



WIMM Methods & Techniques Course 2019

Confocal Microscopy

Christoffer Lagerholm Facility Manager Wolfson Imaging Centre



Wolfson Imaging Centre Oxford



https://www.imm.ox.ac.uk/research/facilities/wolfson-imaging-centre

Microscope Equipment

WIDE-FIELD Microscopes

- 1) DeltaVision Elite Live Cell Imaging System
- 2) Leica DMi8

CONFOCAL Microscopes

- 1) Zeiss 780 Inverted Confocal Microscope
- 2) Zeiss 880 Inverted Confocal Microscope with Airyscan detector
- 3) Zeiss 780 Upright Confocal Multiphoton Microscope
- 4) Zeiss Cell Observer Spinning Disc Confocal
- 5) Leica SPEII Inverted Confocal Microscope

SUPER-RESOLUTION Microscopes

- 1) Leica SP8 STED3X Microscope
- 2) Olympus IX83 TIRF Microscope

Image Analysis and Image Storage

- 1) Huygens
- 2) Imaris
- 3) OMERO 100 TB File Server



Wolfson Imaging Centre

The Wolfson Imaging Centre is an open access light microscopy core facility. Our facility includes a wide range of state-of-the-art widefield and confocal light microscopes suitable for imaging a variety of specimens.







Wolfson Imaging Centre Staff

- Facility Manager
- 2 Assistant Managers Dr. Jana Koth & Dr. Silvia Galiani
- Image Analyst Dr. Ulrike Schulze

Staff Duties

- Training (and managing) users,
- System maintenance and validation
- Implementation of new methods

Microscope Users

- ~ 65-70 users/month
- ~ > 1000+ hrs/month







- What can we learn from light microscopy?
- Why so many microscopes?
- Confocal microscopy





What can we learn from light microscopy?





Example 1: Cell cycle dynamics U2OS cells expressing PCNA-Chromobody-RFP imaged on Widefield/TIRF microscope at 24 z-planes/h





Chromotek https://www.chromotek.com/



Example 2: Cell cycle dynamics Fixed time-point; HeLa cells





3D Immunohistochemistry of fixed HeLa cells expressing H2B-GFP (blue) and stained with antibodies for proliferation marker Ki67 (magenta) and tyrosine $-\alpha$ -tubulin (yellow) (Max Intensity projection of z-stack (112 slices); FOV ~150 µm x 100 µm)





Example 3: Quantitating kinetics and relevance of protein motifs upon DNA damage response.

Induction of DNA damage with 405 nm laser



Characterization of role of *SNM1A*, a 5'–3' exonuclease, in DNA damage response (Lonnie Swift, Ghadir Almuhaini, Christoffer Lagerholm, Peter McHugh)





Example 3: Relevance of protein motifs upon DNA damage response.



Characterization of role of *SNM1A*, a 5'–3' exonuclease, in DNA damage response (Lonnie Swift, Ghadir Almuhaini, Christoffer Lagerholm, Peter McHugh)





Why so many different types of microscopes?





Image Formation

Convolution of microscope optics (PSF) with object = Airy Pattern













Different microscopes have different PSFs



Modified from Schermelleh, L. et al. J. Cell Biol. 2010, 190, 165–175.





Image Comparison of Confocal, SIM, STED, and dSTORM



Trans-golgi network in COS7 cells

(TGN46 (Primary+Alexa488 Secondary IgG))

Imaging cellular structures in super-resolution with SIM, STED and Localisation Microscopy: A practical comparison. E Wegel, A Göhler, BC Lagerholm, A Wainman, S Uphoff, R Kaufmann, IM Dobbie. (2016) Scientific Reports 6, DOI: 10.1038/srep27290





(Laser-scanning) Confocal Microscopy





Conventional Wide-field Microscopy



Problem – fluorescence is emitted along entire illuminated cone, not just at focus





Wide-field Microscopy



Triple-labeled cell aggregate in mouse intestine section (Carl Zeiss. Confocal Laser Scanning Microscopy)







The confocal microscope







Laser Scanning Confocal Microscopy



Triple-labeled cell aggregate in mouse intestine section (Carl Zeiss. Confocal Laser Scanning Microscopy)





What do you get?

Axial PSF Intensity Profiles



Wide-field = No pinhole Confocal = Pinhole







Triple-labeled cell aggregate in mouse intestine section (Carl Zeiss. Confocal Laser Scanning Microscopy)





Laser-scanning Confocal Microscope Componets

- 1) Microscope (Inverted or Upright)
- 2) Lasers (Fluorescence Excitation)
- 3) Confocal Scan Module
 - 1) Relay optics (for Collimation)
 - 2) Dichroic mirrors (Reflect lasers to sample & Reflect fluorescence emission to detectors)
 - 1) Scanning mirrors (Enables multi-point imaging)
 - 2) Adjustable Pinhole(s) (Remove out-of-focus component)
 - 3) Spectral filters (Enables selection of spectral window to send to detectors)
 - 4) Single Point Detectors (PMTs, APDs)
- 4) Electronics (lots)
- 5) Software!





Laser-scanning Confocal optical path







2.3 Optical Diagram of the LSM 710 / LSM 780 (Schematic) Relay optics & Dichroics



Fluorescence Detectors





Point Detectors - PMTs

- Must be fast confocal beam spends only a few μ s on each pixel
 - Photomultiplier tubes







Point Detectors - New vs. Old PMTs



Standard Quantum Efficiencies of Detector Technologies





Practical Laser-scanning Confocal Microscopy



Zeiss 780 Inverted Confocal Microscope





- The Zeiss 780 Inverted Confocal is a conventional laser-scanning confocal microscope that has been optimized for high-sensitivity, multi-color 3D imaging.
- This system is equipped with lasers (405, 458, 488, 514, 561, 594, and 633 nm) and detectors for imaging in the visible spectrum (400 < λ < 700 nm).





Zeiss Confocals – Zeiss Zen Software

Locate / Ocular Mode – Widefield (for focusing on specimen and to select are of interest)



Inverted Microscope

Christoffer Lagerholm Wolfson Imaging Facility Weatherall Institute of Moleg	cular Medicine University of Oxford
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Light Path – Zeiss 780







UNIVERSITY OF OXFORD



Designing the light path in confocal microscopy





Christoffer

Single Imaging Track







Multiple Imaging Tracks





Christoffer Lagerholm

Wolfson Imaging Facility

Weatherall Institute of Molecular Medicine

University of Oxford



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Image optimization on a Confocal Microscope – Image size, Scan speed, Bit depth, Scan Direction, Zoom, Averaging

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Image optimization on a Confocal Microscope – Optical Zoom

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Magnification Zoom



6. Magnification Zoom



Nyquist sampling theorem - lateral

- the size of the pixel should be 2-3x smaller than the lateral optical resolution to realize maximum optical resolution



175 x 175



58 x 58







Consequences of not sampling at Nyquist

Oversampling

- pixels small compared to the optical resolution
- specimen needlessly exposed to light
- image needlessly large

Undersampling

- degraded spatial resolution
- photobleaching reduced
- image artefacts (eg. aliasing)





Image optimization on a Confocal Microscope – Laser Power, Pin hole size, Gain (Master), Digital Offset

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Laser Power
 Pinhole





Adjustment of pin hole size for image optimization

Resolution is limited by the point-spread function



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Y





How big should your pinhole be?



Want pinhole to pass entire Airy disk



Х

Airy disk diameter $\approx 1.22 \lambda /NA$

Width of point spread function at pinhole: Airy disk diameter × magnification of lens





- Width of point spread function at pinhole =
 Airy disk diameter × magnification of lens = 1 Airy unit

 resolution of lens × magnification of lens × 2
 - + 100x / 1.4 NA: resolution = 220nm, so 1 Airy unit = 44 μm
 - 40x / 1.3 NA: resolution = 235nm, so 1 Airy unit = 19 μ m
 - 20x / 0.75 NA: resolution = 407nm, so 1 Airy unit = 16 μm
 - 10x / 0.45 NA: resolution = 678nm, so 1 Airy unit = 14 μm







FWHM=Full Width Half-Maximum

Weak signal > open pinhole > more light but thicker section





But beware – Resolution of confocal is improved with smaller pinhole setting – Airyscan/Lightening







Confocal (pinhole=1AU)



Airyscan (pinhole=0.2 AU)



Dr. Ian Dobbie, Antonia Goehler, Alan Wainman, Rainer Kauffman, Micron, Biochemistry, U. of Oxford Dr. Eva Wegel, John Innes Centre (previously Micron, Biochemistry, U. of Oxford)

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Image optimization on a Confocal Microscope – Laser Power, Pin hole size, Gain (Master), Digital Offset

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- 1. Laser Power
- 2. Pinhole

Gain (Master)
 Digital Offset





Adjustment of detector gain/offset for image optimization







Image optimization on a Confocal Microscope – Image Brightness Scan speed, Zoom, Laser Power, Pinhole size, Gain (Master)

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Multi-dimensional Acquisition



MRC Researc Council

 Multidimensional Acquisition 								
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Z-stack



Time-lapse



Tile-scan



Christoffer Lagerholm

Wolfson Imaging Facility





- Advanced confocal microscopy
 - Spinning Disc (Fast Confocal at fixed pinhole size)
 - Multi-photon (Deep imaging)
 - Airyscan (Confocal with 0.2AU pinhole)
 - FCS/FRAP/IRM/DNA damage.....











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Weatherall Institute of Molecular Medicine





Example 4: Another Cell cycle example; HeLa cells expressing Histone 2B-GFP imaged on spinning disc microscope





DNA damage induced with 405 nm laser





Characterization of role of *SNM1A*, a 5'–3' exonuclease, in DNA damage response (Lonnie Swift, Ghadir Almuhaini, Christoffer Lagerholm, Peter McHugh)







MRC Research

Spatio-temporal dynamics of T-cell activation. Selected time-points from a time-lapse sequence following Jurkat T-cell activation on OKT3 antibody-coated glass coverslips showing the organization of TCR- β (green) and ZAP70 (red) as a function of the size of the contact formed with the coverslip (simultaneously visualized by interference reflection microscopy). The data shows how rapidly ZAP70 is recruited to the contact, and how quickly the TCR begins to form clusters. However, the experiment also emphasizes that these processes are uncorrelated, at least early on, contrary to accepted belief. The image was generated by





Which imaging technique should I use?





Useful online links



• Zeiss – Microscopy from the very beginning

http://zeiss-campus.magnet.fsu.edu/index.html

Molecular Expressions homepage

http://micro.magnet.fsu.edu/

 Alison J. North. Seeing is believing? A beginners' guide to practical pitfalls in image acquisition. JCB Volume 172(1):9-18 January 2, 2006