High throughput, high content cytometry from pathophysiology to pharmacology

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Outline

• Tackling pathophysiology using multiphoton image cytometry
  – Cancinogenesis
  – Cardiac hypertrophy
  – Liver fibrosis

• Higher throughput, higher content
  – Spatial-temporal coherent control, aka temporal focusing
  – Higher content: Improving lifetime imaging
  – Higher speed: New ideas in structured light and 3D imaging

• System biology and pharmaceutical discovery
  – Introduction to system biology & image informatics
  – Pharmaceutical discovery via tissue cytometry: fibrogenesis
From the last episode
Recall the problem of studying carcinogenesis due to recombination.

- Spontaneous mutation is a primary cause of cancer.
- A rapid mutation assay will allow the quantification of the toxicity of new drugs and chemicals.

Recombination rate is 1 in $10^6$ to $10^5$. It is a “needles in a haystack problem.”

Hendricks et al., PNAS 2003
Young Mouse Pancreas Wide-field Imaging
Wide field image is not quantitative in deep tissue

Wikor-Brown, PNAS, 2008,
Kwon, Roy Soc Interface, 2009
3D cell cluster image reveal clonal ex

Distribution of number of cells in each foci

4 weeks vs 70+ weeks old
Recombination still occurs at old age (same rate as animal age)
Majority of recombinant cells are originated from clonal expansion (over 90%)
Identification of stem cells?

Wikor-Brown, PNAS, 2008,
Kwon, Roy Soc Interface, 2009
Genetic Basis for Cardiac hypertrophy

Calcineurin transgenic  Wildtype

Fiber area

http://www.cincinnatichildrens.org

2-D cross section of myocytes

3-D reconstructed myocyte morphology
Multi-Scale Functional modeling of Whole Mouse Heart

Genetic changes $\rightarrow$ Architectural changes $\rightarrow$ Functional biomechanical changes

Ragan et al., JBO, 2007; Huang, JBO 2009
Effect of Desmin Mutation in Mouse Heart

Huang, JBO 2009
Multimodal study of cardiac hypertrophy due to myosin binding protein-C mutation

DSI: MRI tractography
TPM: two-photon microscopy

Fibro-C, a new in vivo index for liver fibrogenesis after bile duct ligation: a path toward less invasive fibrosis staging

Tai et al., Journal of Biomedical Optics, 2009
Improving the clinical utility of fibrosis assay using liver surface imaging

Ting et al., Journal of Biomedical Optics, 2010
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Parallelization is the key to high throughput AND high content biophotonics

Calcium wave propagating through a cardiac myocyte labeled with flo3

800 images at 640 frames/sec : 1.25 sec sequence

Bahlmann et al., OE, 2007
## Ideas and history of parallelizing multiphoton microscopy

<table>
<thead>
<tr>
<th></th>
<th>Single Focus (n=1)</th>
<th>Multiple Foci (n=N×N)</th>
<th>Wide-Field (n=∞×∞)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Illumination</strong></td>
<td><img src="image1" alt="Single focus" /></td>
<td><img src="image2" alt="Multiple foci" /></td>
<td><img src="image3" alt="Wide-field" /></td>
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<tr>
<td>spot shape</td>
<td>Simple spatial focusing</td>
<td>Lenslet array, beam splitter and diffractive optical element with spatial focusing</td>
<td>Temporal focusing</td>
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<tr>
<td><strong>Spot</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>generation</td>
<td>Laser or stage scanning</td>
<td>Laser or stage scanning in parallel</td>
<td>Single shot exposure No actuators</td>
</tr>
<tr>
<td><strong>Excitation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>method</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Depth-</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
State-of-the-art approaches to fast FLIM based on parallelization

Kumar, OE2007

Buranachai JF2008
A new method based on temporal focusing

- Temporal focusing is only available for optical pulse, especially ultrafast pulse
- Temporal focusing cannot occur in free space since light propagating speed is same regardless of wavelengths
  The use of angular dispersion with diffractive optical element gives possibility to realize temporal focusing

Oron et al., “Scanningless depth-resolved microscopy”, Optics Express 13(5), 2005

Zhou et al., “Simultaneous spatial and temporal focusing of femtosecond pulses”, Optics Express 13(6), 2005
Design of a temporal focusing system

- Each color-resolved monochromatic wave is focused at the back aperture of the objective, effective back focal plane.
- Each wave is collimated after the objective, all the wave become overlapped, by approaching focal plane, and they are completely overlapped at focal plane.
- Along optical axis toward focal plane, number of overlapped waves increase, temporal pulse width gets shorter, and TPE efficiency becomes higher.

Time-Bandwidth Product: \( \tau \Delta \lambda = \text{constant} \)
Feasibility of temporal focusing based wide-field multiphoton imaging

- **Quantum Dots and CPNs**
  - Lower power (P = 500 mW)
  - Single QDs are blinking

What about normal dyes and fluorescent proteins?

Abul Rahim, Adv Mat., 2008
Frequency domain heterodyne FLIM

A simple relationships between fluorescence/phosphorescence lifetime, $\tau$, and the measured phase shift, $\phi$, & demodulation, $M$ -- chromophores acts as an electrical low pass filter for light

$$\tan \phi = \omega \tau$$

$$M = \frac{1}{\sqrt{1 + \omega^2 \tau^2}}$$
High Speed 3D Resolved FLIM & PLIM based on Heterodyned Wide Field Imaging

The principle of heterodyne detection

\[ M \sin(\omega t + \phi) \sin((\omega + \Delta \omega)t) \xrightarrow{\text{low pass}} M \sin(\Delta \omega t + \phi) \]

First 3D Resolved Wide-field PLIM (and fast too!)

Marriott et. al., Biophys J, 60 (1992)
Lakowicz et. al., J Fluorescence, 2 (1992)
Oida et. al., Biophys J, 64 (1993)
So et. al., Proc SPIE, 2137 (1994)
Fluorescence lifetime of Rhodamin B in different solvents

<table>
<thead>
<tr>
<th>Solution</th>
<th>Tau (Ph)</th>
<th>Tau (Mod)</th>
<th>Tau (Madge)</th>
</tr>
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<tbody>
<tr>
<td>FL</td>
<td>4.01</td>
<td>4.18</td>
<td>4.00</td>
</tr>
<tr>
<td>RhB/etOH</td>
<td>2.83</td>
<td>2.84</td>
<td>2.93</td>
</tr>
<tr>
<td>RhB/meOH</td>
<td>2.22</td>
<td>2.36</td>
<td>2.46</td>
</tr>
<tr>
<td>RhB/H2O</td>
<td>1.75</td>
<td>1.79</td>
<td>1.68</td>
</tr>
</tbody>
</table>
Cells in matrix loaded with calcein & Syto13

Calcein:
3.85 ns
(Pelet, BJ 2004)
Measured (Ph): 3.5 ns
Tau (Mod): 3.1 ns

Syto13:
1.5-2.5 ns
(Walczysko, AME 2008)
Measured Tau (Ph): 2.9 ns
Tau (Mod): 1.8 ns
Lifetime resolved imaging of regenerated nerve

Immunofluorescent label is localized to a capillary in the regenerated nerve
Oxygen imaging by phosphorescence is a more critical problem in cancer biology.

Typical confocal PLIM frame rates:
- 50 msec per pixel
- 100 x 100 x 100 pixels = 50,000 seconds (13 hours)

Confocal or two-photon (pixel-by-pixel)
- Axially resolved
- Slow

3D resolved, Two-Photon Wide-field PLIM
- Fast
- Axially resolved

Wide-field (1-photon)
- Fast
- No axial resolution

Probes:
- Ruthenium based (~600 ns)
- Palladium based (~113 μs)

Sudh et al, Opt Exp, 2006
Yaseen et al, Opt Exp, 2009
Jain, Science, 2005

Probes:
- Ruthenium based (~600 ns)
- Palladium based (~113 μs)
Mapping $O_2$ distribution is important to study hemodynamics and cognitive activity of the brain.

Regeneration matrix properties control clinical outcome; spontaneous angiogenesis affects metabolism of healing wounds.

Sakadiz, Nat. Neu. 2010

Wang, OL 2011

Regeneration matrix
Oxygen sensing: Ruthidium Phosphorescence Lifetime Calibration

Stern—Volmer relationship of phosphorescence quenching

\[ \frac{\tau^0}{\tau} = 1 + k_q \tau^0 [Q] \]
Signal to noise of oxygen measurement

Ruthidium concentration:
1.2 mM (Sud, OE 2006)

Data Acquisition time:
1.2 sec to 6 sec

Average photon # per pixel:
480 to 2400

Lifetime noise:
13% to 37%

Expected data acquisition time for 10% lifetime noise:

6 sec
3 sec
1.5 sec
Resolving phosphorescence & fluorescence components in the specimen

RhB-DHPE labeled cell in Ruthidium buffer

Intensity

Lifetime - Phase

Lifetime - Modulation

Photon per pixel

Lifetime (ns)
Resolving phosphorescence & fluorescence components in the specimen

RhB-DHPE labeled cell in Ruthidium buffer

Intensity Fraction

Intensity scaled lifetime

RhB

Ruthidium

0 1
Potential for high throughput minimally invasive diagnosis?

Tai et al., Journal of Biomedical Optics, 2009
Ting et al., Journal of Biomedical Optics, 2010

Assembled temporal focusing endomicroscope
Size of current design: 8mm (diameter), 35mm (length)
FOV: 100 x 150 um, WD: 180um, NA: 0.8
Can wide-field multiphoton microscope image deep?

The scattering of emission photons results in drastic signal to background degradation.

It is the common perception that wide field 3D imaging system CANNOT go deep.
Spatial temporal lock-in detection with structured light

The IP landscape for structured light imaging is equally well trodden ground

The COMBINATION of WF-MM + structured light is novel

SMART/NUS provisional patent is filed
Maximize photon usage based on maximum likelihood

Optical transfer function – information of images in spatial frequency space

Wide field (missing cone) ≠ structured illumination (no missing cone) ≠ deconvolution
Single-shot Multi-plane Temporal Focusing Imaging

Why stop at fast 2D frames? Why not fast 3D image cubes?

Single shot multi-plane imaging without mechanical scanning
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Emergence of system biology

System biology – Multiple factors analysis

\[
\frac{dN_1}{dt} = k_{11}N_1 + k_{12}N_2 + \cdots + k_{1m}N_m
\]

\[
\vdots
\]

\[
\frac{dN_m}{dt} = k_{11}N_1 + k_{12}N_2 + \cdots + k_{1m}N_m
\]

Conventional single factor analysis

Multiple factor analysis using microarray
Emergence of image informatics

Perlman et al., Science 2004

Heat map

Perturbation: Known & unknown “drug” at different dosages

Readout: morphology & protein distribution
Most drugs that target the same pathway have similar heat map independent of their chemical structure.

Perlman et al., Science 2004
Classifying unknown drug & drug interaction

Blue: unknown drug

Perlman et al., Science 2004
Image informatics = System biology + Structure

Bakal, Science, 2007 (Started with Perlman et al., Science 2004,)
Bakal – Gene regulation of cellular morphology

Down/up regulation of 200+ genes

Results:
* Clustering of interacting genes
* Linking gene cluster with their functions

Future?

Genes/proteins ↔ Structure

Pattern formation

Structure ↔ Function

Force, adhesion, migration

\[
\frac{dN_1(x,t)}{dt} = D_1 \nabla^2 N_1 + \ldots + D_m \nabla^2 N_m + k_{11}N_1 + \ldots + k_{1m}N_m
\]

\[
\frac{dN_m(x,t)}{dt} = D_1 \nabla^2 N_1 + \ldots + D_m \nabla^2 N_m + k_{11}N_1 + \ldots + k_{1m}N_m
\]
Starting studies:
(1) Liver fibrosis (in collaboration with Hanry Yu, Colin Sheppard, NUS)
(2) Nerve regeneration (in collaboration with Ioannanas Yannas, MIT)

Collagen Scaffold properties affects regeneration efficacy

Fibroblast – myofibroblast transformation and their reorganization is a key step in tissue regeneration

Regeneration:
Fewer & disorganized myofibroblast

Scar:
Numerous & organized myofibroblast

Yannas, JRS-Interface, 2005

Meyer-ter-vehn et al., IVOS, 2006
Using image informatics to elucidate molecular mechanisms of fibrogenesis disease processes in liver fibrosis & nerve regeneration

HSC activation is a key step toward fibrosis

With collaborators: H. Yu (NUS) , I. Yannis (MIT), B. Roysam (RPI), X. Wei (Fudan University)

Chia et al., J. Hepatology, Submitted
Tzeranis et al., Roy. Soc. Interface, In press
High content study of HSC pharmacological responses
Typical HSC drug response data
Correlate ex vivo HC imaging responses to drug in vivo responses

Drug efficacy is based on in vivo results reported in literature

Correlation between in vivo drug efficacy and ex vivo drug induced morphological changes provides information on the mechanisms of drug action

Most effective drugs targets apoptosis, contraction, and proliferation
Image Informatics: Quantitative model of pathophysiology

Quantitative Multiscale Model of pathophysiology

Targeted perturbation of signaling network

Pharmaceutical intervention

Regulate protein expression

Regulate matrix property

Smad7

RhoA

cofilin

Data clustering & interpretation

High throughput, high content Image cytometry

Image segmentation/classification
Acknowledgement