Molecular Nanoprobes for Multiphotonics as New tools for Bioimaging

2nd Annual Symposium on Integrating Nanotechnology with Cell Biology and Neuroscience
The University of New Mexico, August 22nd 2008
UNM ICNCBN-IGERT
Multiphotonics: Two-photon absorption (TPA) ⇒ Two-photon excited-fluorescence (TPEF)

Fluorescence excited by:

- 1 photon
- 2 photons

Two-Photon Excited Fluorescence (TPEF or 2PEF)

⇒ Advantages in biological imaging:

- intrinsic 3-D resolution
- increased penetration in tissues
- reduced photodamage
- reduced background fluorescence
Two-photon excited fluorescence

\[ \propto \sigma_2 \Phi I^2 \]

- pulsed lasers + focusing

\( \Phi \): fluorescence quantum yield

\( \sigma_2 \): two-photon absorption cross section

(in GM = 10^{-50} \text{ cm}^4 \cdot \text{s. photon}^{-1})

\( \sigma_2 \Phi \): TPEF cross-section (in GM)

\[ \Rightarrow \text{molecular engineering of fluorophores with high } \sigma_2 \text{ in the biological spectral window (700-1200nm)} \]
Fluorescent Markers and Probes for 2PEF imaging

- **Endogenous biological chromophores:**
  
  NADH, riboflavins, retinol : \(10^{-5} < \sigma_2 \cdot \Phi < 1 \text{ GM}\)

- **Classical one-photon fluorophores:**
  
  DAPI : \(\sigma_2 \cdot \Phi < 1 \text{ GM}\)
  
  Coumarin 307, Bodipy : \(\sigma_2 \cdot \Phi < 20 \text{ GM}\)
  
  Fluorescein : \(\sigma_2 \cdot \Phi < 40 \text{ GM}\)
MOLECULAR ENGINEERING FOR MULTIPHOTONIC BIOIMAGING:

examples and applications

What is needed?

• high fluorescence quantum yield
• very large TPA cross-sections in the target spectral range
• without residual one-photon absorption

⇒ 3D imaging
⇒ enhanced sensitivity
⇒ reduced photodamage
⇒ selective photo-addressing
Molecular engineering of quadrupoles

- Increase length
- Adjust $\theta$
- Type of spacer
- D/A strength

- TPA enhancement
- Spectral tuning
- (photo)stability

Rod-like and banana-shaped quadrupolar fluorophores

Measurement of two-photon absorption cross sections by TPEF

\[
\frac{\left( \sigma_2 \Phi \right)}{\left( \sigma_2 \Phi \right)_R} = \frac{\eta_{\text{spectral},R}}{\eta_{\text{spectral}}} \frac{n^2}{n_R^2} \frac{C_R}{C} \frac{n_R}{n} \left( \frac{F}{P^2} \right) \left( \frac{F}{P^2} \right)_R^{-1}
\]

Molecular optimization of quadrupolar fluorophores

<table>
<thead>
<tr>
<th>Fluorophore</th>
<th>(\lambda_{\text{abs}}^{\text{max}}) (nm)</th>
<th>(\Phi) ((\tau)) (ns)</th>
<th>(\sigma_{2}^{*}) (GM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hex(_2)N</td>
<td>431</td>
<td>0.85</td>
<td>2115</td>
</tr>
<tr>
<td>Bu(_2)N</td>
<td>429</td>
<td>0.78</td>
<td>3470</td>
</tr>
<tr>
<td>Oct(_2)N</td>
<td>470</td>
<td>0.47</td>
<td>5480</td>
</tr>
</tbody>
</table>

*at 705 nm (toluene)

major TPA amplification in the NIR fluorescence is maintained

From model lipidic membranes.....

TPEF cross-sectional image of a GUV labeled with BAQ1. The giant unilamellar vesicles (GUV) were prepared from 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC).

Angew. Chem., Int. Ed. 2001, 40, 2098
APPLICATION: 3-D BIOIMAGING

To non-damaging cell imaging

TPEF image of LLC-PK1 cells labeled with BAQ1, (excitation at 740 nm with less than 1mW excitation power)

FROM IMAGING TO SENSING
Towards Medium Responsive Two-Photon Nanoprobes

⇒ sensitive two-photon pH probes in the NIR

⇒ sensitive two-photon micropolarity probes

⇒ fast voltage probes
Recent examples of TP probes

Metal-ion sensor 
(Mg$^{2+}$)

J. W. Perry and coworkers, JACS, 2004, 12, 9291-9306

Metal-ion sensor 
(Ca$^{2+}$, Ba$^{2+}$, Mg$^{2+}$, Na$^{+}$, K$^{+}$)

B. R. Cho and coworkers, JOC, 2004, 5749-5751

$H^+$ sensors ⇒ 2-photon pH probes?
TP probes for metal-ions have been recently synthesized.
This fluorophore with crown ether is a sensor for Mg2+.
This another one is based on the same design and can sense different ions :.....
At last, this quadrupole is sensitive to the pH of solution.
SMART TWO-PHOTON FLUOROPHORES AS SENSITIVE pH PROBES IN THE NIR

**Graphical Abstract**

- **Low TPA**
  - Chemical structure: [Image]
  - Description: 
    - Reaction: \( + \text{H}^+ \rightarrow - \text{H}^+ \)
    - Low TPA

- **High TPA**
  - Chemical structure: [Image]
  - Description: 
    - Reaction: \( + \text{H}^+ \rightarrow - \text{H}^+ \)
    - High TPA

**References**

Two-photon pH sensing at membrane interface: two-photon microspectroscopy

2 photon excitation @780 nm

2PEF emission when the membrane is stained under neutral and basic conditions
Towards Medium Responsive Two-Photon Nanoprobes

Towards sensitive two-photon pH probes in the NIR

Towards sensitive two-photon micropolarity probes

Towards fast voltage sensitive probe
Incoherent processes

TPEF

Visualization localization

\( \sigma_2 \)

Coherent processes

Second Harmonic Generation : SHG

Local order Assymetry

Molecular engineering of NLO molecular probes

\( \sigma_{\text{SHG}} \)

Multiphotonics : Second harmonic generation (SHG)
SHG requires asymmetric source

Amphiphilic Push-pull Chromophores

⇒ objectives: from static imaging of cells to dynamic imaging of membrane processes
molecular engineering of chromophores for combined TPEF and SHG imaging of membrane dynamics

\[ \Rightarrow \text{Amphiphilic Polyenic Push-pull Chromophores (APPC)} \]

\[ n \uparrow \Rightarrow \sigma_{\text{SHG}} \uparrow \quad (\text{Chem. Comm., 2000, 353}) \]

\[ \sigma_2 \uparrow \text{in the NIR (Optics Lett., 2000, 25, 3220)} \]
Wavelength (nm)

- 400
- 450
- 500
- 550
- 600
- 650

Power (a.u.)

- 0.0
- 0.5
- 1.0

SHG Power (a.u.)

- 0.0
- 0.5
- 1.0

SHG 2PEF

 fluorescense

Power (a.u.)

- 0.0
- 0.5
- 1.0

Wavelength (nm)

- 400
- 450
- 500
- 550
- 600
- 650
Simultaneous TPEF and SHG cross-sectional images of isolated Ncad1 cells labeled with an amphiphilic push-pull polyenic chromophore. Internalized dye molecules become randomly oriented in the cytoplasm and generate no SHG, whereas they continue to generate fluorescence.

APPLICATIONS: NON-LINEAR IMAGING OF “FLIP-FLOP” DYNAMICS

Biophys.J. 2001, 80, 1568.
Measurement of intermembrane distance at subwavelength resolution

SHG produced by two labeled GUV membranes. When they are separated by 0.6 times the excitation beam waist ($w_0$), SHG constructively interferes resulting in “hot spots”.
SHG for membrane voltage sensing?

(Lewis, Loew, 1993)
Voltage sensors?

Membrane ~ 50-100 Å
Potential 100 mV

Membrane potential $10^5$ V/cm

SHG probes for imaging of neuronal activity

Imaging ~400 µm deep into intact neurons
⇒ No photodamage

Optically recording of action potentials
⇒ linear dependence of SHG on E
⇒ 0.833 ms temporal resolution
⇒ 0.6 µm spatial resolution

Collab. W. Webb

J. Neuroscience, 2004, 24, 999
**Soft substitutes for semiconductor QD's?**

quantum dots (QDs) ⇒ bright nanoobjects
⇒ tuneability, photostability
⇒ very large one ($\varepsilon \Phi$) and two-photon ($\sigma_2 \Phi$) brilliance*

Soft substitutes for semiconductor QD's?

quantum dots (QDs) ⇒ bright nanoobjects
⇒ tuneability, photostability
⇒ very large one (ε Φ) and two-photon
    (σ₂Φ) brilliance*

but toxicity, clearance, degradation ?…

Soft All-Organic Alternative to QD's?
• biocompatibility / degradability
• environmental friendly
An "organic" alternative? : modular route towards organic nanodots:

⇒ optimized fluorophore

⇒ phosphorous-based dendrimers

JP Majoral (Toulouse)
Nanodots: Modular approach

Controlled Grafting of Optimized Fluorophores on a branched (dendritic) platform

⇒ nano-object of controlled size, geometry, number of fluorophores.

Organic nanodots: a valid strategy towards bright nano-objects

<table>
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<tr>
<th>$n_F$</th>
<th>$\varepsilon$ (M$^{-1}$ cm$^{-1}$)</th>
<th>$\Phi$</th>
<th>huge extinction coefficients</th>
<th>fluorescence is maintained</th>
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<tr>
<td>1</td>
<td>1</td>
<td>85 000</td>
<td>0.8</td>
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</tr>
<tr>
<td>G1</td>
<td>12</td>
<td>1 000 000</td>
<td>0.75</td>
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<tr>
<td>G2</td>
<td>24</td>
<td>2 000 000</td>
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<td>G3</td>
<td>48</td>
<td>3 800 000</td>
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<tr>
<td>G4</td>
<td>96</td>
<td>7 100 000</td>
<td>0.5</td>
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</table>
Organic nanodots: a valid strategy towards bright nano-objects

⇒ "super" bright nano-objects: record brilliance: $\varepsilon \cdot \Phi$

- $\Phi_F > 50\%$ (QD 30-50\%)
- $\varepsilon \rightarrow 7\,000\,000\,M^{-1}\,cm^{-1}$

PCT Int. Appl. 2007, WO 2007080176
Fluorescent nanodots with giant TPA cross-sections.

⇒ "super" bright nano-objects:
record two-photon brilliance: \( \sigma_2 \Phi \)
\( \sigma_2 \rightarrow 60\,000\,\text{GM} \) (QD 700-10\,000 GM)*

* Lin, Chou *Small*, 2006, 2, 1308.
**Nanodots Two-Photon Brilliance as compared to QD’s**

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<thead>
<tr>
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<td>2.4</td>
<td>508</td>
<td>2.44</td>
<td>0.192</td>
<td>75</td>
<td>390</td>
<td>150</td>
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<td>2710</td>
<td>6190</td>
<td>4530</td>
<td>10300</td>
</tr>
</tbody>
</table>

Lin, Chou *Small*, 2006, 2, 1308-1313
biocompatible biphotonic contrast agents for *in vivo* imaging

Two-photon imaging of the vascular network in the dorsal part of the rat olfactory bulb. Vessels were labeled after injecting intravenously a small bolus of 500 µM G2 nanodots in water. The image was taken at ~200 µm depth. No obvious toxic effects were observed during the experiment.


Collab. S. Charpak, L. Moreaux (INSERM, Paris Descartes)

\[ \sigma_2 = 130 \text{ GM} \]

\[ \lambda_{em} = 440 \text{ nm} \]
2-photon in vivo small animal imaging

Xenopus laevis (stage 53)
Gaëlle Recher, François Tiaho (Rennes)

\[ \sigma_2 = 1000 \text{ GM} \]
\[ \lambda_{em} = 530 \text{ nm} \]

Nandots: versatile nano-objects

- **Fluorescence Tuning:**
  quantum dots: size

- **Shape Modulation:**

  *nanodots*: fluorophore

- **Modular approach**
  ⇒ Water solubility:

- **Surface functionalization**
  ⇒ targeting


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