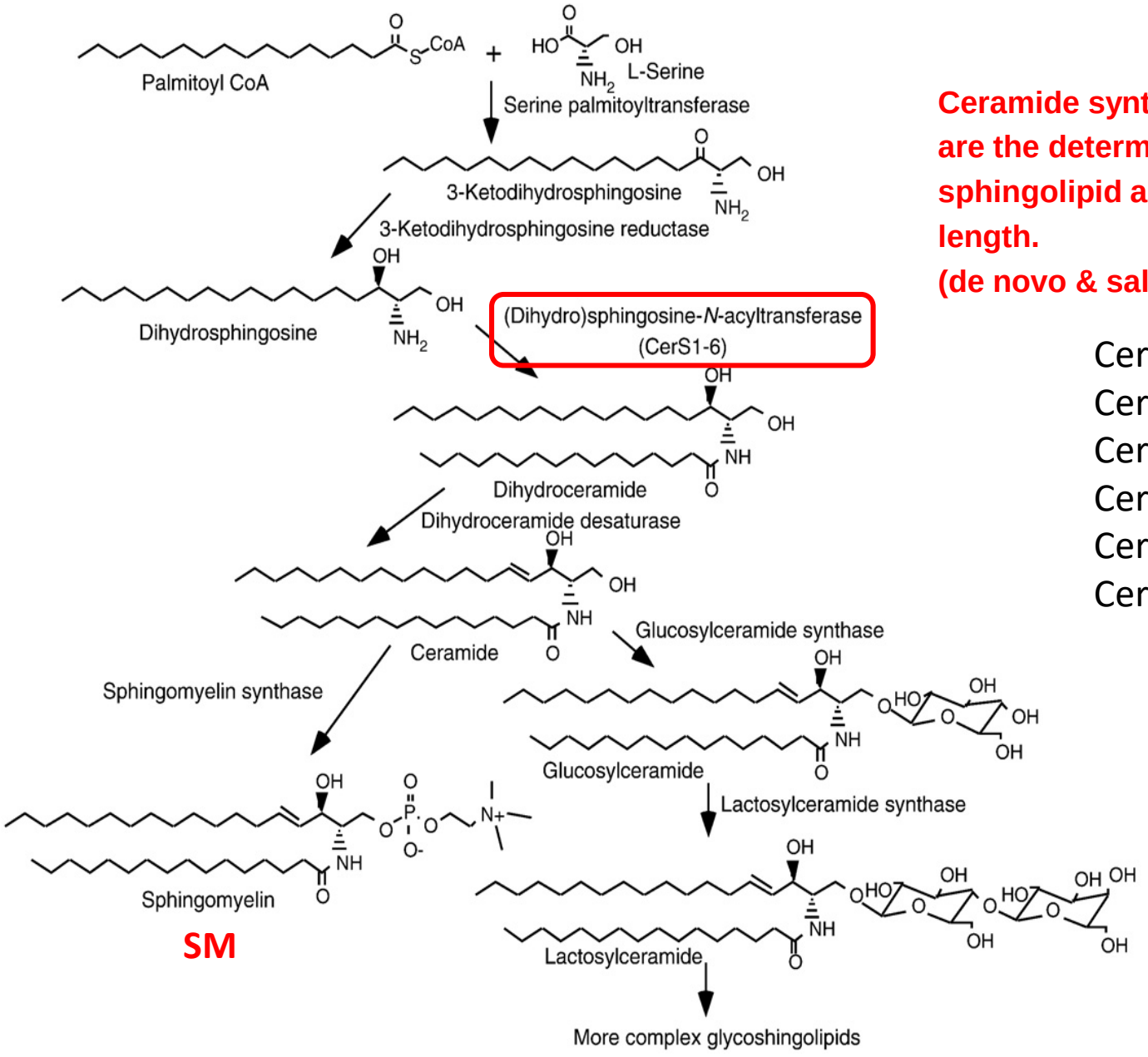


Interdigitation of lipids could link inner leaflet lipids to the outer leaflet lipids



Effect of SM chain length on the Gag assembly?

De novo synthesis pathway of sphingolipids

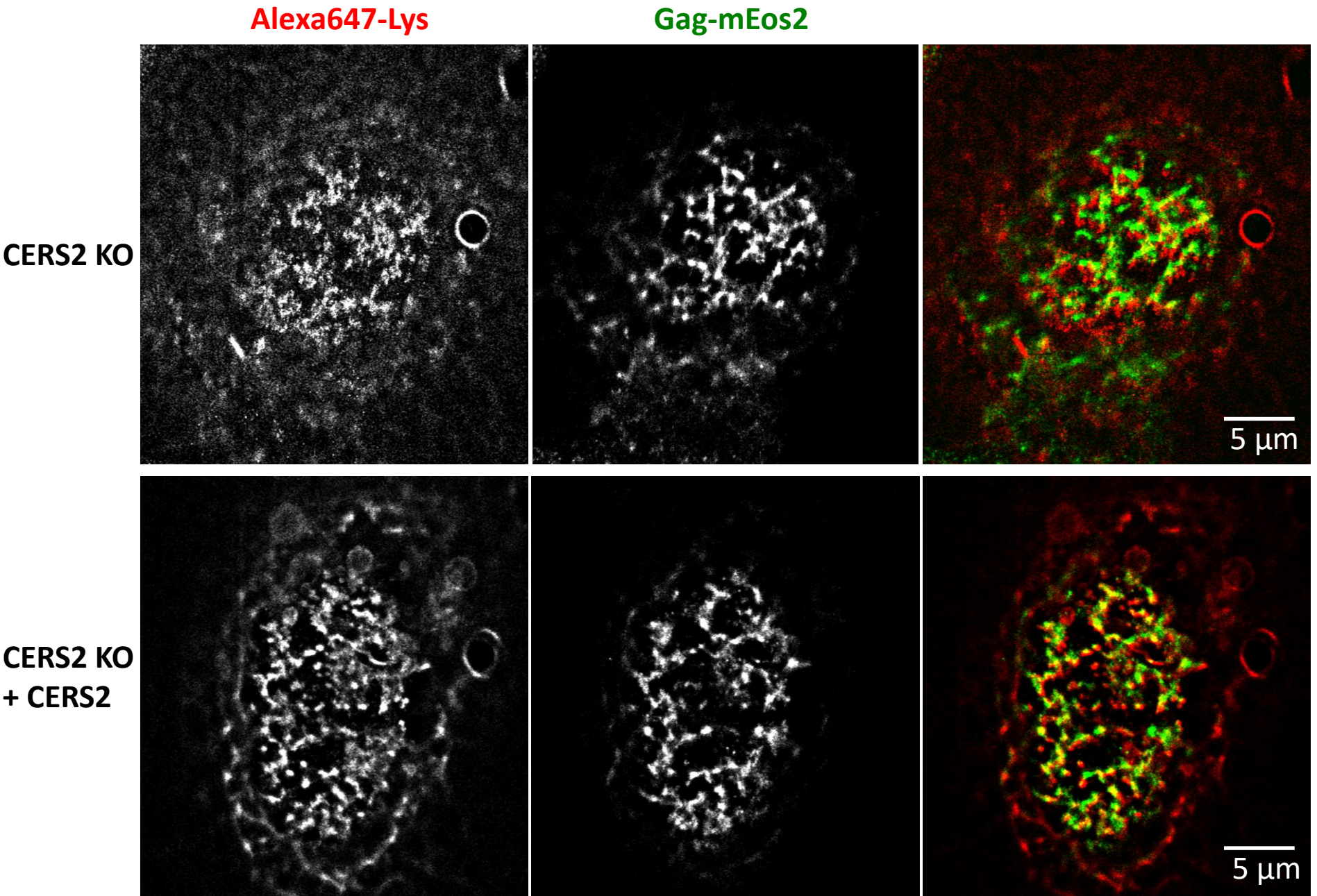


Ceramide synthases (CerS) are the determinant factor of sphingolipid acyl cain length.
(de novo & salvage pathway)

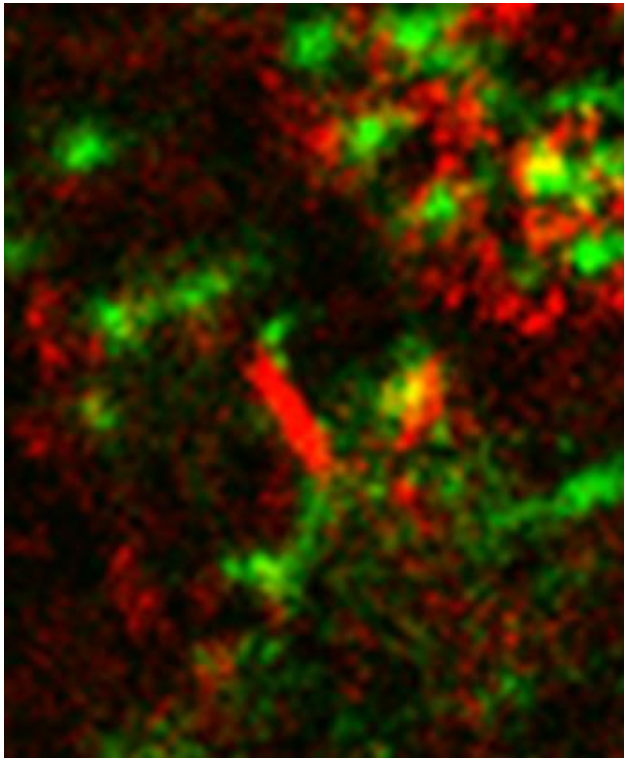
CerS1	C18
CerS2	C22-24
CerS3	C26
CerS4	C18-22
CerS5	C16
CerS6	C14/C16

K. Hanada, K. Kumagai, N. Tomishige, T. Yamaji, *Biochim Biophys Acta* **1791**, 684 (2009).

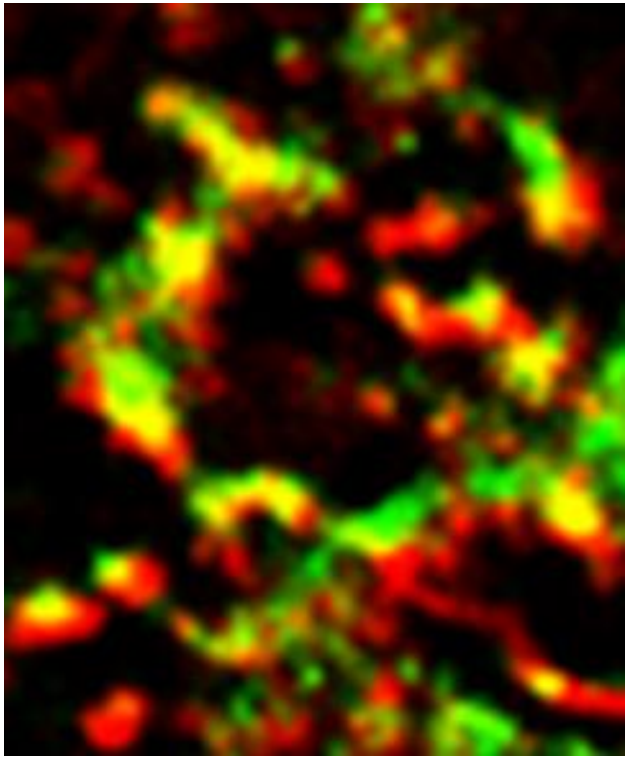
PALM/STORM imaging of Gag and SM in CERS2 KO cells



CERS2 KO



CERS2 KO
+ CERS2



Summary 5

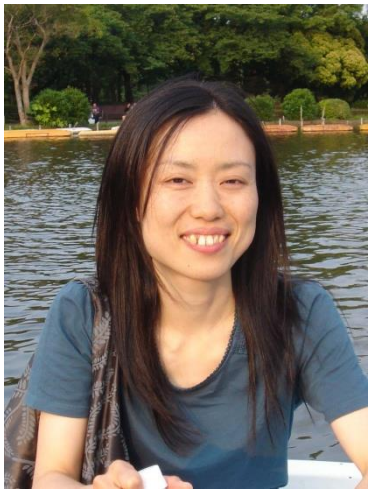
-Outer leaflet sphingomyelin and inner leaflet Gag co-localize on the plasma membrane.

-Expression of Gag alters the lateral diffusion of cell surface SM.

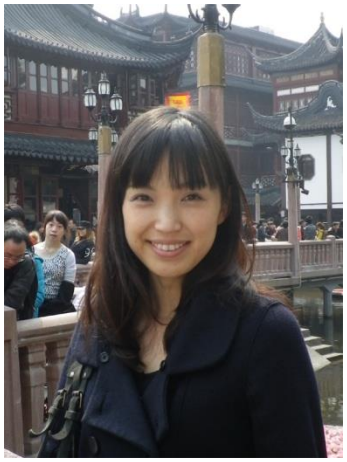
-Co-localization seems to be dependent on the fatty acid length of sphingomyelin.

Tomishige unpublished

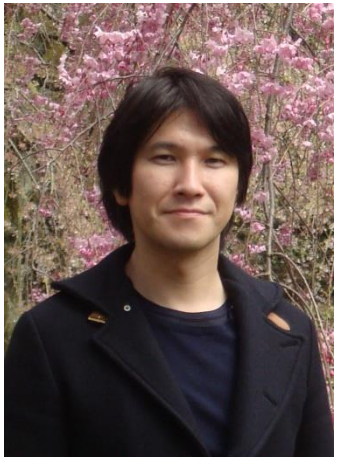
Neval Yilmaz



Reiko Ishitsuka



Asami Makino



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Motohide Murate