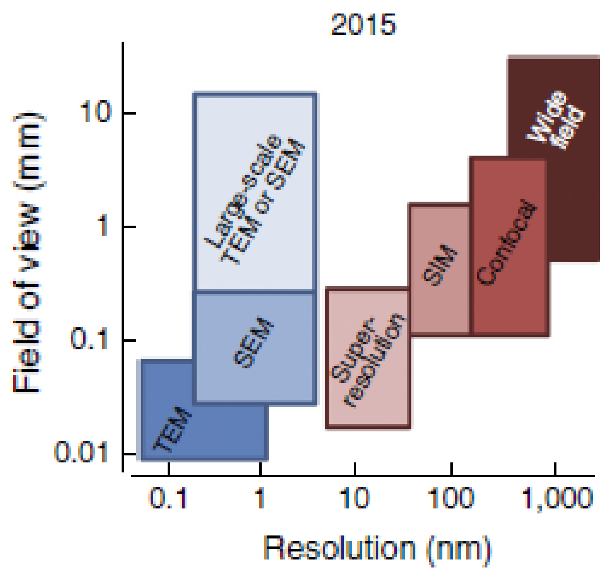
Correlative imaging: Because two microscopes are better than one.

Yvette Zarb
Department of Neurosugery

Fluorescence microscopy

- Identifies specific molecules
- Enables the study of the molecules' biological role
- Limitations:
  - A large fraction is unlabelled
  - Lowest resolution is 10nm



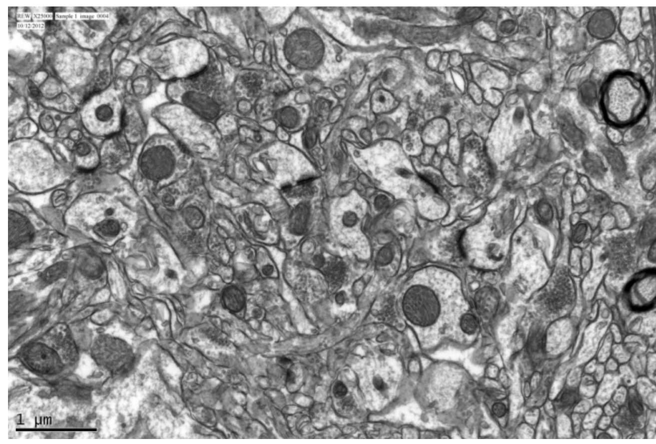
Adapted from: deBoer, Hoogenboom & Giepmans, 2015

Electron microscopy (EM)

- To study molecules in their biological context and at high resolution

- Limitations:
  - Ultrastructural analysis is done in grayscale
  - Biological samples are in a fixed state
  - Molecules are hard to define
  - Finding rare events is hard

- Immuno-EM
  - Difficult to master
  - Destruction/ inacessability to antigens
  - Lack of suitable antibodies
  - Size of antibodies limits the resolution



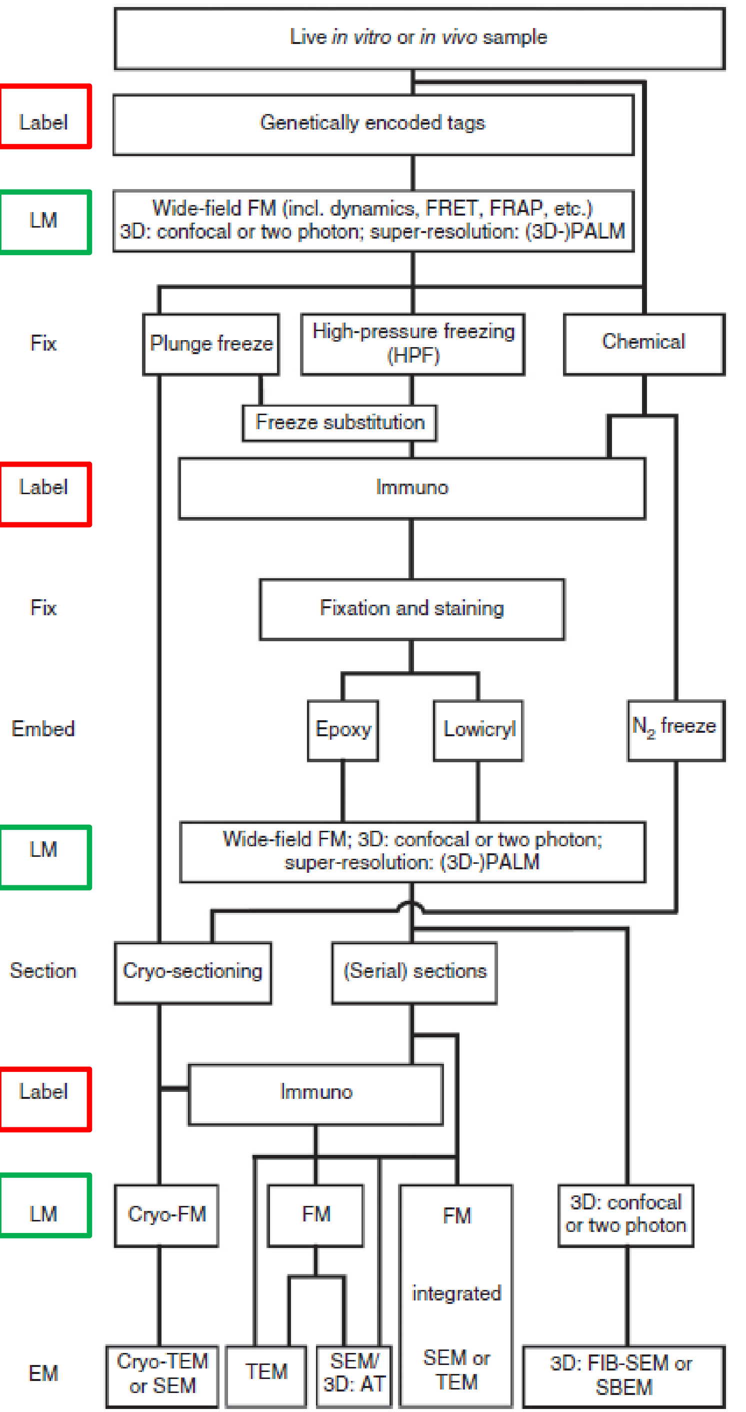
http://www.researchgate.net/

Correlative Light and Electron Microscopy (CLEM)

- Combines the strengths of the two imaging techniques

- Recent developments in several aspects of this technique has made it a powerful tool

- Combinations of these methods can be:
  - Samples are analysed by fluorescence imaging and then processed for EM
  - Ultrathin sections prepared for EM which are imaged with LM



Taken from: deBoer, Hoogenboom & Giepmans, 2015

Paper 1 - CLEM Optimisation

NATURE METHODS | ARTICLE

Protein localization in electron micrographs using fluorescence nanoscopy

Shigeki Watanabe, Annedore Punge, Gunther Hollopeter, Katrin I Willig, Robert John Hobson, M Wayne Davis, Stefan W Hell & Erik M Jorgensen

CLEM optimisation

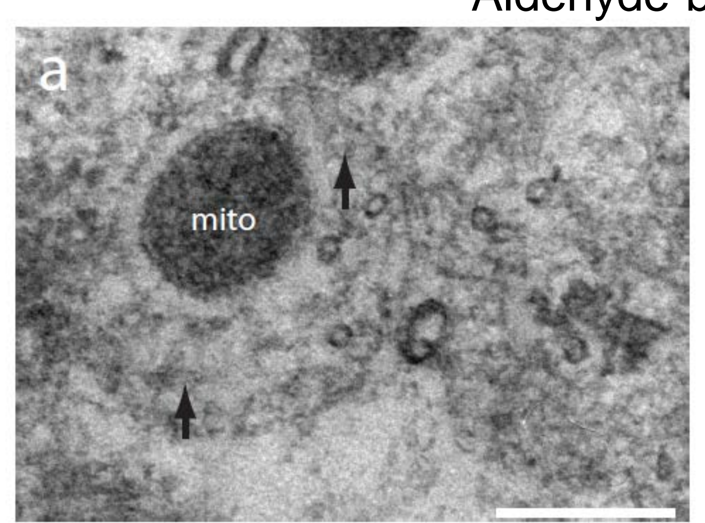
- Cryo-sections lack tissue contrast
- EM techniques quench fluorophores by acidic, dehydrated and oxidizing conditions.
- In this paper: optimisation of each step of sample preparation

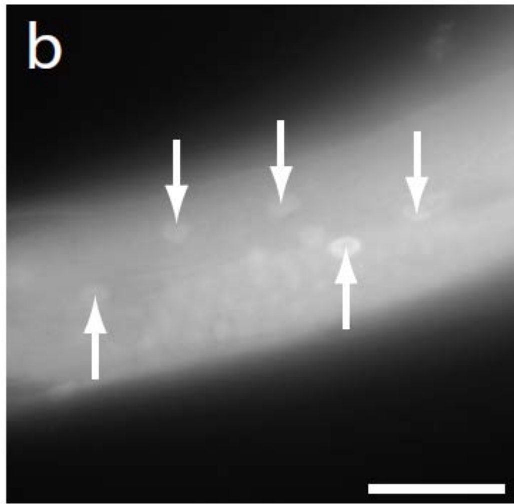
Study principle

- Use *C.elegans*
  - Fluorescent proteins stably expressed
  - EM methods are well established
- Target proteins: Citrine and Eos/Dendra
  - **H2B** – nucleus easily visualized
  - **TOM20** – mitochondrion cross section is a good test of super-resolution technologies
  - **α-liprin** – neurons are the most sensitive to fixation

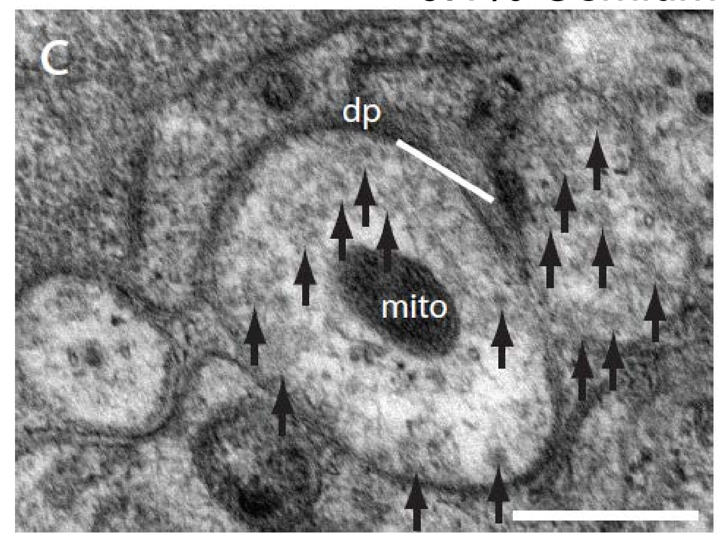
Optimization of fixatives

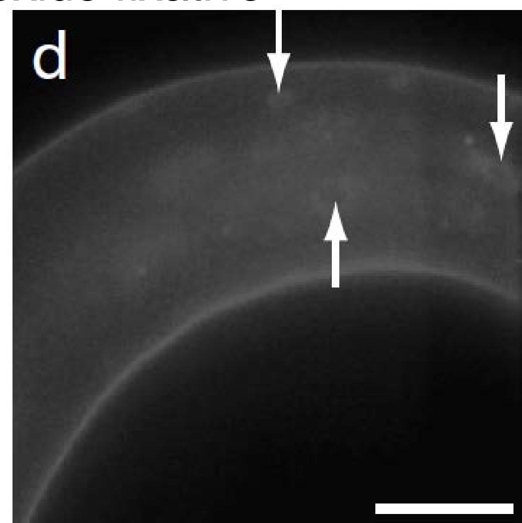
Aldehyde-based fixatives





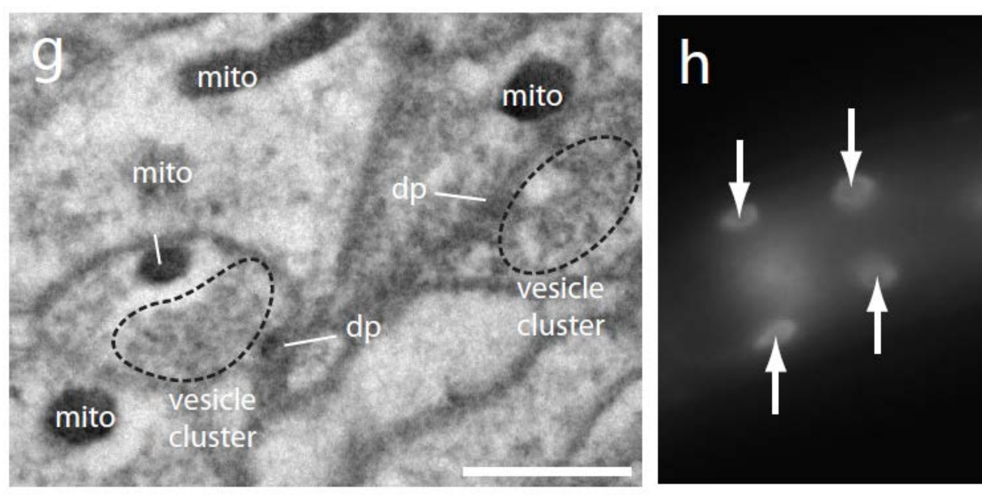
0.1% Osmium tetroxide fixative

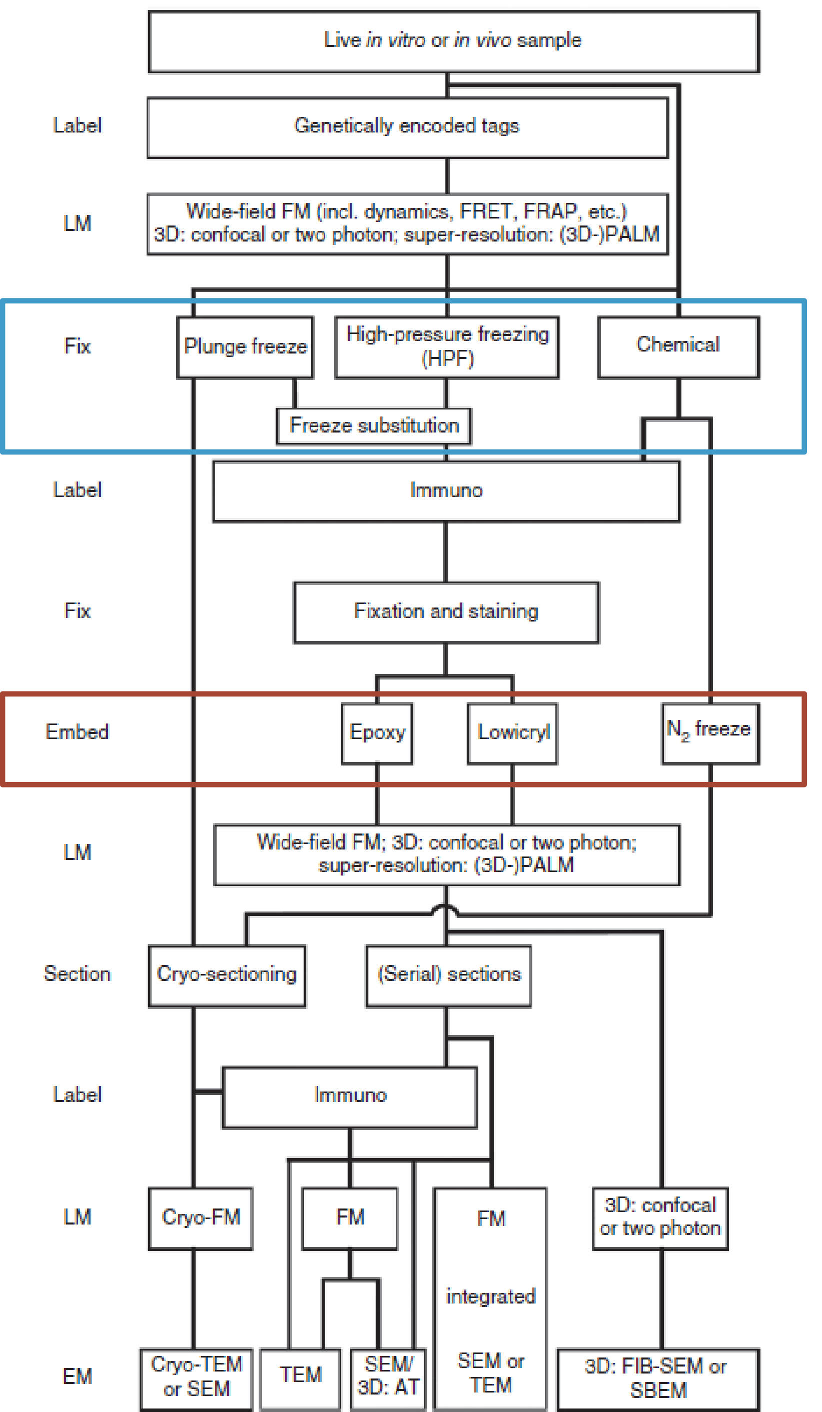




Optimization of fixatives

0.1% Potassium permanganate 0.001% Osmium tetraoxide





Taken from: deBoer, Hoogenboom & Giepmans, 2015

Optimization of Plastic

- Embed tissue in plastic resin for ultra-thin sectioning
- Polymerization requires dehydration and heat
  - Denaturing the proteins & fluorophores
- In this paper: Hydrophilic, low-temp resins
  - Included 2 – 5% water

Optimization of Plastic

- Lowicryl K4M
  - Most hydrophilic resin
  - 5% inclusion of water led to poor polymerisation
- LR Gold
  - Polymerized rapidly
  - Did not penetrate the tissue
- LR White
  - pH is too acidic for fluorophores
  - Neutralized: good preservation, irregular polymerisation

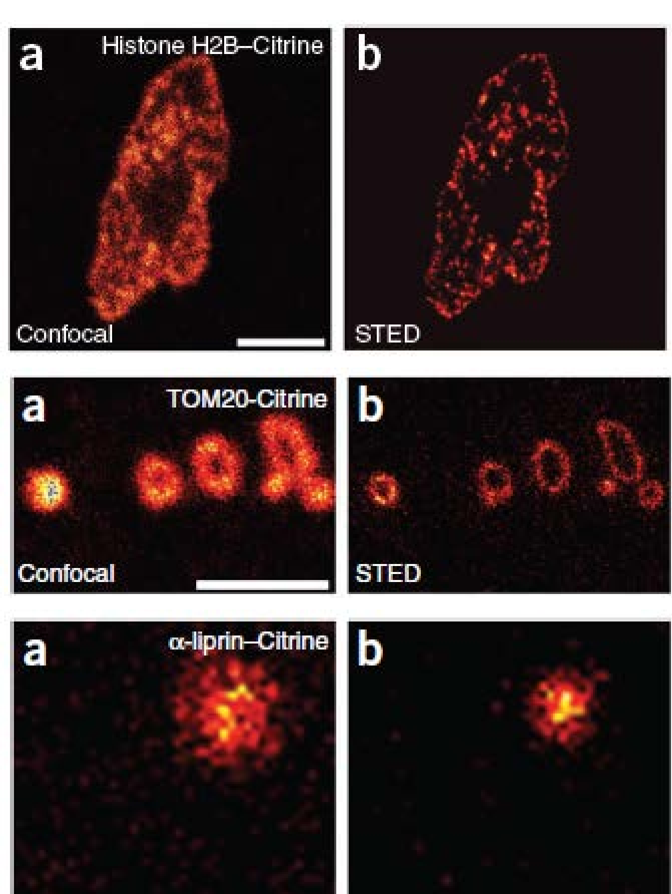
Optimization of Plastic

- Glycol methylacrylate (GMA)
  - 3% water
  - Polymerisation at pH8
  - Fluorescence brighter than LR White

- Limitations
  - Does not cross-link to the cuticle
  - Tissue sectioned thicker than 70nm (approx 100nm)

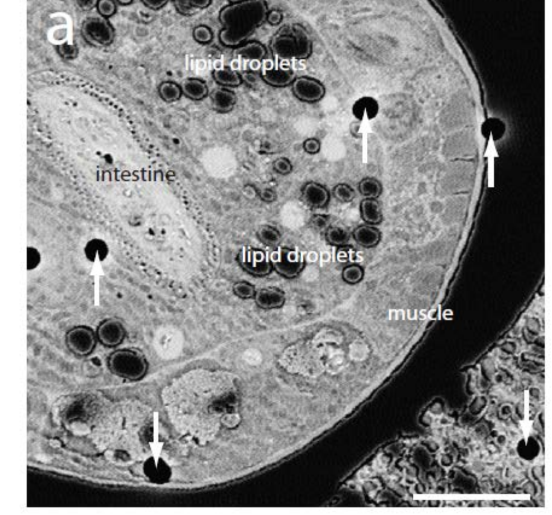
Confocal & STED

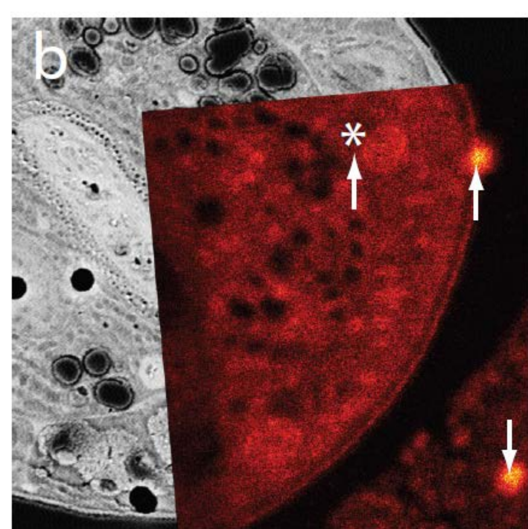
- Confocal is diffused
- STED has an improved resolution
- α-liprin was not resolved, as expected



Alignment of LM to EM

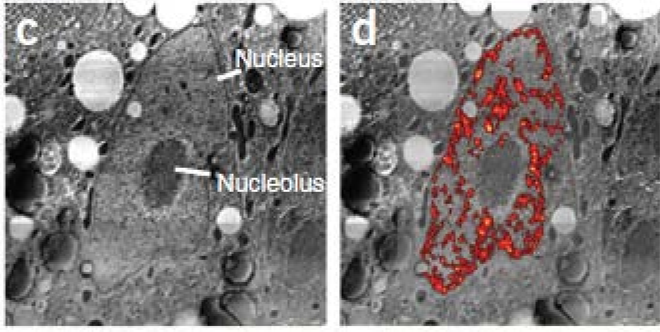
- Silica beads as fiduciary marks
- Fluorescent in UV light
- Reflect electrons



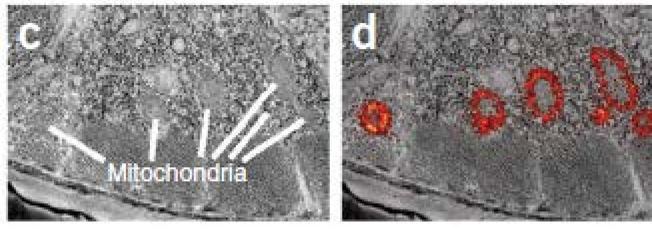


Correlation fluorescence and electron microscopy

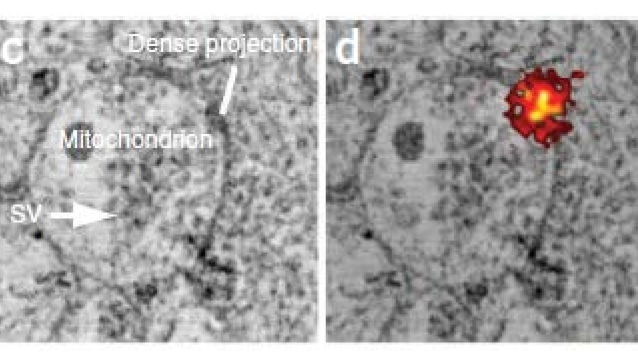
Histone 2B



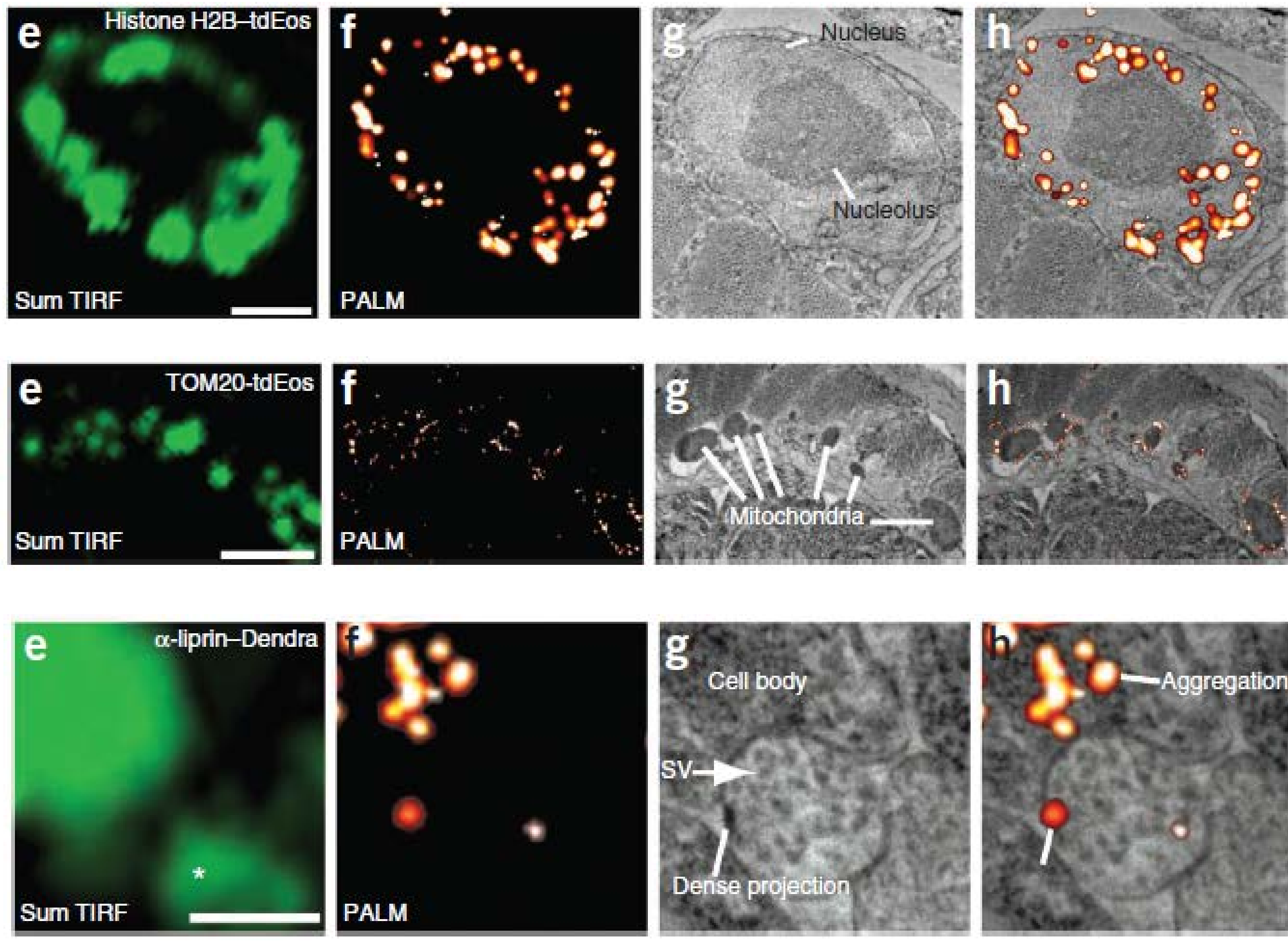
TOM20



α-liprin



CLEM using PALM



Conclusion – paper 1

- CLEM can be used to study a molecule in its biological context

- For this method SEM is preferred due to the thickness of the section
    - Thickness needed for the generation of an adequate fluorescence signal

- For high resolution in SEM requires a good production of back-scattered electrons
    - High atomic stains quench fluorescence
    - Alternative (uranyl acetate) images not as crisp

Paper 2 – Fluorophore optimization

NATURE METHODS | BRIEF COMMUNICATION

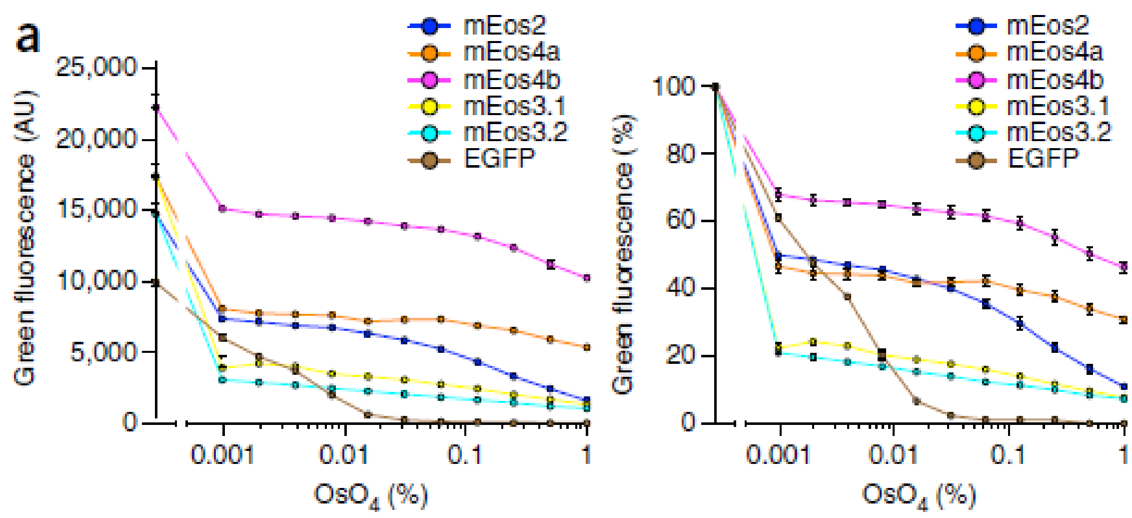
Fixation-resistant photoactivatable fluorescent proteins for CLEM

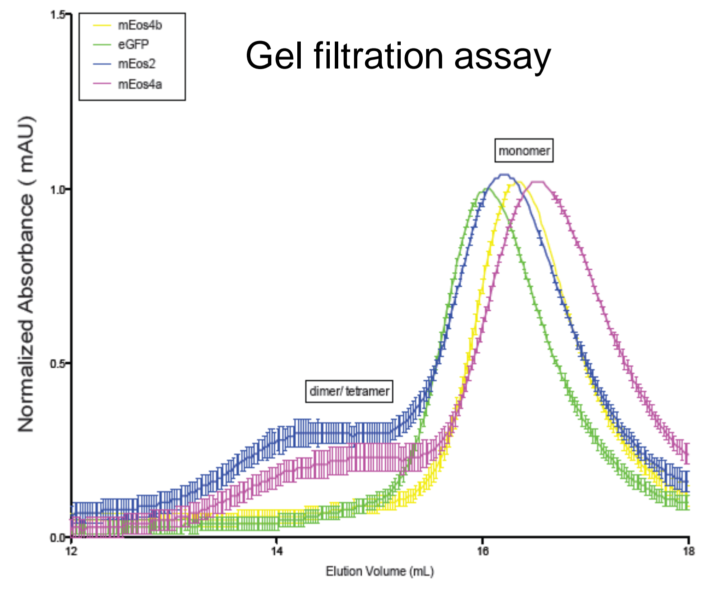
Maria G Paez-Segala, Mei G Sun, Gleb Shtengel, Sarada Viswanathan, Michelle A Baird, John J Macklin, Ronak Patel, John R Allen, Elizabeth S Howe, Grzegorz Piszczek, Harald F Hess, Michael W Davidson, Yalin Wang & Loren L Looger

Fluorophore optimization

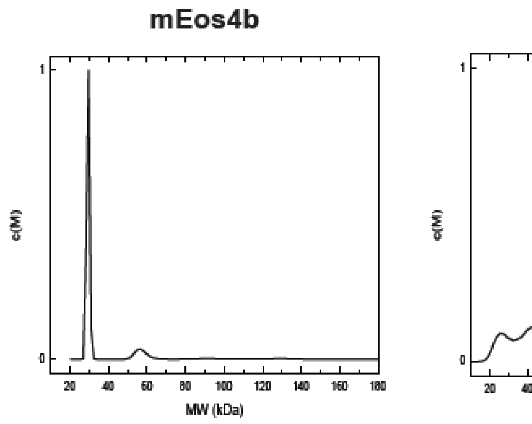
- Compromise between fluorescent signal and preservation of ultrastructure architecture
- In this paper: demonstrate two mEos4 variants, better survive OsO_4 fixation

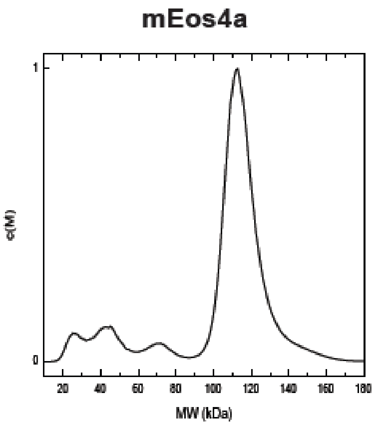
Testing of mEos2 mutants



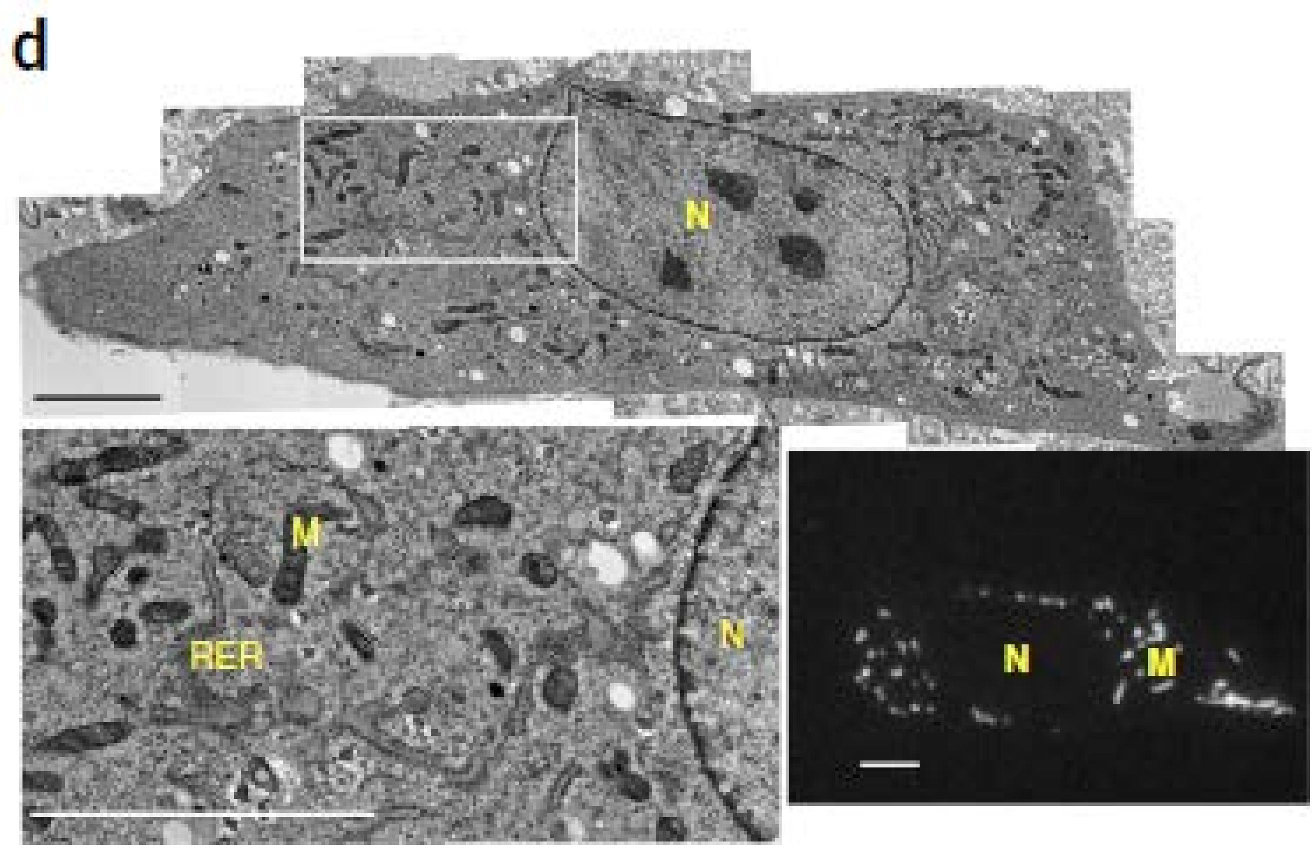


Analytical ultracentrifugation

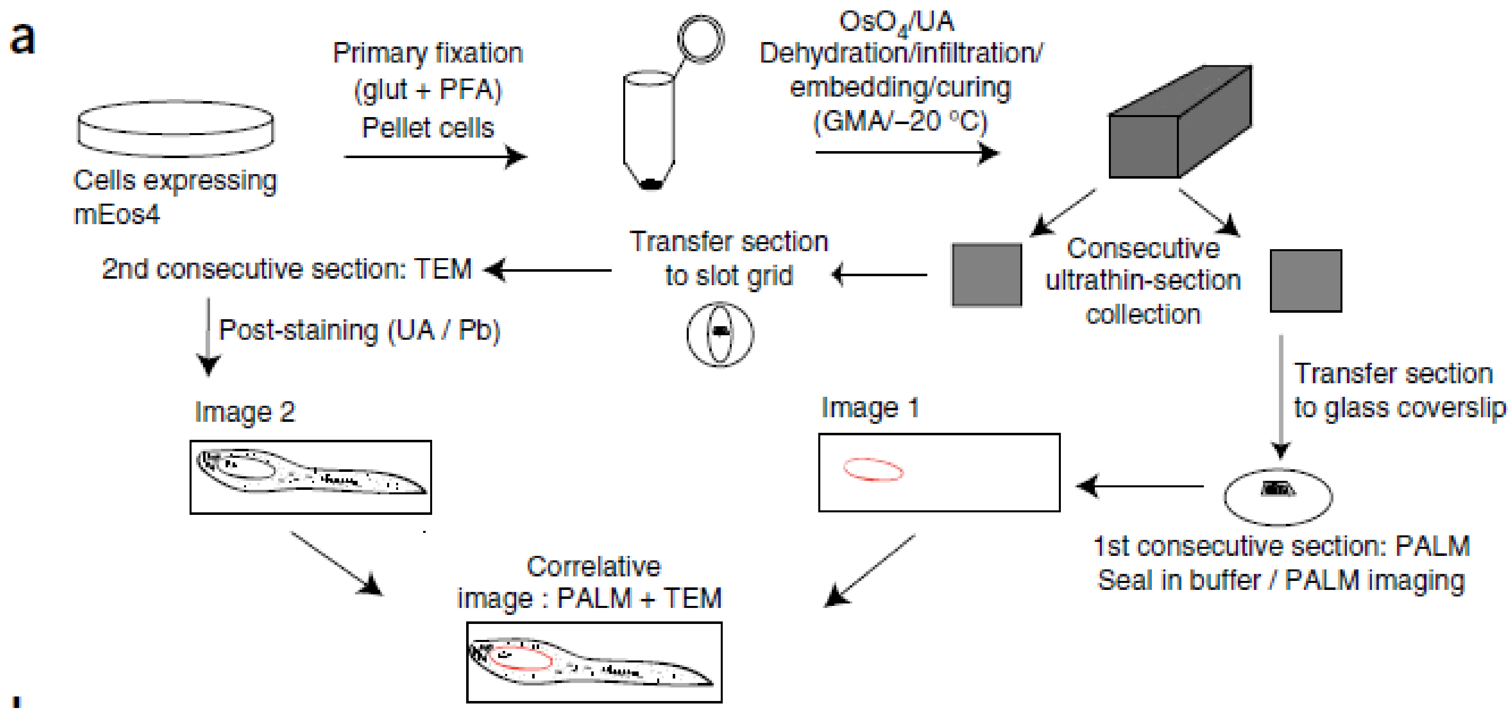




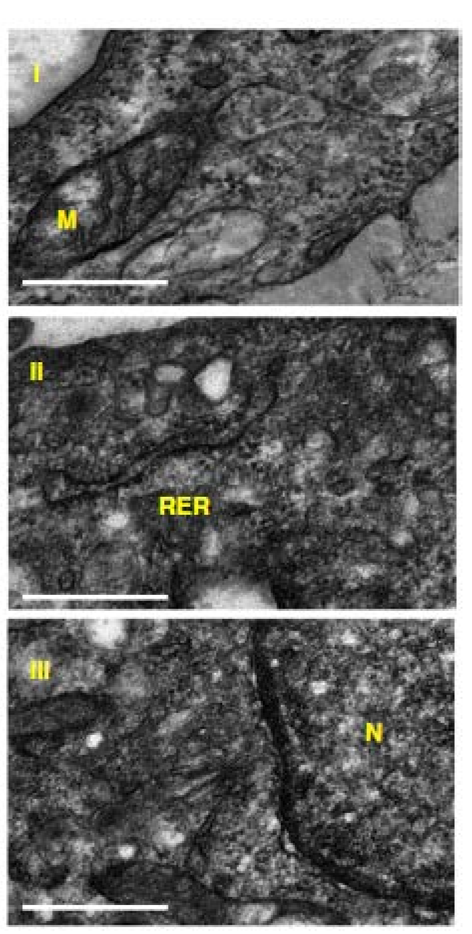
Fluorescence retention

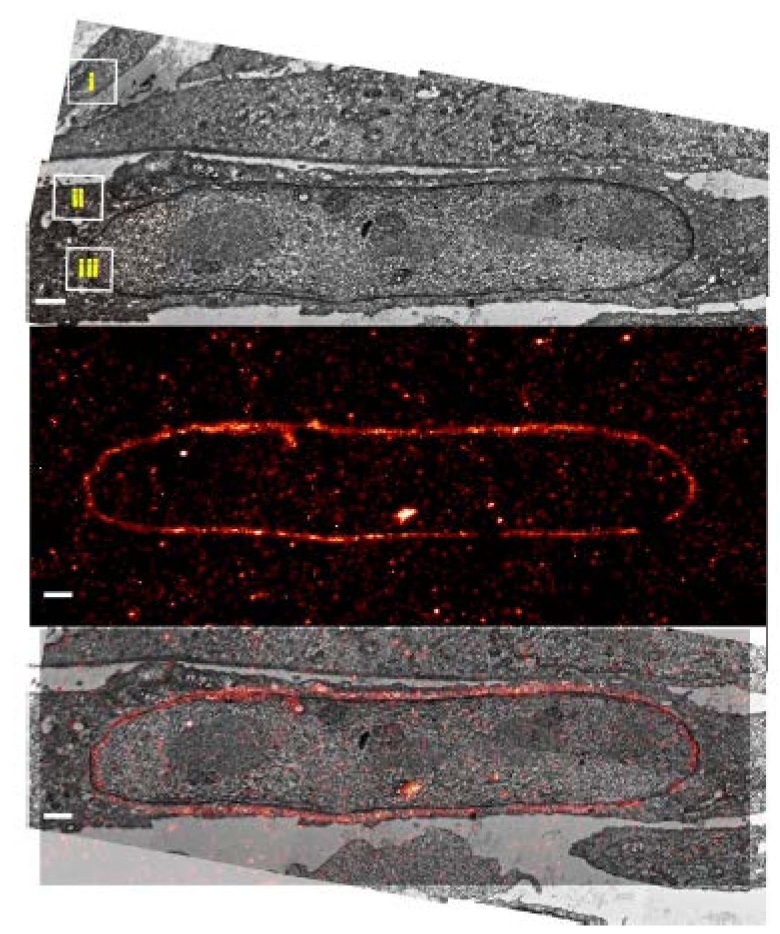


Consecutive-section approach



Correlation imaging



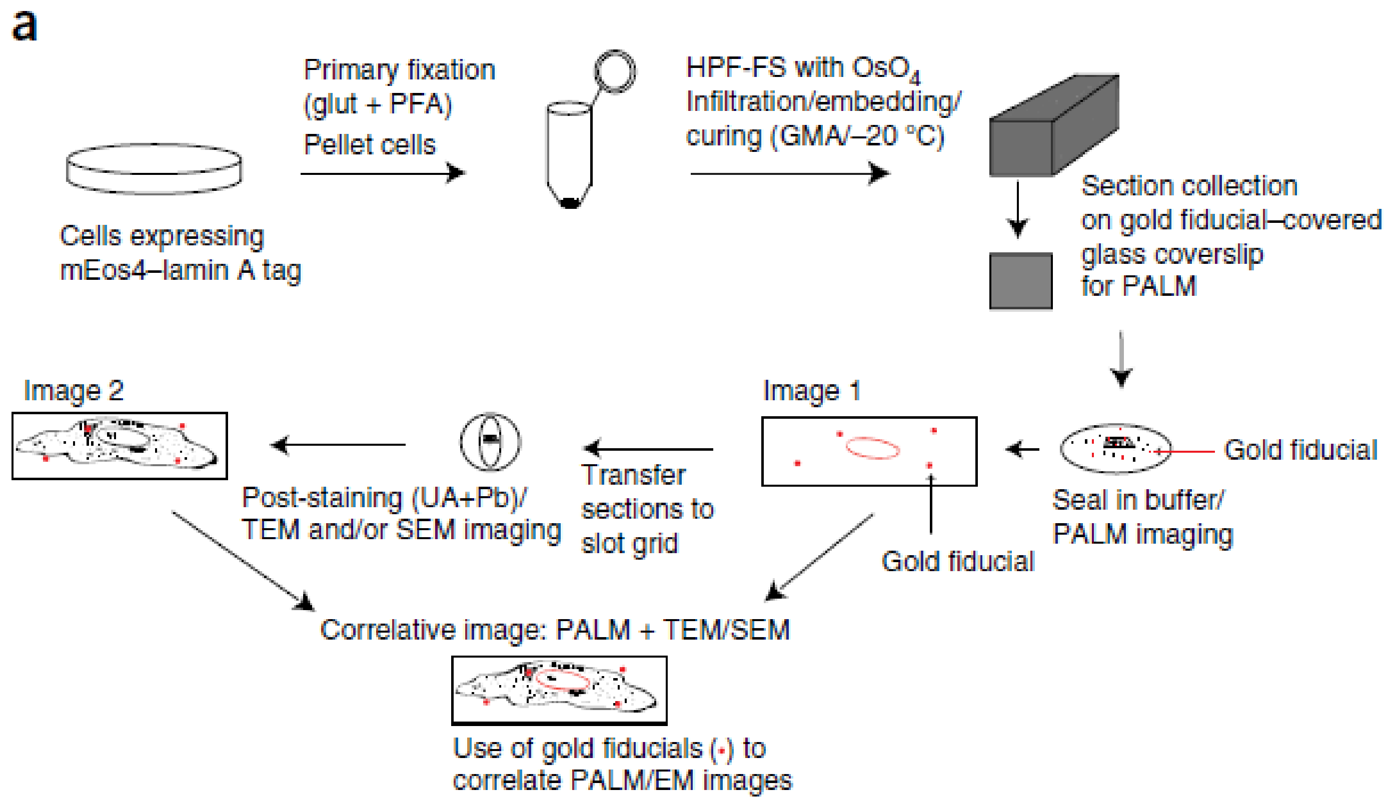


TEM

Lamin-A

Correlation

Same-section approach



Correlative PALM and TEM/SEM

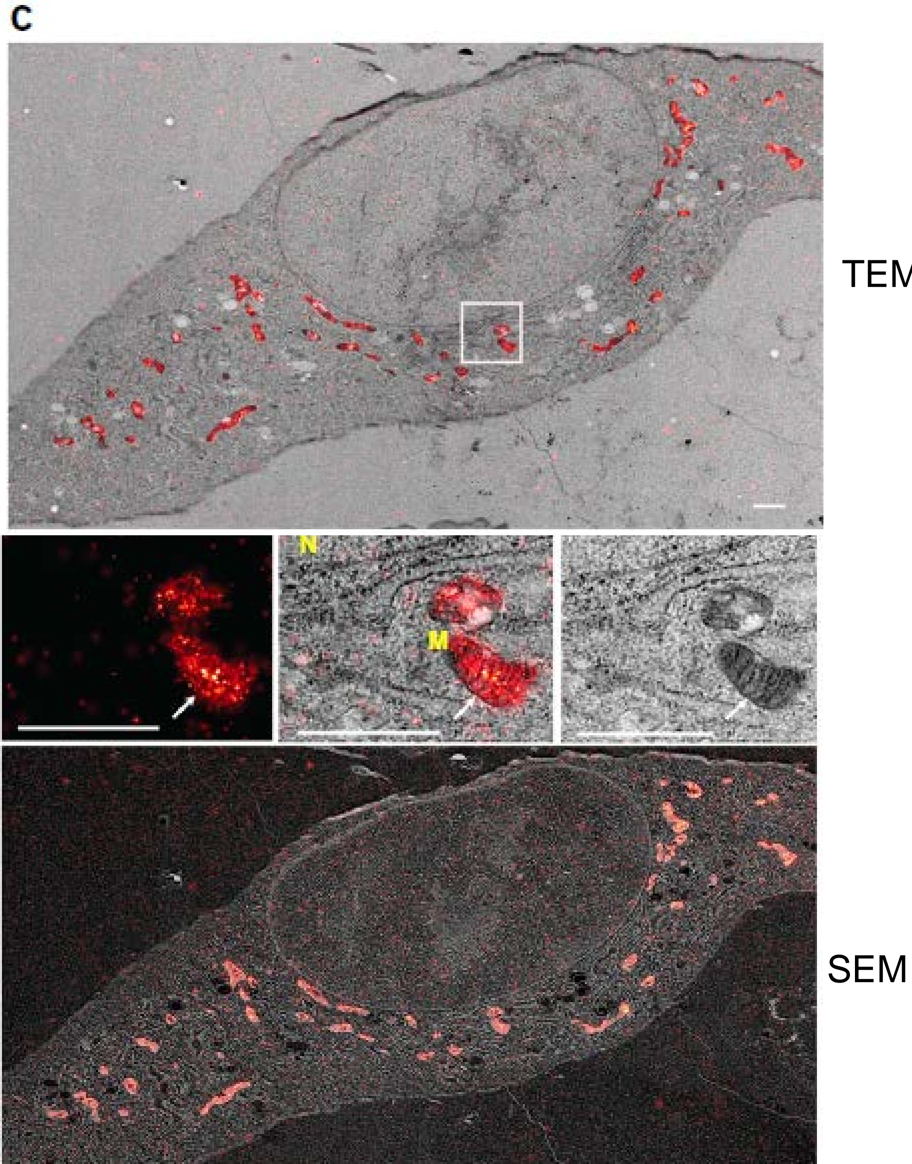
b

N

M

RER

Lamin-A



Conclusion – Paper 2

- Ultrastructure is preserved
- Staining for both approaches was similar
- Same-section approach:
  - Allows more precise, quantitative registration (fiduciary marks)
- Consecutive-section approach:
  - easier and minimizes sample handling
- mEos4b is effectively a pure monomer
  - Target proteins fuse reluctantly
- mEos4a is appropriate for broader staining
  - Whole cells or organelles

Limitations of CLEM

- Implementation of CLEM is preceded by several considerations
    - Research questions, models & microscopes available
- Always LM before EM
- Only samples that work in plastic resin can be scaled up
    - Enables serial sectioning
- CLEM is an improvement over immuno-EM
    - Does not depend on antibodies
    - Some proteins do not tolerate fluorescent tags
    - May disrupt function or localization
- LM looses its 3-dimensiality
    - Z-dimension resolution is lower than x- and y-axis resolution
    - Reconstructing the volume of the tissue
    - 3D imaging

Developments

Introduction of correlative light and *airSEM*™ microscopy imaging for tissue research under ambient conditions

Inna Solomonov[1]*, Dalit Talmi-Frank[1]*, Yonat Milstein[2], Sefi Addadi[2], Anna Aloshin[1] & Irit Sagi[1]

Correlative light and electron microscopy enables viral replication studies at the ultrastructural level

Kirsi Hellström[a], Helena Vihinen[b], Katri Kallio[a], Eija Jokitalo[b], Tero Ahola[a],

Correlative light-electron microscopy (CLEM) combining live-cell imaging and immunolabeling of ultrathin cryosections

Carolien van Rijnsoever, Viola Oorschot & Judith Klumperman

Correlative in-resin super-resolution and electron microscopy using standard fluorescent proteins

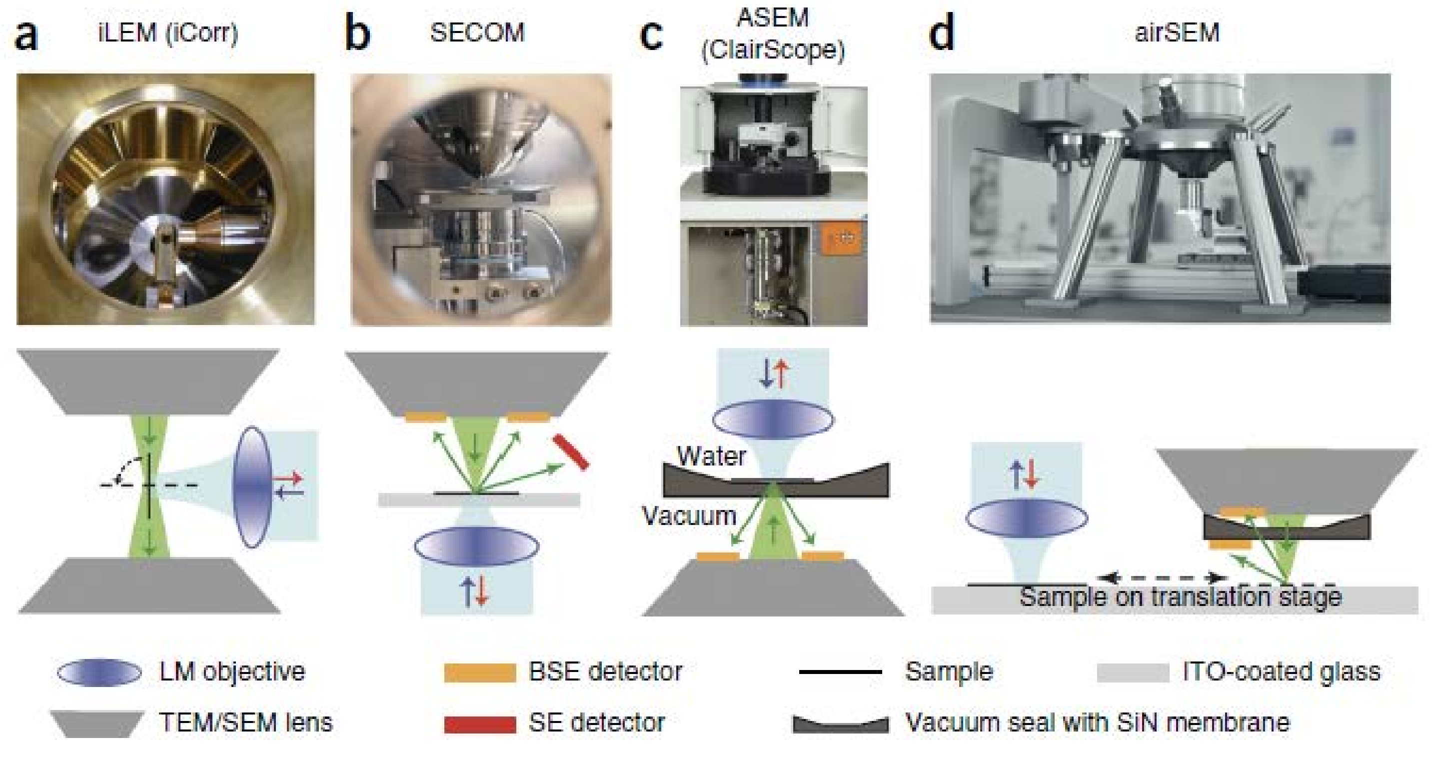
Errin Johnson[1], Elena Seiradake[2], E. Yvonne Jones[2], Ilan Davis[3], Kay Grünewald[2] & Rainer Kaufmann[2,3]

Visualizing viral protein structures in cells using genetic probes for correlated light and electron microscopy

Horng D. Ou[a, b], Thomas J. Deerinck[c], Eric Bushong[c], Mark H. Ellisman[b, c, d], Clodagh C. O'Shea[a, b],

Thanks!!

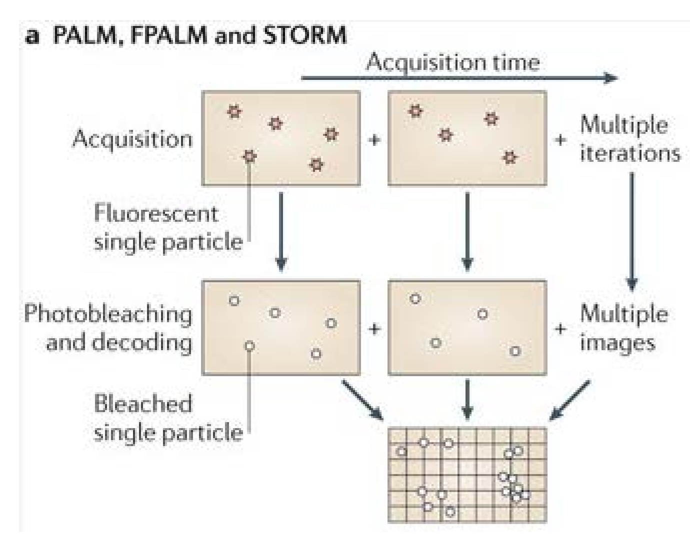
Commercial integrated CLEM microscopes



Taken from: deBoer *et al.*, 2015

PALM principle

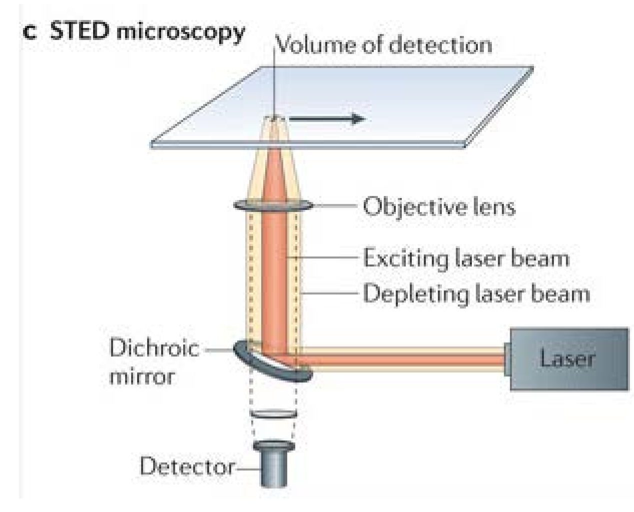
- Photoactivated light microscopy
- controlled activation of sparse subsets fluorescent molecules
- The PALM image is a composite of all the single molecule coordinates



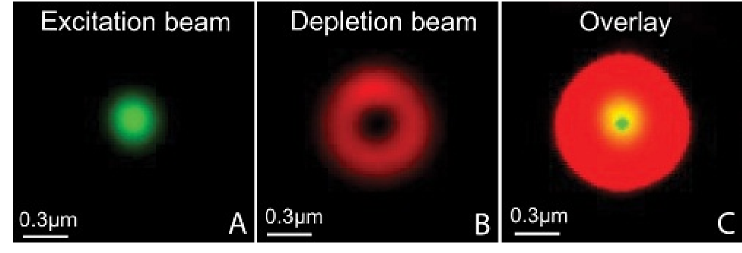
Balagopalan et al., 2011

STED principle

- stimulated emission depletion
- built on the basis of a confocal laser scanning microscope
- inhibit fluorescence emission



Balagopalan et al., 2011



http://www.anes.ucla.edu/sted/principle.html

Steps in sample preparation for EM

- Rapid freezing
    - Water molecule immobilization, no ice crystals
- Freeze-substitution
    - Fixatives dissolved in organic solvent to replace water
- Infiltration with the plastic resin
- Polymerization
- Sectioning