Zoom-in beyond light microscopy:

new approaches for biological structure research - correlative light and electron microscopy on one and the same sample

“Small is beautiful....”

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Correlative light-electron microscopy (CLEM)

Aim:

A) identify on LM and EM level the identical structure (morphomics) - “retain context information"

B) image and locate specific fluorescent labelled molecules in living cells, record dynamical processes by LM and investigate them at EM level

Fluorescent Light Microscopy

Transmission Electron Microscopy & Tomography/ Scanning EM

fluorescent labels

no labels or e-dense labels

The precision depends on the structure preservation ..... 

See lecture: Heinz Schwarz, the “poor mans” confocal way....

“ Imaging Space“ - Time vs. Spacial Resolution:

EM...electron microscopy; LM...light microscopy

EM & LM covers a range of

x,y -> mm-nm; z -> 10-1μm - <10nm-
t -> μsec-min

-> only a correlative approach allows not to get lost in details & keep the large picture „in-focus“
Correlative light & electron microscopy

The context matters...

"The Art lies in the context..."

(Ultra-) structural research: the classical approach

CLEM is more than 1&1.....

<table>
<thead>
<tr>
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<th>(Fluoro) LM</th>
<th>EM</th>
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<tr>
<td>Observation of living cells</td>
<td>+++</td>
<td>-</td>
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<tr>
<td>Identification of labeled structures</td>
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<td>Identification of unlabeled structures</td>
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<tr>
<td>Context space</td>
<td>+</td>
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<td>Reference space (for labels)</td>
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(Ultra-) structural research: the classical approach

The classical way - correlate data from different approaches...

One preparation for a microscope-independent approach for tissue & cells...

question

unique preparation protocol

answers

correlative microscopy

different microscopic techniques

correlation of specific data

comparable image

Data quality
One preparation for a microscope-independent approach for tissue & cells....

Prerequisite: Structure preservation & fluorochrome - The idea...

A) Integrated/ In-Situ solutions
- FLM/TEM FEI iCorr (cryo-)
- Delmic FLM/SEM
- Jeol ClairScope

B) Off-line solutions (Transformation "2" Coordinate Systems):
- Array Tomography (brutal force)
- Zeiss Shuttle& Find LM-SEM, FEI MAPS CorrSight-SEM, Jeol Map & manual correlation etc...

A Compromise: a modified „Freeze Substitution“ (or embedding) for correlative LM/EM

1. transfer frozen sample into substitution media at -110°C or -90°C
   Media (organic solvent saturated with fluorochrome and Uranyl Acetate)
2. substitution according to tissue experience (-90/-70/-50°C)
3. wash excess fluorochrome and fixative until no colour bleeding (-50°C)
4. start infiltrating with resin (-50°C HM20, 0°C Epon...)
5. polymerization under UV or with heat
6. trimming for CLSM or SEM, sectioning for LM, TEM....
**Dyes, penetrating or surviving during freeze-substitution...**

- Uranylacetate
- Sudan III
- Safranin T
- Nile red
- Nile blue sulfate
- DiIC₁₈
- DiOC₆
- Acridin Orange
- Nonyl-Acradin Orange
- 1,8 ANS
- DCVJ
- Bodipy 560
- Oregon Green
- GFP / YFP...

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**Fluorescence Protein surviving during freeze-substitution or fixation (GFP, EoS...)**

Correlative and integrated light and electron microscopy of in-resin GFP fluorescence, used to localize diacylglycerol in mammalian cells


Kawaschinski, 2000 "Vergleichende Untersuchungen an identischen Hautproben mittels der konfokalen LSM und der Transmissions-Elektomikroskopie" FH Hamburg Bergedorf

Nat Methods, 2015 Jan 12. doi: 10.1038/nmeth.3225. [Epub ahead of print]

- ...“photoconvertible Eos fluorescent protein that fluoresces and photoconvert normally in heavily fixed (0.5-1% OsO₄), plastic resin-embedded samples...”

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**One biopsy: for LM, CLSM & EM**

modified freeze-substitution with fluorescence dyes....


"From tissue to cellular ultrastructure: closing the gap between micro- and nanostructural imaging"

Katja Kawaschinski; 2000 "Vergleichende Untersuchungen an identischen Hautproben mittels der konfokalen LSM und der Transmissions-Elektomikroskopie" FH Hamburg Bergedorf

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**Correlating light and electron microscopy**

Miriam S. Lucas, Philippe Geiser, Maga M. Günther, Roger Wacht, Jason Mercer, Ari Helenius, Andreas Schertel; Correlative 3D Microscopy: CLSM and FIB/SEM Tomography: A Study of Cellular Entry of Vaccinia Virus; Imaging & Microscopy, GIT Publ.; EMCO308; Vol. 10, Iss 3, p 30-31
One biopsy: for LM, CLSM & EM in-vivo 2photon selection

- High pressure freezing
- Freeze substituted
- HM20 embedded
- CLSM 3D imaging on bloc
- Section staining

Correlating light and electron microscopy

TEM:
- Tedious
- Support grid masking...
- Higher throughput...???

Biel et al., J Microsc 212 (2003)

3D Electron Microscopy

The LM/SEM (FIB) world

- "Thick" not e-transparent
  - Serial section real section or "en-bloc"

The LM/TEM world

- "Thin" e-transparent => "Tomography"
  - Virtual image stack
  - Various angle views...

=> TEM "Tomo"

CLEM & Large Volume 3D EM methods...

K.L. Briggsman&D.D. Bocks 2012;
Large Volume 3D EM methods...

**FIB/SEM**
(focused ion beam SEM)

Sample preparation

- High-pressure Freezing
- Freeze substitution & low temp. embedding
- Determination of ROI & 3D imaging
- 3D FIB-SEM tomography

![Sample preparation diagram](image)

**Acquisition of 3D image stacks with FIB-SEM**

1. Deposition of protecting C-layer
2. Milling of a trench, milling current 6.5 - 13nA
3. Polish the cross section, milling current 1.5nA
4. Imaging with SEM (ESB)

Pre-selection by CLSM & recognition of ROI in SEM

- Fluorescence signal
- Reflection of surface
- SEM image

![Pre-selection diagram](image)
Acquisition of 3D stack by FIB / SEM

Vaccinia interaction with host-cell....

FIB lamella for TEM/STEM Tomography..

TEM Tomography on ROI:
multilamellar bodies in the Stratum Granulosum..

Stratum Corneum lipids are synthesised in the TGN (GluCer) and exported in Multivesicular lamellar bodies into the intercellular space (Cer)....
Bacteria in legume root nodules

Mung bean

- Root nodules are colonized by nitrogen-fixing bacteria
- This symbiosis provides the host plant with nitrogen, which in return provides nutrients for the bacteria

Images of plant and roots courtesy of Prof. H.-M. Fischer

Choosing the ROI...

CLSM

SEM

FIB-SEM

Correlation of CLSM and FIB-SEM stacks

Some statistics for system biology...

- Volume: 260 µm³
  - 39% symbiosomes
  - 12.5% bacteria
- 31 symbiosomes
- Ø 3.5 bacteria per symbiosome
- From CLSM data: ~50% of cells invaded by bacteria

Tool to study e.g. invasion process of bacteria into root cells
  - Quantitatively
  - In spatial context
Large Volume 3D EM methods...

- array tomography (CAT - correlative/computer controlled)

3D data with an in situ ultramicrotome.....

(Denkatome....Denk&Horstmann 2004 PLOS Vol 2)

May be combined with CLSM of pre-embedding antibody labeling, or diffusible dyes....
- fast for large volumes
- destructive
- large amount of heavy metal needed
- e-beam induced hardening during operation

Fluorescence Protein surviving during freeze-substitution or fixation (GFP...)

Correlative Microscopy:
on high pressure frozen, substituted and embedded samples...(passive labeling...)

- photoconvertible Eos fluorescent protein that fluoresce and photoconvert normally in heavily fixed (0.5-1% OsO4), plastic resin-embedded samples...

Ultramicroscopy 143 (2014) 3–14

GFP, mCherry
Short FS protocol:
- HM20
- K4M
- LRW

Resin embedded samples are a "Storage" device for "morphomic data"

-> “Data block” & “Data slices”
Correlative light-electron microscopy (CLEM)

**Fluorescent Light Microscopy**
- fluorescent labels, live imaging
  (ideally GFP-like labels)
- Videomicroscopy
- Confocal/Spinning disk
- Multiphoton
- FRAP
- FRET, BRET, FLIM
- STED
- PALM

**Transmission Electron Microscopy & Tomography**
- no labels or e- dense labels
- Classic EM
  (Room temperature)
- Cryo-EM
  (LN₂ temperature)

Resolution: 16 nm

Perfused cell membrane are helpful for import of "Marker" ...but what does it mean for the localisation

Marker size & distance....

**GFP** (3-5nm) 238AA; 26,9kDa
**minisOG** (3nm) 106AA; 15kDa
**Llama AB** (3-5nm) 238AA; 15/30kDa

**3D views of an IgG molecule 160kDa**

Is two more than one? Or greater than the sum....

**Correlative Life cell LM - EM CLLEM**

Katia Cortese, Alberto Diaspro and Carlo Tacchetti, 2009 JHC, Vol 57(12)

Some slides with different approaches..

**3D LM/EM Tomo - sections (GFP, YFP, photoconversion)**

1. in vivo observation LM...
2. chem. fix with sucrose
3. photooxidation...
4. wash
5. poststaining
6. embedding in resin
7. TEM at <200-300nm thin sample areas
8. TEM

G. record tomogram....

**New fluorescence protein (miniSOG, photoconversion)**

1. in vivo observation LM...
2. chem. fix with sucrose
3. photooxidation...
4. wash
5. poststaining
6. embedding in resin
7. TEM at <200-300nm thin sample areas
8. TEM

G. record tomogram....
GFP et al. at cryo-LM conditions much more stable -> C-Cryo-LEM...

**3D LM/ EM Tomo - Data from cryo-layers/sections..**

(GFP, YFP, in-vivo marker....)

A. in vivo observation LM...
B. rapid freezing...
C. cryo-FLM (ROI)
D. Cryo Phako
E. ROI identification - select thin area
F. TEM at <200-300nm thin sample areas
G. record tomogram....


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**Correlating FM and cryo-ET: Full Correlation Cycle**

LM

- Transfer coordinates
- area of interest

EM

- Cryo-Electron Microscopy
- NG108 neuroblastoma/glioma hybrid cell line

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Can SEM replace classical TEM application for ultrastructure research?
Large Volume 3D EM methods...

- array tomography (CAT - correlative/computer controlled)

3D LM/SEM - Data from sections....

Serial section array
SEM imaging:
K. D. Micheva, S.J. Smith
Neuron 55, 2007

1. Chemical Fixation
2. Dehydration
3. Embedding
4. Serial sections
5. Post-embedding labeling
6. Multilabeling...
7. Histo or SEM
-> Au-BSE localisation???
TEM or SEM???

Tissue...

3D LM/SEM - Data from sections...

Preparation... & Localisation...

Wilke et al; 2008

TEM (Grid...)

Serial section vs. FIB/SEM.....

Prerequisite transparent, conductive support -> ITO

Casino 3.2.01 (freeware)

Topview

70-100nm: 1.8 - 2 kV ideal
kV adapted to Section Thickness - induced conductivity

70-100nm HM20/ Epon: 1.8 - 2 kV ideal

ITO-coating (grainless)

cover slide C/S - 150-170µm (LM)

FEI Solution - MAPS: Correlative Microscopy

Light Microscopy  •  Corr. Workbench

Electron Microscopy  •  SEM

LM Acquisition  EM Acquisition

- compatible with any kind of LM brand’s images..

Bridging Microscopes....
Fluorescence after Fixation: a. Dehydration

- EtOH after CPD after FD
- DAPI ++ ++ + ++
- Alexa 488; FITC + - - -
- Cy3 ++ ++ + ++

3h: 40-50 different ROI on 10 different samples - matching SEM found & vice versa
Correlative Microscopy 2 - Array Tomography:
- combine Shuttle&Find with automatic section detection - ROI definition and automatic section imaging in a SEM

Matrix of 4 x 3 images each 16384 x 16384 pixels
=> 3'221'225'472 pixel per ROI ! => Acquisition time is per ROI 4h 9min!

Array Tomography & Immunolocalisation (lipids):
- primary antibody against Glucosyl-Ceramid 3; secondary antibody Cy3

PSF correction....:
- superresolution in Z (50-70nm)
Correlative microscopy: advantages

unique preparation protocol

- all samples viable for LM, CLSM, TEM, REM
- number of necessary samples is decreased
- more time for imaging
- comparable image data from different microscopes
- parallel imaging on identical structures possible
- combination with super-resolution possible...

Sample preparation

In-vivo

block-face

serial section

labelling/staining

LM/CLSM

biopsy / extraction

Sample Preparation

CLSM

FLM

FIB/SEM (->TEM)

CAT

FLM

BF/DF LM

LVSEM

3D correlative (SR)LM & SEM
-> higher localisation precession - Au-IgG

Variable surface glycoprotein (VSG) 6nm Au & alpha-Tubulin 12nm Au

H. Schwarz/B. Humble (MAPS - FEI)...

Some slides with different approaches..

Integrated FLM & SEM: An other solution from a supplier.....

Correlating FM, Superresolution (PALM) and SEM imaging

Correlating FM (fiducial markers) and TEM Tomography
Fluorescence Protein surviving during freeze-substitution or fixation (GFP, EoS...)

- photoconvertible Eos fluorescent protein that fluoresces and photoconverts normally in heavily fixed (0.5-1% OsO₄), plastic resin-embedded samples...

Short FS protocol with OsO₄:
- HM20
- K4M
- LRW

PET
XCT
MRI
OCT

→ Tof-MS: Radioisotopes; metabolites; drugs...
→ File Format: DICOM for Micros.
**Benefit from multimodal correlative microscopy:**

- 3D information on all structural level can be integrated on the same specimen area/feature as sub-volume at higher magnification....

You never can image a whole body at max resolution......1x10^26 voxels.....you need a clever selective working strategy......

(10^12 terra; 10^15 peta; 10^18 exa; 10^21 zetta, 10^24 yotta)
What are we dealing with...
  (water, molecules and bioorganisation)
- Living "systems" - dynamic and crowdiness...
- Consequences for EM...& sample preparation...
- 3D & Correlative Microscopy (CL-"S"EM)...
- One Biopsy for various Imaging Modalities...
- 3D EM techniques & new alternatives...
- CLEM alternatives...& Future

Future:
- Improvements for data handling & Processing ...
- Correlative labels (Click-iT,GFP,EoS,APEX...)...
- Automatized interfaces to various imaging modes...
- Automatized sample preparation LM-> EM...
- Use of histopathology samples

Call to Action - Open opportunity:
What’s required to solve the problem...
- Instrumentations/ Interfaces
- Computer Science
- Biology
- Chemistry/ Labels - Markers
- Engineering
- Politics & Policy/ Media
- Fund Raising
- Entrepreneurs

Thank you for your attention
"...we are only a few steps away from studying the same sample in a semi- or fully automated way by using a combination of four-dimensional light-optical live cell imaging techniques, followed by plunge- or high-pressure freezing techniques at a well chosen experimental end point, in order to perform electron tomography of the vitrified sample on the nanoscale in the cryoelectron microscope all in one run, and within one working day...."

F. Braet and W. Geerts 2009 J. Microsc. Vol. 235 Correlative Microscopy...