Imaging lipids using lipid-binding toxins

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Lipids are not randomly distributed in the cell
Lipids are not randomly distributed in the cell

Sphingolipids
Cholesterol

LBPA (BMP)
Late endosomes

Cardiolipin
Mitochondria
Lipids are not randomly distributed in the cell

- Sphingolipids
- Cholesterol
- Mitochondria
- Sphingomyelin
- Cardiolipin
- LBPA (BMP)
- Late endosomes
- Phosphatidyl-ethanolamine
- Phosphatidylcholine
- Glycolipid
- Phosphatidylserine
- Phosphatidylyethanolamine
Lipid raft hypothesis

Glycolipid

GPI-anchor protein

Sphingomyelin

Cholesterol

Glycerolipids

outside

Plasma membrane

inside

Acylated protein

Lipid raft hypothesis

Proposed function:
Signal transduction
Membrane traffic
Virus and bacteria infection

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$^2$

$= 10^6$ Å$^2$

20,000 lipid molecules in 100 nm$^2$
Difficulty of imaging lipids

1. Small size domains

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

20,000 lipid molecules in 100 nm^2
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.

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

Lysenin, a novel sphingomyelin-specific binding protein
MBP (Maltose-binding protein)-Lysenin specifically recognizes sphingomyelin in ELISA

Kiyokawa 2004
Structure of Membrane Lipids

Phosphatidyl-ethanolamine

Phosphatidyl-choline

Sphingomyelin

Lysobisphosphatidic 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

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Recognition</th>
<th>Toxicity</th>
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<tbody>
<tr>
<td>WT 1-297</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1-246</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1-207</td>
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<tr>
<td>61-297</td>
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<td>61-246</td>
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<td>161-271</td>
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<tr>
<td>161-246</td>
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</tr>
</tbody>
</table>

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
Imaging Lipid Asymmetry

Glycerolipids

Cholesterol

Sphingomyelin

GPI-anchor protein

Glycolipid

Acylated protein

inside

Plasma membrane

outside

Glycerolipids

Sphingomyelin

Cholesterol
Asymmetric distribution of lipids in plasma membrane

Problems of the biochemical method

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

phospholipase

Only outer leaflet lipids are hydrolysed when the membrane is intact

Inner leaflet lipids are hydrolysed when the membrane is broken
SDS-digested Freeze-fracture Replica Labeling (SDS-FRL) method

1. quick freezing

2. fracturing and metal coating

3. digestion by SDS

4. labelling by a specific probe

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.
The images show various clusters with different compositions:

- **PIP₂/PC (0.1:99.9)**
- **PIP₂/PC (1:99)**
- **PIP₂/PE/PS (1:49.5/49.5)**
- **PS/PC (1:99)**

A graph below displays the densities (particles/μm²) of clusters:

- **PC**
- **SM**
- **PE**
- **PS/PI**
- **PIP₂**

The x-axis represents the cluster type, and the y-axis represents the densities. The graph includes error bars indicating variability. The colors blue and red differentiate between clusters in PC/SM and PE/PS, respectively.
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

Textbook

Our result

outer leaflet
inner leaflet
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.
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.
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

Control

+SMase

2 μm
- PIP₂ domains are located to the opposite side of sphingomyelin-rich domains.

- Sphingomyelin domains are required for the formation of PIP₂ domains.
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.

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

+SMase

0 min 180 min

Cell division inhibited

Cell division inhibited

Nuclear division inhibited

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

+SMase +PC

0 min 180 min

+SMase +SM

0 min 180 min

Cells divided

Cell division inhibited

Nuclear division inhibited
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
Sphingomyelinase treatment abolishes the accumulation of PIP\textsubscript{2} to the cleavage furrow.
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.
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 infection.

- 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

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

Lipidomic analyses revealed that HIV-1 viral particle are rich in SM and PIP₂.
**Gag protein in virus formation**

Gag is a 55 kDa multidomain protein involved in virus formation. It interacts with PI(4,5)P2 (= PIP2), cholesterol, and sphingomyelin. During maturation, gag protein interacts with gRNA, MA, CA, NC, and p6 proteins. budding and assembly occur, resulting in the formation of mature, infectious virus particles.
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
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?
SM cluster in proximity of Gag cluster is larger than that without Gag cluster

Gag cluster gathers SM domains?
FRAP (fluorescent recovery after photobleaching)

**Fluorescent intensity**

- 100%
- 0%
- $1/2F_\infty$
- 0

**Time**

- $t = 0$
- $t_{1/2}$

**Fluorescence recovery process**

- Bleached molecule: **out**
- Fluorescent molecule: **in**

**Immobile fraction**

- $F_\infty$

**Mobile fraction**

- = the percentage of molecule moving into bleached area

**Diffusion coefficient ($D$)**

- = status of environment, size of particle, etc
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
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?
- Decrease of sphingomyelin
  - decreases PIP$_2$
  - no change in sphingomyelin

- Crosslinking PIP$_2$
  - merges sphingomyelin domains
Sphingomyelin has unique fatty acid composition

- **Fatty acid composition of SM (HeLa)**

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>% of total SM fatty acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0</td>
<td>50%</td>
</tr>
<tr>
<td>18:0</td>
<td>30%</td>
</tr>
<tr>
<td>22:0</td>
<td>10%</td>
</tr>
<tr>
<td>24:0</td>
<td>5%</td>
</tr>
<tr>
<td>24:1 n-9</td>
<td>5%</td>
</tr>
</tbody>
</table>

- **Fatty acid composition of total lipid (HeLa)**

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>% of total fatty acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>10%</td>
</tr>
<tr>
<td>16:0</td>
<td>30%</td>
</tr>
<tr>
<td>16:1 n-7</td>
<td>10%</td>
</tr>
<tr>
<td>18:0</td>
<td>10%</td>
</tr>
<tr>
<td>18:1 n-9</td>
<td>30%</td>
</tr>
<tr>
<td>18:1 n-7</td>
<td>5%</td>
</tr>
<tr>
<td>18:2 n-6</td>
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<tr>
<td>20:0</td>
<td>5%</td>
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<tr>
<td>20:1 n-9</td>
<td>5%</td>
</tr>
<tr>
<td>20:4 n-6</td>
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</tr>
<tr>
<td>20:5 n-3</td>
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</tr>
<tr>
<td>22:0</td>
<td>10%</td>
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<tr>
<td>22:5 n-3</td>
<td>10%</td>
</tr>
<tr>
<td>22:6 n-3</td>
<td>10%</td>
</tr>
<tr>
<td>24:0</td>
<td>5%</td>
</tr>
<tr>
<td>24:1 n-9</td>
<td>5%</td>
</tr>
</tbody>
</table>

**C24 - Sphingomyelin**

\[(\text{SM})_2 \text{N}^+ \]

**Head group**

**Sphingosine back bone**

**Fatty acid**
Interdigitation of lipids could link inner leaflet lipids to the outer leaflet lipids

Effect of SM chain length on the Gag assembly?
Ceramide synthases (CerS) are the determinant factor of sphingolipid acyl chain length. (de novo & salvage pathway)

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

De novo synthesis pathway of sphingolipids

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

CERS2 KO

Alexa647-Lys  Gag-mEos2

CERS2 KO + CERS2

5 µm  5 µm
- 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
Mitsuhiro Abe
Akiko Yamaji-Hasegawa
Peter Greimei
Nario Tomishige
Motohide Murate