Analysis of receptor oligomerization by FRAP microscopy

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Outline

✓ Introduction to FRAP
✓ Oligomerization and its importance
✓ Target proteins for study
✓ Overall Methodology
✓ Results
FRAP

Principle

A) The bilayer is uniformly labeled with a fluorescent tag

B) This label is selectively photobleached by a small fast light pulse

C) The intensity within this bleached area is monitored as the bleached dye diffuses out and new dye diffuses in

D) Eventually uniform intensity is restored
1. How much light returns relative to the amount of light that was there before the photobleaching? This is the percent recovery.

\[
\frac{Y}{X} \times 100
\]

2. How fast did the fluorescent molecules migrate back into the photobleached area.

This is a measurement of the "diffusional mobility" which is usually called lateral mobility since this experiment is usually done is a planar lipid bilayer.

GPCR Protein

Biogenic amines
- Noradrenaline
- Dopamine
- 5-HT
- Histamine
- Acetylcholine

Amino acids and ions
- Glutamate
- Ca²⁺
- GABA

Lipids
- LPA
- PAF
- Prostaglandins
- Leukotrienes
- Anandamide
- S1P

Peptides and proteins
- Angiotensin
- Bradykinin
- Thrombin
- Bombesin
- FSH
- LH
- TSH
- Endorphins

Others
- Light
- Odorants
- Pheromones
- Nucleotides
- Opiates
- Cannabinoids
- Endorphins

G-protein-independent effect molecules
- Adenylyl cyclases
- Inhibition of cAMP production
- Ion channels
- Phosphodiesterases
- Phospholipases

Orthosteric binding
- α, β, γ
- GTP

G-protein coupled receptors
- PLC-β
- DAG
- Ca²⁺
- PKC
- Adenylyl cyclases
- Increase in cAMP concentration

Others
- RhoGEFs
- Rho

Ion channels
- Pi3Kγ
- PLC-β
- Adenylyl cyclases

Biological responses
- Proliferation
- Differentiation
- Development
- Cell survival
- Angiogenesis
- Hypertrophy
- Cancer

Transcription factors
- Regulation

Gene expression
- Nucleus
Role of GPCR Dimer formation

1. Ontogeny
2. Ligand-promoted regulation
3. Pharmacological diversity
4. Signal transduction
5. Internalization

EMBO reports 5, 1, 30–34 (2004)
### Distribution and Physiologic Effects of Different Adrenergic Receptors

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Receptor Type</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood vessels</td>
<td>$\alpha_1$ and $\alpha_2$</td>
<td>Constriction</td>
</tr>
<tr>
<td></td>
<td>$\beta_2$</td>
<td>Dilatation</td>
</tr>
<tr>
<td>Heart</td>
<td>$\beta_1$</td>
<td>Tachycardia; increased contractility</td>
</tr>
<tr>
<td></td>
<td>$\alpha_1$</td>
<td>Increased contractility</td>
</tr>
<tr>
<td>Bronchi</td>
<td>$\beta_2$</td>
<td>Relaxation</td>
</tr>
<tr>
<td>Thrombocytes</td>
<td>$\alpha_2$</td>
<td>Aggregation</td>
</tr>
<tr>
<td>Kidneys</td>
<td>$\alpha_1$ and $\alpha_2$</td>
<td>Vasoconstriction</td>
</tr>
<tr>
<td></td>
<td>$\beta_1$ and $\beta_2$</td>
<td>Renin release; inhibition tubular sodium reabsorption</td>
</tr>
<tr>
<td>Adipocytes</td>
<td>$\alpha_2$</td>
<td>Inhibition lipolysis</td>
</tr>
<tr>
<td></td>
<td>$\beta_1$, $\beta_2$, and $\beta_3$</td>
<td>Lipolysis</td>
</tr>
</tbody>
</table>
CD 86 and CD28

Nature Reviews Immunology 3, 939-951, 2003
Methodology

Antibody treatment

Cells grown on cover slips

- Polyclonal – goat anti-YFP
- Monoclonal – mouse anti-YFP

For cross linking- goat antimouse Igγ

FRAP Microscopy

- Excitation of CFP and Cerulean: 405 nm diode laser
- Excitation of YFP and Cerulean: 514 nm argon laser

- Emission – 461-488nm
- Emission – 518-600nm

Determination of expression ratios of fluorophores

- CFP and YFP images were collected at fixed parameters
- Analyze the ratio of CFP/YFP in region of interest
- Compared to control cells expressing fusion proteins containing extracellular YFP and intracellular CFP in a single protein
Immobilization of proteins by anti-YFP

Confocal image of HEK293T cells

CD86 cells labeled with YFP and antibody
Polyclonal and Monoclonal Antibodies

Restricted Mobility only after adding secondary Ab

Tag-Protein = Extracellular
Example YFP-CD86, CFP-CD28, YFP-β₁-AR

Protein-Tag = Intracellular
Example CD86-CFP, β₂-AR- Cerulean
Influence of polyclonal anti-YFP - HEK 293 T cells
Intracellular or extracellular tagged CD28 and CD86
Specificity of immobilization

Immobilized using unrelated activin receptor IIb (ARIIb)
Marginal or slight reduction in mobilities
Minor changes with non-interacting proteins
**Validation of dual fluorescence FRAP – CD86 Monomer**

- **CD86 - CFP > YFP - CD86**
- **CFP/YFP concentration = ~1.2**
- **Recovery comparable to cells containing YFP-ARIIB**
Relative fluorescence recovery of CD86

1. Immobilization efficiency
2. Non specific immobilization

Relative expression ratio of CFP and YFP tagged CD86 had no influence on the mobility of intracellular receptors

Confirms with monomeric nature of CD86
Relative fluorescence recovery calculations and Theoretical FRAP

Based the kinetic parameters of theoretical FRAP curves of dimers on the experimentally determined values of CD28 variants

Theoretical extent of recovery of intracellular fluorescent tag by CFP/(CFP+YFP)

For example, at a 1:1 expression ratio of intracellular CFP tagged and extracellular YFP tagged protein

a. 1/4 dimers are YFP-YFP dimers
b. 1/4 dimers are CFP-CFP dimers
c. 2/4 dimers are CFP-YFP heterodimers

Since all dimer combinations containing a protein with an extracellular YFP are immobilized
Only CFP-CFP homodimers are mobile

For CFP recovery = 50% fluorescence from CFP-CFP dimers and 50% from CFP-YFP dimers
Validation of dual florescence FRAP – CD28 Dimer

55.6% of CD28-CFP receptors were mobile over immobilized YFP CD28
82.5% of CD28-CFP expressed in YFP-ARIIB
Relative fluorescence recovery of CD28 dimer

Mobility restriction of CD28

Ratio CFP/YFP

a) 1.4 ~ 61.2% of CD28-CFP
b) 0.4 ~ 39.6% of CD28-CFP

Specific interaction – CD28 monomers

Theoretical calculations for a dimer
Mobile Fractions predicted

a) For 1.4 = 58.3%
b) For 0.4 = 28.6%
**β₁-AR homomeric interactions**

Increase in β₁-AR-CFP
Not much in YFP-β₁-AR
β₂-AR homomeric interactions

Marginal FRAP of both β₂-AR-CFP and YFP-β₂-AR
Relative recovery of $\beta_1$-AR

Immobilization of YFP- $\beta_1$-AR restricted the mobility by 15%
Relative recovery of $\beta_2$-AR

Immobilization of YFP-$\beta_2$-AR restricted $\beta_2$-AR-CER
Oligomerization in native tissue

Mobility of β₂AR Cerulean

Antibody induced immobilization
Stability of receptor complexes

Immobilization of YFP- $\beta_1$-AR had no effect on the extent of immobilization of $\beta_1$-AR-CFP

But the fluorescence recovery time was substantially increased with decreasing CFP/YFP

At CFP/YFP of $\sim 0.3$ $\beta_1$-AR-CFP/ $\beta_1$-AR-CFP homodimer is unlikely

Time constant for $\beta_1$-AR-CFP increased to 155s compared to 22s in cells co-expressing YFP-ARIIB and preincubated with Ab.
Dimerization versus oligomerization of $\beta_2$-AR

Cells expressing a 3.5 fold excess – No detection
78% of $\beta_2$-AR should be mobile – Dimer

The mobility of $\beta_2$-AR restricted

Suggests that one YFP $\beta_2$-AR restrict mobility of 4(3.5+) $\beta_2$-AR CFP
Summary

Predictions about the stability of interactions

Quantify the fraction of interacting proteins

Dimerization and higher order complex formation can be distinguished

$\beta_2$-AR formed higher order oligomers which were relatively stable

$\beta_1$-AR formed unstable dimers
Thank you
Supplementary information
### S F1 e. Mean FRAP of constructs – Influence of polyclonal anti-YFP

<table>
<thead>
<tr>
<th>Construct</th>
<th>Recovery (%) ± s.e.m.</th>
<th>Recovery (%) ± s.e.m.</th>
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</thead>
<tbody>
<tr>
<td>CD86-CFP</td>
<td>86.9 ± 10.2</td>
<td>CD28-CFP</td>
</tr>
<tr>
<td>CD86-YFP</td>
<td>87.6 ± 6.1</td>
<td>CD28-YFP</td>
</tr>
<tr>
<td>YFP-CD86</td>
<td>82.5 ± 5.9</td>
<td>YFP-CD28</td>
</tr>
<tr>
<td>YFP-CD86 and anti-YFP</td>
<td>26.6 ± 3.0</td>
<td>YFP-CD28 and anti-YFP</td>
</tr>
<tr>
<td>CD86-CFP and anti-YFP</td>
<td>84.0 ± 3.6</td>
<td>CD28-CFP and anti-YFP</td>
</tr>
<tr>
<td>CD86-YFP and anti-YFP</td>
<td>85.3 ± 4.0</td>
<td>CD28-YFP and anti-YFP</td>
</tr>
</tbody>
</table>
S F2. Influence of antibody binding to extracellularly YFP labelled proteins on the mobility of unrelated proteins.
S F2. Influence of antibody binding to extracellularly YFP labelled proteins on the mobility of unrelated proteins – Unspecific immobilization

\[
\begin{array}{|c|c|c|}
\hline
\text{Sample} & \text{Recovery} & \text{[\%]} \\
\hline
\text{ARIIB-CFP and YFP-CD86, } n = 23 & 84.4 \pm 1.5 & 13 \\
\text{ARIIB-CFP and YFP-CD86 and anti-YFP, } n = 44 & 73.4 \pm 1.3 & \\
\text{ARIIB-CFP and YFP-CD28, } n = 11 & 85.2 \pm 3.0 & 13 \\
\text{ARIIB-CFP and YFP-CD28 and anti-YFP, } n = 20 & 74.5 \pm 1.3 & \\
\text{CD86-CFP and YFP-\(\beta_1\), } n = 22 & 89.9 \pm 2.1 & 16 \\
\text{CD86-CFP and YFP-\(\beta_1\) and anti-YFP, } n = 41 & 75.4 \pm 2.0 & \\
\text{CD86-CFP and YFP-\(\beta_2\), } n = 24 & 89.3 \pm 2.1 & 18 \\
\text{CD86-CFP and YFP-\(\beta_2\) and anti-YFP, } n = 21 & 73.2 \pm 3.2 & \\
\hline
\end{array}
\]
**S F3.** Immunoblot of intra and extracellularly tagged CD28 fusion proteins

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>5</th>
<th>3</th>
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<tbody>
<tr>
<td>55 kDa</td>
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<td>130 kDa</td>
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<td>250 kDa</td>
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Non-reducing

<table>
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<td>-130 kDa</td>
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<td>-250 kDa</td>
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</table>

Reducing
S F4. Functional and expression properties of β-AR fusion compared to β WT

### a

<table>
<thead>
<tr>
<th>DNA</th>
<th>EC$_{50}$ ± s.e.m. [nM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 HEK TSA-WT</td>
<td>118.2 ± 39.3</td>
</tr>
<tr>
<td>2 β₁-WT</td>
<td>22.4 ± 4.9</td>
</tr>
<tr>
<td>3 β₁-CFP</td>
<td>7.5 ± 2.6</td>
</tr>
<tr>
<td>4 YFP-β₁</td>
<td>8.9 ± 3.6</td>
</tr>
<tr>
<td>5 β₂-WT</td>
<td>37.2 ± 13.3</td>
</tr>
<tr>
<td>6 β₂-CER</td>
<td>4.9 ± 2.3</td>
</tr>
<tr>
<td>7 YFP-β₂</td>
<td>5.6 ± 1.4</td>
</tr>
<tr>
<td>8 YFP-β₂</td>
<td>8.1 ± 3.8</td>
</tr>
</tbody>
</table>

### b

![Graph showing max stimulation counts for different DNA constructs.]

### c

Expression levels of indicated cDNA-amounts of β-AR encoding constructs in fmol/μg total protein ± s.e.m.

<table>
<thead>
<tr>
<th>DNA</th>
<th>0.25 μg</th>
<th>0.5 μg</th>
<th>0.75 μg</th>
<th>1.0 μg</th>
</tr>
</thead>
<tbody>
<tr>
<td>β₁-WT</td>
<td>3.2 ± 1.6</td>
<td>5.5 ± 3.2</td>
<td>5.8 ± 2.9</td>
<td></td>
</tr>
<tr>
<td>β₁-CFP</td>
<td>6.1 ± 1.5</td>
<td>5.9 ± 1.8</td>
<td>6.6 ± 0.9</td>
<td></td>
</tr>
<tr>
<td>YFP-β₁</td>
<td>3.4 ± 1.9</td>
<td>4.1 ± 2.0</td>
<td>5.0 ± 2.5</td>
<td></td>
</tr>
<tr>
<td>β₂-WT</td>
<td>0.3 ± 0.1</td>
<td>0.8 ± 0.5</td>
<td>1.3 ± 0.9</td>
<td>1.6 ± 0.4</td>
</tr>
<tr>
<td>β₂-CER</td>
<td>1.3 ± 0.3</td>
<td>1.8 ± 0.8</td>
<td>1.6 ± 0.9</td>
<td>2.2 ± 0.5</td>
</tr>
<tr>
<td>β₂-CFP</td>
<td>0.8 ± 0.5</td>
<td>1.1 ± 0.9</td>
<td>1.7 ± 0.8</td>
<td>1.8 ± 0.7</td>
</tr>
<tr>
<td>YFP-β₂</td>
<td>3.1 ± 1.2</td>
<td>3.3 ± 1.5</td>
<td>1.9 ± 0.1</td>
<td>3.1 ± 0.8</td>
</tr>
<tr>
<td>endogenous receptor (HEK TSA-WT cells)</td>
<td>0.01 ± 0.008</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
S F5. Influence of anti-YFP treatment on HEK 293T cells either expressing intracellular or extracellular fl tagged β₁-AR or β₂-AR.
S F5. Influence of anti-YFP treatment on HEK 293T cells either expressing intracellular or extracellular fl tagged β₁-AR or β₂-AR
S F5. Influence of anti-YFP treatment on HEK 293T cells either expressing intracellular or extracellular fl tagged β₁-AR or β₂-AR
**S F5. Influence of higher temperature and of exchange of fluorophores on $\beta_2$-AR**

<table>
<thead>
<tr>
<th></th>
<th>Recovery (%) ± s.e.m.</th>
<th></th>
<th>Recovery (%) ± s.e.m.</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\beta_1$-CFP</td>
<td>78.9 ± 8.0</td>
<td>$\beta_2$-CFP</td>
<td>80.8 ± 12.3</td>
</tr>
<tr>
<td>YFP-$\beta_1$</td>
<td>73.7 ± 6.2</td>
<td>$\beta_2$-CER</td>
<td>75.3 ± 6.4</td>
</tr>
<tr>
<td>YFP-$\beta_1$ and anti-YFP</td>
<td>21.6 ± 11.9</td>
<td>YFP-$\beta_2$</td>
<td>76.5 ± 3.9</td>
</tr>
<tr>
<td>$\beta_1$-CFP *</td>
<td>79.3 ± 8.4</td>
<td>$\beta_2$-CER *</td>
<td>80.6 ± 11.4</td>
</tr>
<tr>
<td>$\beta_1$-CFP and anti-YFP *</td>
<td>84.0 ± 7.1</td>
<td>$\beta_2$-CER and anti-YFP *</td>
<td>73.6 ± 2.9</td>
</tr>
<tr>
<td>$\beta_1$-YFP **</td>
<td>82.3 ± 8.7</td>
<td>$\beta_2$-YFP **</td>
<td>79.3 ± 8.4</td>
</tr>
<tr>
<td>$\beta_1$-YFP and anti-YFP **</td>
<td>75.1 ± 4.0</td>
<td>$\beta_2$-YFP and anti-YFP **</td>
<td>76.4 ± 1.1</td>
</tr>
</tbody>
</table>

*, ** measurement of same cells prior and after treatment with anti-YFP
S F6. Influence of anti-YFP treatment on HEK 293T cells either expressing intracellular or extracellular fltagged β₁-AR or β₂-AR
S F7. Data analysis of FRAP measurement

(a) Relative intensity over time for different samples: A, whole cell; B, ROI; C, ROI_corrected.

(b) Recovery (%) over time for immobile and mobile fractions.

(c) Recovery (%) over time for different samples: M, I, E.

(d) Relative recovery over time for M - E and I - E.