Multidimensional Tracking of GPCR Signaling with Proximity-Labeling

Technical Journal Club
Anna Henzi, 11.07.2017
Proximity-dependent labeling methods

- Enzymes create radicals
  - Proximity-dependent biotin identification (BioID)
  - Horseradish peroxidase (HRP)
  - Ascorbate peroxidase (APEX)
- covalently tag neighbouring proteins with biotin
- identify labeled proteins by mass spectrometry (MS)
- Cells remain intact for labeling

Enzymes for proximity labeling

- Horseradish peroxidase (HRP)
- Ascorbate peroxidase (APEX)
- Proximity-dependent biotin identification (BioID)

## Enzymes for proximity labeling

### TABLE 1 | Comparison of the Different Proximity Labeling Methods

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>BioID</th>
<th>HRP</th>
<th>APEX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzymatic activity</td>
<td>Biotin ligase based</td>
<td>Peroxidase based</td>
<td>Peroxidase based</td>
</tr>
<tr>
<td>Labeling target</td>
<td>Lysine</td>
<td>Electron-rich amino acids</td>
<td>Tyrosine and potentially other electron-rich amino acids</td>
</tr>
<tr>
<td>Size (kD)</td>
<td>35</td>
<td>44</td>
<td>27</td>
</tr>
<tr>
<td>Labeling time</td>
<td>15–24 h</td>
<td>5–10 min</td>
<td>1 min</td>
</tr>
<tr>
<td>Incubation time with substrate</td>
<td>15–24 h</td>
<td>5–10 min</td>
<td>30–60 min</td>
</tr>
<tr>
<td>Activation by H₂O₂</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Substrates for protein labeling</td>
<td>Biotin</td>
<td>Biotin-phenol (and biotin- or fluorescein-acylazide)</td>
<td>Biotin-phenol</td>
</tr>
<tr>
<td>Half-life of generated radicals</td>
<td>Mins</td>
<td>&lt;1 ms</td>
<td>&lt;1 ms</td>
</tr>
<tr>
<td>Active region</td>
<td>Intracellular</td>
<td>Extracellular, secretory pathway (inactive in cytosol)</td>
<td>Intracellular</td>
</tr>
<tr>
<td>Available variants</td>
<td>BioID2 (27 kD)</td>
<td>Split HRP</td>
<td>APEX2</td>
</tr>
<tr>
<td>Note</td>
<td>Reduced activity below 37 degrees</td>
<td>Can be used as an EM tag; HRP-conjugated antibodies available</td>
<td>Can be used as an EM tag</td>
</tr>
<tr>
<td>Organisms</td>
<td>Mammalian cells, xenograft tumors in mice, <em>Trypanosoma brucei</em>, <em>Toxoplasma gondii</em>, <em>Dictyostelium discoideum</em>, <em>Plasmodium berghei</em></td>
<td>Mammalian cells</td>
<td>Mammalian cells, <em>Schizosaccharomyces pombe</em>, <em>Saccharomyces cerevisiae</em>, <em>Drosophila melanogaster</em></td>
</tr>
</tbody>
</table>

APEX, engineered ascorbate peroxidase; BioID, proximity-dependent biotin identification; EM, electron microscopy; HRP, horseradish peroxidase.  
Chen, 2017
SILAC (Stable Isotope Labeling by Amino Acids in Cell Culture)

- Approach for expression proteomics: simultaneous identification and quantitation of protein mixtures
- Non-radioactive isotopically labelled amino acids in cell culture medium

Shao-En Ong et al. Mol Cell Proteomics 2002;1:376-386
Proteomic Mapping of Mitochondria

- 94% with mitochondrial annotation
- Coverage 80-90%
- 31 orphans
Paek et al, 2017

**GPCR**

- Many GPCR targeted drugs
- Many aspects of signalling incompletely characterized

**GPCR signaling assays: limitations**

- Single pathway readout
- Limited time resolution
- Overexpression or modification of effectors
Multidimensional Tracking of GPCR Signaling via Peroxidase-Catalyzed Proximity Labeling

**GPCR-APEX**

Peroxidase-based proximity labelling
Isobaric tagging and MS

- Quantitative, time-resolved measurement of GPCR agonist response
- Parallel quantification of all interacting effectors
- Native cellular environment, endogenous expression level

**Graphical Abstract**

![Multidimensional signaling and internalization kinetics](image-url)
Multidimensional Tracking of GPCR Signaling via Peroxidase-Catalyzed Proximity Labeling

APEX2
- Biotin-phenoxy radicals (< 1ms)
- Labeling radius 20nm

Angiotensin II type 1 receptor (AT1R)
Multidimensional Tracking of GPCR Signaling via Peroxidase-Catalyzed Proximity Labeling

Proximity-labeling experiment

Biological replicates

Biotin-phenol
1 hr before quenching

Ang II
20 min

Ang II
1 min

Losartan
20 min

Losartan
1 min

No ligand

No ligand

No H₂O₂

H₂O₂
1 min

Quench & freeze

Proteomic analysis

Frozen cell pellet

Lysis & streptavidin pull-down

Tryptic peptides

Isobaric tag labeling

Labeled peptides

MS¹

MS²

MS³
Multidimensional Tracking of GPCR Signaling via Peroxidase-Catalyzed Proximity Labeling

Western blot

Quantification by MS

A

<table>
<thead>
<tr>
<th>AngII 21 min</th>
<th>AngII 2 min</th>
<th>Los 21 min</th>
<th>Los 2 min</th>
<th>/No H2O2</th>
<th>/No ligand</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
</tbody>
</table>

Streptavidin-HRP blot

Ponceau staining

B

Relative protein abundance (replicate 2, arbitrary units) vs. Relative protein abundance (replicate 1, arbitrary units)

R² = 0.99
Multidimensional Tracking of GPCR Signaling via Peroxidase-Catalyzed Proximity Labeling

Characterize local milieu of AT1R

Enrichment of G-proteins

No difference for antagonist treatment (losartan)

Differential enrichment of endocytosis-related proteins after agonist treatment (ATII)
Multidimensional Tracking of GPCR Signaling via Peroxidase-Catalyzed Proximity Labeling

Time resolution

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**Figure B**

![Graph showing % Max. enrichment over Time (s)]

**Figure C**

Matrix showing % Max. enrichment over Time (s) for various proteins.

**Figure D**

Graph showing Fold-enrichment (normalized to 0 s) over Time (s) for different Gα subunits.

**Figure E**

Matrix showing % Max. enrichment over Time (s) for proteins TFRC, VPS29, SNX1.
Multidimensional Tracking of GPCR Signaling via Peroxidase-Catalyzed Proximity Labeling

biased agonist TRV027
Multidimensional Tracking of GPCR Signaling via Peroxidase-Catalyzed Proximity Labeling

Generalizability?

β2 adrenergic receptor (β2AR)

Agonist: BI167107
Multidimensional Tracking of GPCR Signaling via Peroxidase-Catalyzed Proximity Labeling

Potential to discover novel mediators of GPCR signalling: LMBRD2
Multidimensional Tracking of GPCR Signaling via Peroxidase-Catalyzed Proximity Labeling

**Summary**

- Local milieu of GPCR in ligand-free state
- G proteins separated from activated receptor during internalization, but not adenylyl cyclase 3 (B2AR)
- Differences in internalization kinetics
- Bystander proteins
An Approach to Spatiotemporally Resolve Protein Interaction Networks in Living Cells

Lobingier et al, 2017

Simultaneously capture spatial and temporal context

High background by APEX proximity labelling

> deconvolve: **timing, location and interactions**
Spatiotemporally Resolve Protein Interaction Networks in Living Cells

GPCR Protein Network: B2AR
Spatiotemporally Resolve Protein Interaction Networks in Living Cells

GPCR Protein Network: B2AR

**E**

<table>
<thead>
<tr>
<th>Time with agonist (min)</th>
<th>B2AR-APEX2</th>
<th>Pulldown</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Arrestin3-GFP</td>
<td>Clathrin (CLTC)</td>
</tr>
<tr>
<td>1</td>
<td>Arrestin3-GFP</td>
<td>Retomer (VPS35)</td>
</tr>
<tr>
<td>2</td>
<td>Arrestin3-GFP</td>
<td>GAPDH</td>
</tr>
<tr>
<td>3</td>
<td>Lysate</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**F**

![Graph showing protein intensity over time](image)

- **Arrestin3**
- **CLTL** (clathrin heavy chain)
- **VPS35** (retromer complex)
Spatiotemporally Resolve Protein Interaction Networks in Living Cells

“bystanders” as compartmental markers
- Plasma membrane: radixin (RDX), occluding (OCLN)
- Endosomes: early endosomal antigen 1 (EEA1), VTI1B
Spatiotemporally Resolve Protein Interaction Networks in Living Cells

Generate spatial APEX references

APEX profiles from same subcellular compartment quantitatively similar (bystander), but differ for interacting proteins
Spatiotemporally Resolve Protein Interaction Networks in Living Cells

Identify unknown interacting proteins?

delta-opioid receptor (DOR): mechanisms of downregulation
Spatiotemporally Resolve Protein Interaction Networks in Living Cells

Identify unknown interacting proteins?

delta-opioid receptor (DOR): mechanisms of downregulation
Spatiotemporally Resolve Protein Interaction Networks in Living Cells

**Figure A:**
- **Control** 0, 6
- **WWP2 siRNA** 0, 6
- **10 μM DADLE (hr)**
- FLAG (DOR)
- WWP2
- Loading Control (CLTC)

**Figure B:**
- **Control** 0, 6
- **TOM1 siRNA** 0, 6
- **10 μM DADLE (hr)**
- FLAG (DOR)
- TOM1
- Loading Control (CLTC)

**Figure C:**
- Graph showing percent DOR remaining over time with siRNA control and WWP2 siRNA.

**Figure D:**
- Graph showing percent DOR remaining over time with siRNA control and TOM1 siRNA.
Spatiotemporally Resolve Protein Interaction Networks in Living Cells

• Allows resolving of protein networks according to locating and timing
• Established using GPCR, validated with capture of known binding partners
• Discovery of new components
Proximity labeling

Advantages
- No need for organelle purification
- Labeling in intact cells > preserve weak/transient interactions, reduce false positives
- Labeling in living cells
- Proteomic analysis of other subcellular regions

Limitations
- Limitations for labelling
- Cannot distinguish direct binding vs proximity
- Further fine-tuning by split APEX, faster kinetics