2019 SBI2 educational session

3D optical microscopy methods, techniques, developments

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Microscopic imaging

Seeing is believing

Seeing is understanding
Outline

1. Biological models
2. Imaging methods
3. Considerations for 3D imaging
4. Technical development
5. 3D Image analysis
Biomimetic model systems

- Cell Monolayer
  - Trans-well
  - Spheroids/Organoids
  - Organ chips
  - Patient-derived Xenograft (PDX)

<table>
<thead>
<tr>
<th>2D</th>
<th>3D</th>
</tr>
</thead>
<tbody>
<tr>
<td>In vitro</td>
<td>In vivo</td>
</tr>
<tr>
<td>Simple (Reproduibility)</td>
<td>Complex Physiology (Relevance)</td>
</tr>
<tr>
<td>Easy (Maintenance)</td>
<td>High Cost</td>
</tr>
</tbody>
</table>
3D organoids

(1) Embryonic stem cell-derived organoids
(2) Adult stem cell-derived organoids
(3) iPSC-derived organoids

- 3D growth (Organotypic)
- Genetic & Phenotypic features
- Genetic modification
- Matched normal controls
- Tissue-Stromal interactions
- Drug screening
- Biobanking

Nat. Rev. Cancer
Imaging can provide more information on phenotypic changes

Biochemical assay
- Target-based
- Enzyme
- Receptors
- Channels
- Hormones...
- Kinetics
- Binding

**Cell-based assay**
- Phenotype-based
  - Transcription
  - Protein expression
  - Cell viability
  - Proliferation...
- MTT
- Cell-Titer Glo

**3D imaging**
- Spatial information
- Cell and Organoids level
- Microenvironment...
- Immunostaining
- Live imaging

<table>
<thead>
<tr>
<th>3D imaging</th>
<th>Drug response</th>
</tr>
</thead>
<tbody>
<tr>
<td>![Image 1]</td>
<td>![Image 2]</td>
</tr>
</tbody>
</table>

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Biological imaging methods for 3D models

<table>
<thead>
<tr>
<th></th>
<th>CLSM</th>
<th>SDCM</th>
<th>2PM</th>
<th>3D-SIM</th>
<th>SPIM/LSFM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resolution</td>
<td>High</td>
<td>Moderate</td>
<td>Moderate</td>
<td>Highest</td>
<td>Moderate</td>
</tr>
<tr>
<td>Z-range</td>
<td>Limited</td>
<td>Limited</td>
<td>Longer than CLSM</td>
<td>Longer than 2PM</td>
<td>Longest</td>
</tr>
<tr>
<td>Scan speed</td>
<td>Slow (Fast)</td>
<td>Faster than CLSM</td>
<td>Slow</td>
<td>Slower than CLSM</td>
<td>Fastest</td>
</tr>
<tr>
<td>Phototoxicity</td>
<td>Highest</td>
<td>Less than CLSM</td>
<td>Less than CLSM</td>
<td>Higher than CLSM</td>
<td>Lowest</td>
</tr>
</tbody>
</table>

Confocal laser scanning microscope (CLSM)
Spinning-disk confocal microscope (SDCM)
2-photon microscope (2PM)
3D-structural illumination microscope (3D-SIM)
Selective plane illumination microscope / Light-sheet fluorescence microscope (SPIM/LSFM)

Fischer RS et al. TRENDS in Cell Biology, 2011
Galvano-Resonant hybrid scanner can improve scan speed in CLSM

<table>
<thead>
<tr>
<th>Scanner type</th>
<th>Galvano</th>
<th>Resonant</th>
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</thead>
<tbody>
<tr>
<td>Speed</td>
<td>4 fps (512x512)</td>
<td>30 fps (512x512)</td>
</tr>
<tr>
<td>Image quality</td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>Zoom</td>
<td>1x-1,000x</td>
<td>1.5x-8x</td>
</tr>
</tbody>
</table>

Galvano scan

Resonant scan
Biological imaging methods for 3D models

CLSM

2-PM/FLIM

SPDC

3D-SIM

LSFM
Considerations for 3D imaging
1. Sample preparation

- **Fixed**
  - Section
  - Whole mount
  - **Formalin**
    - FFPE
    - Microtome
  - **PFA**
    - Frozen
    - Cryostat

**Live**

- **Cell**
  - Organoids (+Gel matrix)
  - Tissue explant

- **Considerations for 3D imaging**
  - Tissue damage
  - Missing parts
  - Time consuming

- **3D reconstruction from serial images**
2. 3D Imaging depth

(1) Light Source

LED

Laser

(2) Sample thickness and Imaging direction

Upright

Gel thickness

Height

Inverted

(3) Plate bottom thickness and shape

Whole plate accuracy

Max Volume: 300μL, 200μL, 300μL

U Shape, Spindle Shape, V Shape

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Considerations for 3D imaging
Tissue clearing can improve imaging depth

See Deep Brain (SeeDB)
3. Plate format

- Materials, Stiffness, Clearness
- Shape, Surface area, Color
- Attachment, Cell density
- Culture volume
- Evaporation, Edge effect
- Pipetting error

<table>
<thead>
<tr>
<th>Materials</th>
<th>Chemical Resistance</th>
<th>Binding</th>
<th>Characteristics</th>
<th>Applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glass</td>
<td>High</td>
<td>Very low</td>
<td>Hard, Clear, Flat</td>
<td>Fluorescence, High resolution imaging</td>
</tr>
<tr>
<td>Polystyrene</td>
<td>Low</td>
<td>Low-High</td>
<td>Hard, Clear</td>
<td>ELISA, Fluorescence, Luminescence</td>
</tr>
<tr>
<td>Polypropylene</td>
<td>High</td>
<td>Very low</td>
<td>Soft, Opaque</td>
<td>Storage, Fluorescence, Luminescence</td>
</tr>
</tbody>
</table>
4. Labeling and Dyes

Transient labeling: Dyes
- Nuclear/DNA - Hoechst, DRAQ5, Sir-DNA
- Cell - Cell tracker
- Organelles - Mitotracker, Lysotracker
- Cytoskeleton - Sir-actin, Sir-tubulin
- Live/Dead cells – Propidium iodide (PI), DRAQ7

Stable labeling: Lentiviral H2B-GFP/RFP, CRISPR

- Signal reduction
- Bleaching
- Toxicity
- Signal bleed through
- Stable labeling but takes time
- Repeated addition
- Optimization
- Sequential scan, Far red dye

Considerations for 3D imaging
5. Object resolution

Labeled (3D)  VS  Unlabeled (2D MIP)

H2B-GFP  VS  Brightfield, DRAQ7

High Resolution (Single cell)  VS  High Throughput (Organoids)

Cellular details  VS  Image processing, Data analysis, Imaging speed

9/17/2019  Considerations for 3D imaging
Expansion microscopy can increase resolution and sample clearness

Chojinski TJ et. al., (2016) Nat. Method
Super resolution microscopy can provide ultrastructural resolutions

3D-Photoactivated localization microscopy (3D-PALM) / Stochastic optical reconstruction microscopy (STORM)
- Resolution <20nm, 50-100nm for STORM
- Proteins tagged with photoactivable fluorophores
- Cylindrical lens
- Limitation on the imaging speed

Interferometric Photoactivated localization microscopy (iPALM)
- Axial resolution <20nm
- Samples up to ~300nm in thickness
- Technically complex

Fischer RS et al. TRENDS in Cell Biology, 2011
6. Temporal resolution
6. Temporal resolution

**Static time points**

- Low temporal resolution
  - 0 hour
  - 4 hour

**Time lapse**

- High temporal resolution

**Considerations for 3D imaging**

- Less phototoxicity
- Image processing
- Data analysis
- Dynamic information
- Kinetics, Tracking
- Imaging time
- Data size
Temporal resolutions in drug response

50μM Irinotecan

Day1

Day3

Day4

Cytotoxic

5μM 5-FU

Day1

Day3

Day4

Cytostatic

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Considerations for 3D imaging
Technical development
Autofocus and Z-drift correction (ZDC) for consistent imaging

- Fixed position
- Sample signal-based

Plate bottom
Well bottom

Infra-red light
**Immersion objectives**

Water immersion objective

- Resolution
- Z information
- Light detection
- Multi-area imaging

PerkinElmer

**Effects of Refractive Index Mismatch on Sample Shape**

- Olympus

20x Air
NA=0.4

20x Water
NA=1.0

NA: Numerical aperture
Pre-scan for sample re-positioning

Trask OJ et. al., PerkinElmer
3D targeted pre-scan to narrow down ROI

- Increases scan speed and accuracy
- Removes unnecessary scanning area (XYZ)
- Reduces file size

XY targeting

Z targeting

Different ROI size (?)

Different Z range (?)
3D Presci-scan reduces scan time and file size

96 well plate, organoids in gel with 3 channels, 20x objective, 5µm z-step size, Bin 2

Gel height ~ 1,000µm
Organoids size 100-400 µm

Without PreciScan

With PreciScan in XY

With PreciScan in XY and Z

Scan time

139 hrs

9 hours

2 hr
Presci-scan of 3D organoids

5x Pre-scan  Fast analysis  Select objects  20x Post-scan
Light-field microscopy can image 3D samples faster

→ Computational deconvolution

Prevedel R. et. al. (2014) Nat. Method
3D Image analysis
3D Image analysis software

Commercial

- Easy to use
- Performance
- Established algorithms
- Batch analysis
- Limited flexibility
- Cost

Free open source

- MATLAB, Python, C++
- Fiji-imageJ
- Training & Optimization
- Flexible
- Batch analysis
- Free
- Limited applications
- Data format & Imports
Imaris 3D data analysis - Tracking organoids

1. Upload multiple time points as a single file

2. Organoid Surface Detection

3. Individual Cell Detection

4. Individual Organoid Tracking

5. Exporting Cell Counts & Morphological Data

6. Generate Growth Curves

Growth rate vs. drug concentration
High-throughput image analysis tools

Organoseg
- 4x objective 2x2 binning
  - Raw grayscale image
  - Open-dose morphology by reconstruction
  - Adaptive threshold with multiple window sizes
  - Superimpose thresholds
  - Binarize, filter noise, fill holes, and smooth
  - Identify regions of interest
  - Sort aggregates, remove debris (optional)
  - Extract metrics

OrgaQuant
- EVOS 8-bit tiff
  - Transmitted Light
  - Single plane

CALYPSO
- 512x512 resolution
- Confocal pinhole at 500µm
- 4µs/pixel scan speed
- 6.4mm² central area of well


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3D Image analysis
Questions and further discussions?

- **How can we scale up?**
  - Different plate format → Different cell behaviors?
- **Accuracy, Resolution vs. Throughput**
- **Assay validation**
- **Quality control**
  - Culture: Media/FBS, Passage number…
  - Imaging: Controls, Temperature, Humidity…
  - Analysis: Standard, Training set…
  - Automation: Liquid handling, Integration…
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