Detectors for imaging
How PMT’s work

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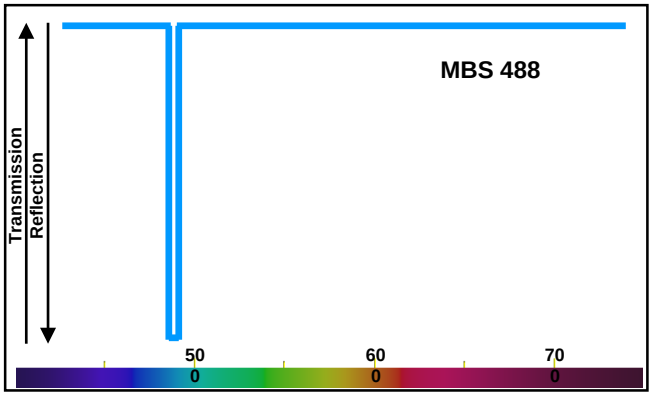
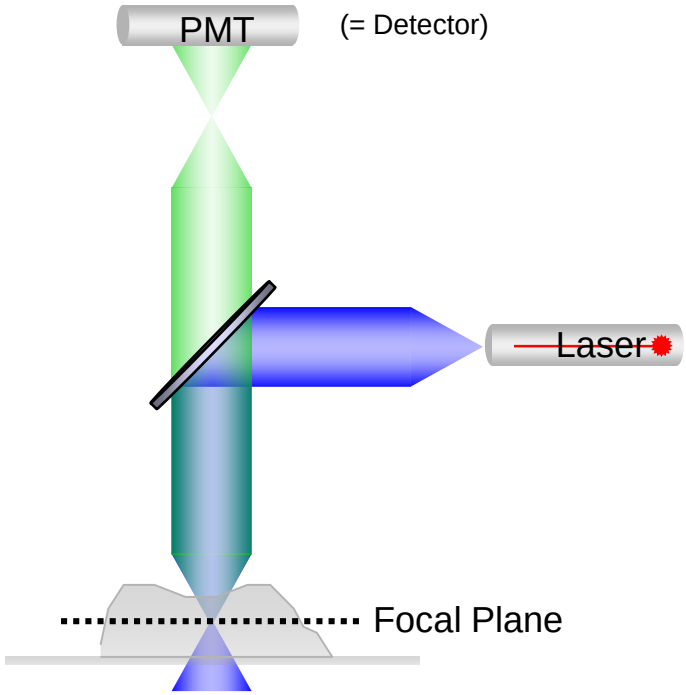
Confocal Point Scanning
Laser – Scanning - Confocal

Lasers are:

- Monochromatic so better for specific fluorescence
- Focusable to a single spot

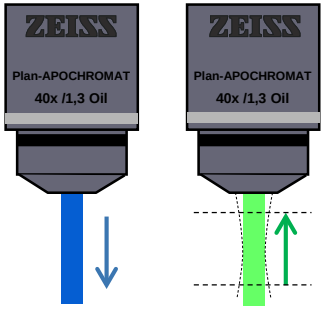


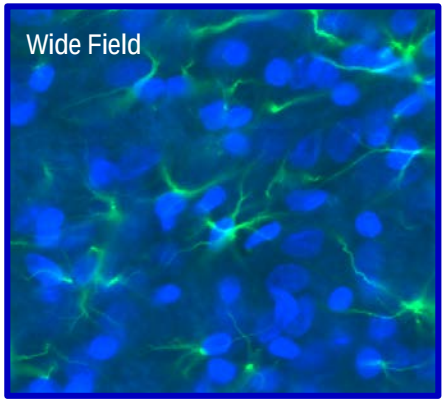


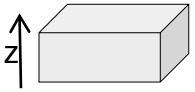
Widefield versus Confocal
Laser – **Scanning** - Confocal

Wide Field

camera



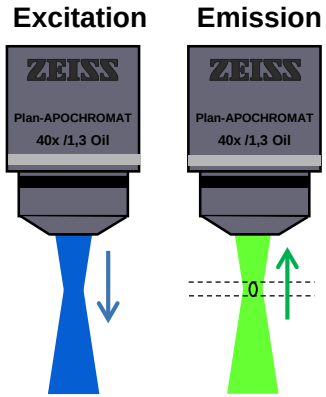


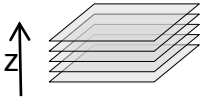


limited z-resolution
thick sections
(out-off-focus haze)

Confocal

scanning

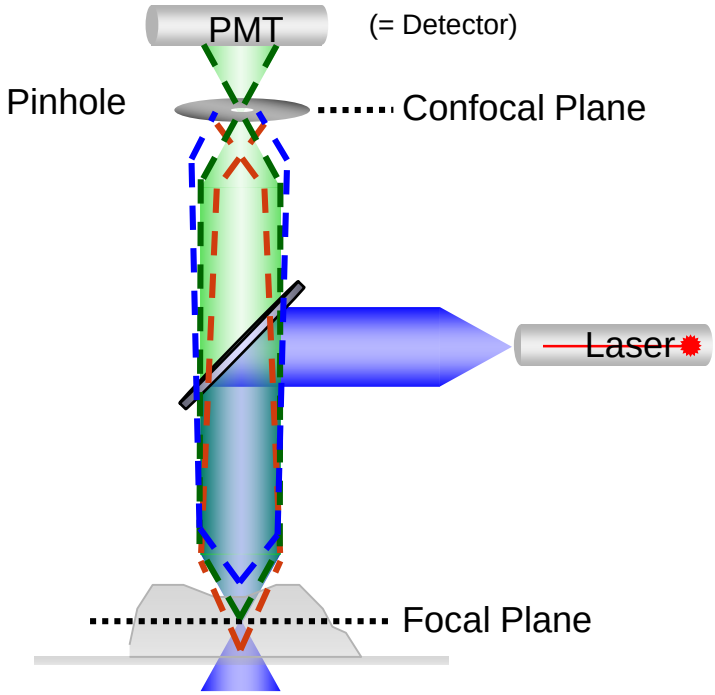




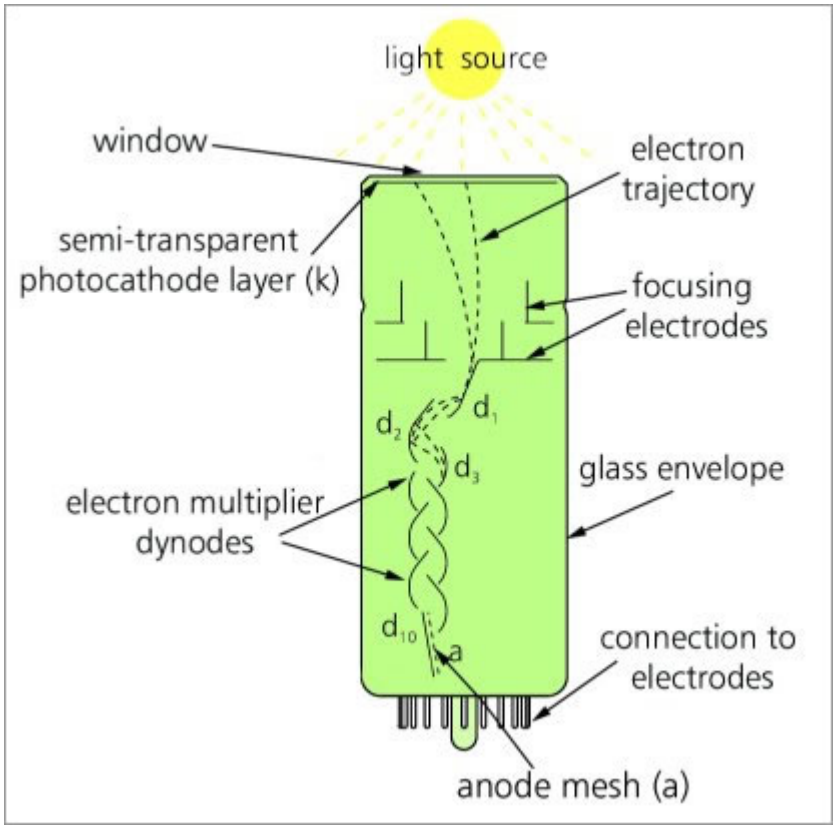
high z-resolution
3D via sectioning
(haze removed)

Confocal Point Scanning
Laser – Scanning - **Confocal**

A minute diaphragm, situated in a **con**jugated **focal** plane, prevents out of focus light from being detected.



How PMT's work
The basic principles



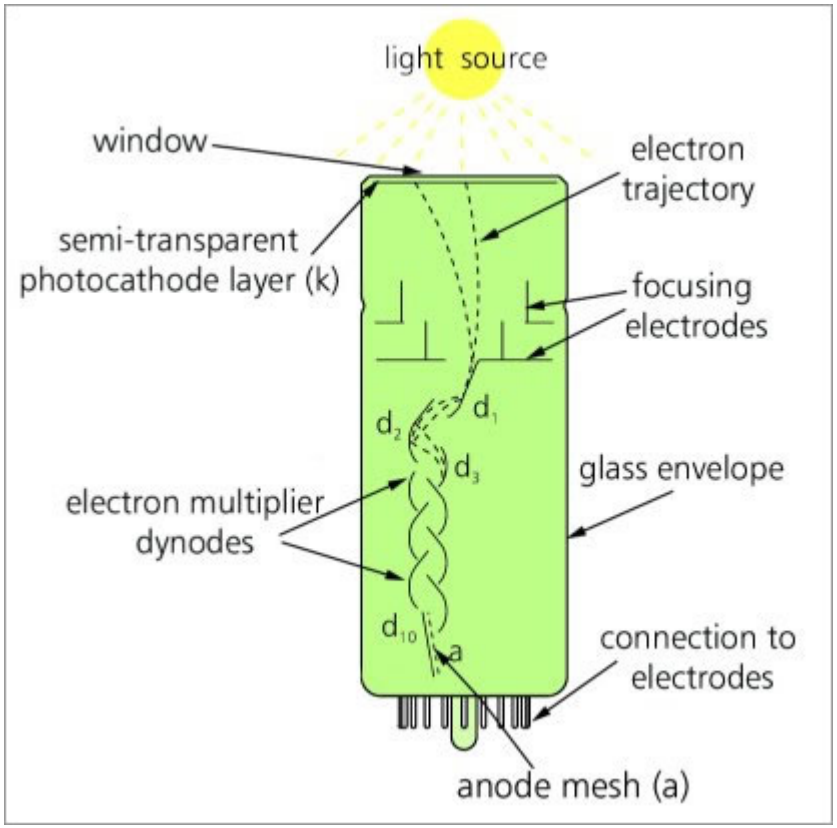
PMT's utilise 2 effects:

• **Photoelectric effect,** which is that electrons can be emitted from materials when they absorb energy from light.
Discovered 1887 by Heinrich Heinz but used experimentally by Albert Einstein in 1905 leading to the 1921 Nobel prize for "The discovery of the law of the photoelectric effect".

• **Secondary emission** – A high energy particle can induce the emission of secondary particles.
Discovered in 1902 - Austin and Starke

• Both effects combined in 1934 by RCA – Harrison NJ.

How PMT's work
The basic components



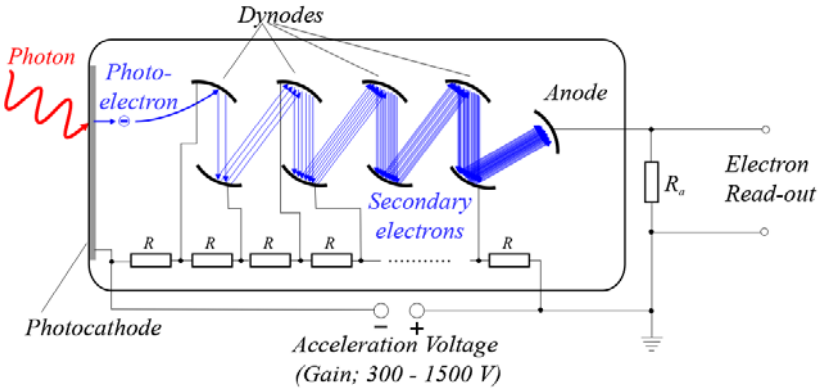
Photons hit the **Photocathode** which emits electrons by the photoelectric effect

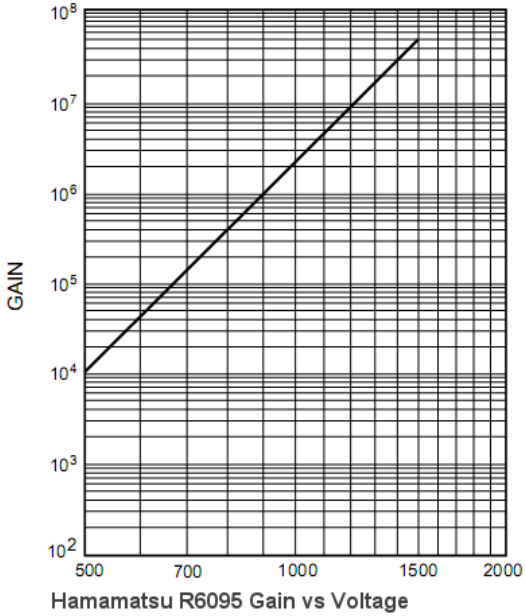
The photoelectrons are electrostatically accelerated and focused (and sometimes shuttered) by **focussing electrodes.**

The electrons impact the **dynode** and liberate a number of secondary electrons which in turn are electrostatically accelerated and focussed onto the next **dynode** in the chain. The max gain per dynode is typically about 25x

The secondary electrons from the last dynode are collected at the **anode** where they can be measured.

How PMT's work
Adjustments





Typically only 2 adjustments can be made:

1. **Gain (Actually voltage)** - The ratio of secondary to primary electrons emitted at each dynode depends on the energy of the incident photons and is controlled by inter-electrode potentials.

Changing the gain in the middle ranges does not change sensitivity, it just changes amplification

2. **Offset –** A residual background current at the anode is usually always present but can be subtracted. Some modern LSM's calibrate this automatically

Also:
Analogue gain *– Old fashioned multiplication of signal using analogue electronics which introduces noise. Phased out about 15 years ago.*
Digital gain *– Multiplication of signal after signal digitised, useful in multi array detectors.*

Key factors effecting performance of PMT's

Factors effecting sensitivity and gain:

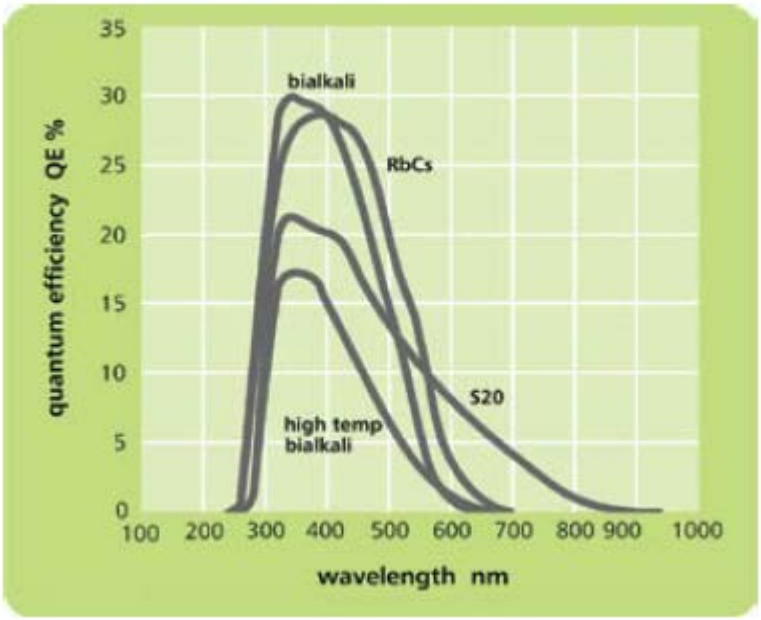
- Size of Photocathode = Large is easy to hit so good for NDD's but has a higher background
- Fill factor – If light missed the detector, a factor in older multi array detectors
  e.g. LSM 510 Meta only 80% versus 98% on the LSM 980
- Window material = Usually borosilicate glass, only worth changing for deep UV work.
- Photocathode compounds = (More detail on coming slide)
- Window arrangement = 'Head on' versus 'side on'. Side on designs are more robust and typically used in LSM
- Number and arrangement of dynodes
- Age = (More detail on coming slide)

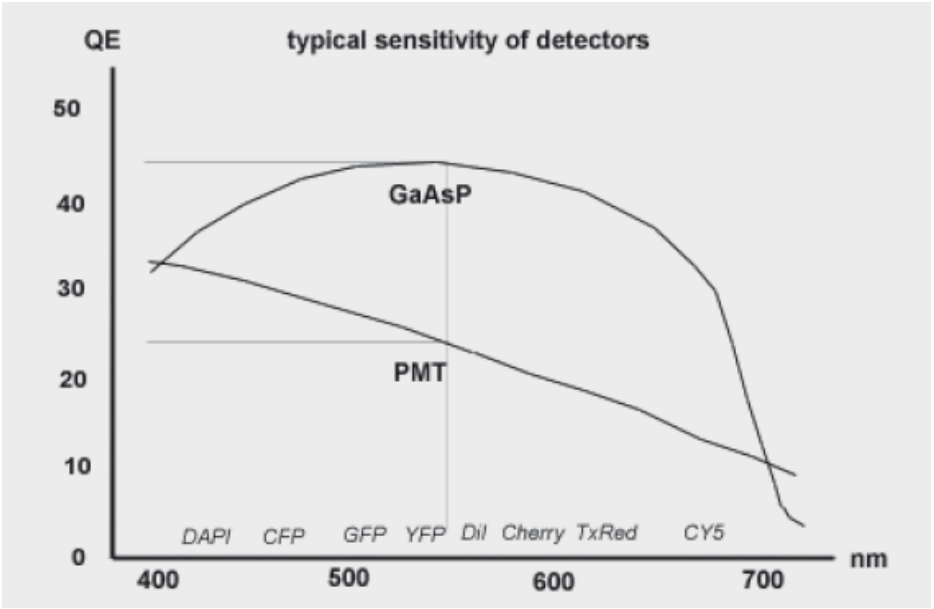
Contributors to noise:

- Dynode design
- Strong magnetic fields, or weak shielding – noise when mobile phones near
- Absorbed Helium
- High temperatures (More detail on coming slide)
- Cosmic rays
- Previous exposure to bright light

Key factors effecting performance of PMT's
Photocathode compounds

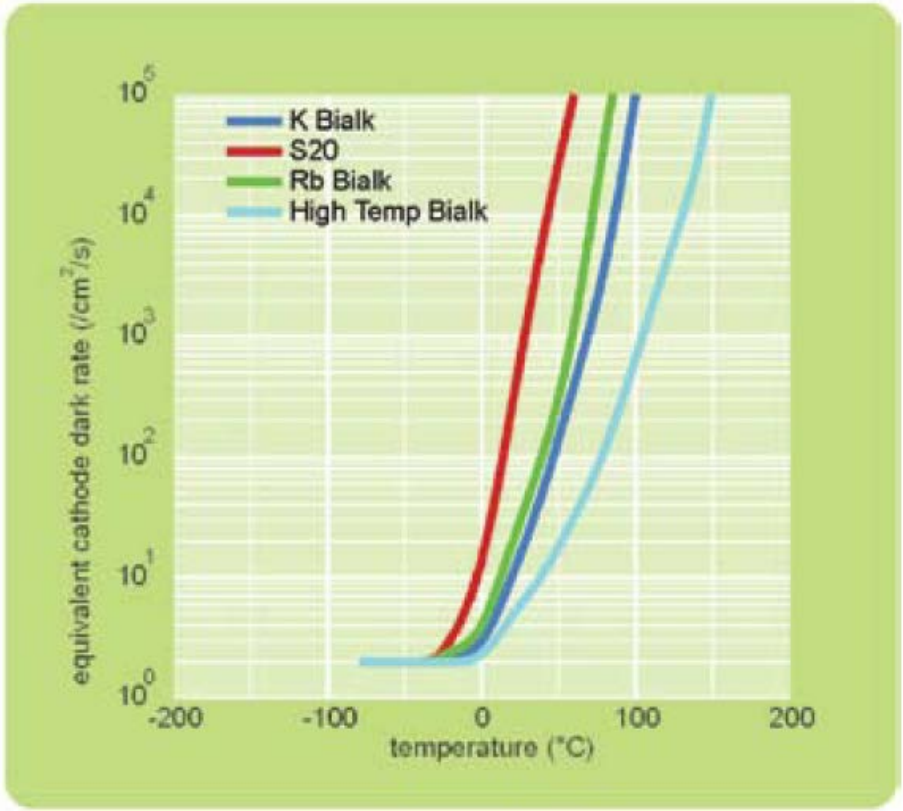
- Photocathode compounds = Different combinations of materials have different band gaps and sensitivities
- In 1935 the peak QE was 0.4% at 800nm (silver Oxide-caesium)
- In 1936 changing to caesium-antimony gave 12% at 400nm (1st commercial design)
- 1953 Hamamatsu founded
- Since 2003 GAllium ArSenide Phosphide (GaAsP) designs yield 45 to 56% QE





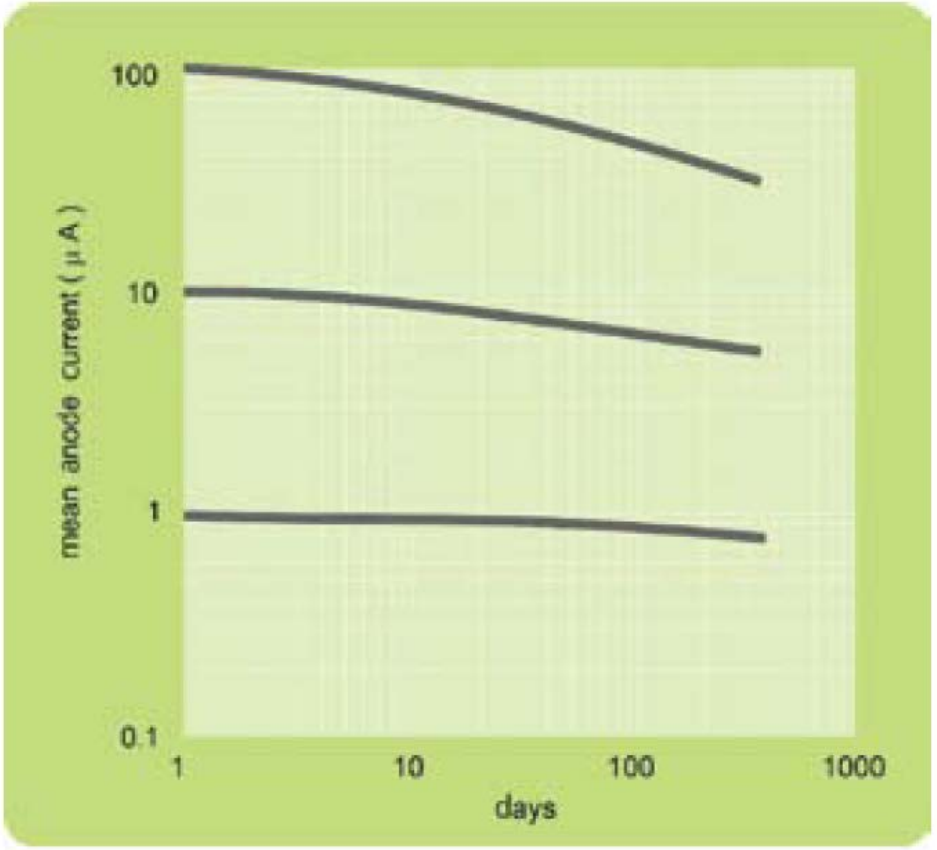
Key factors effecting performance of PMT's
Temperature

- At room temperature thermally generated electrons from the cathode dominate
- For all cathode types cooling below -25 gives no advantage
- For LSM work with a small photocathode area even working at room temperature will yield almost undetectable noise levels



Key factors effecting performance of PMT's
Age

- Its not so much the age as the mileage
- Illustrating long term stability (1 year) for SbCs dynodes as a function of mean anode current, under conditions of constant applied voltage and illumination.
- PMT sensitivity can be tested and checked.



Practical advice

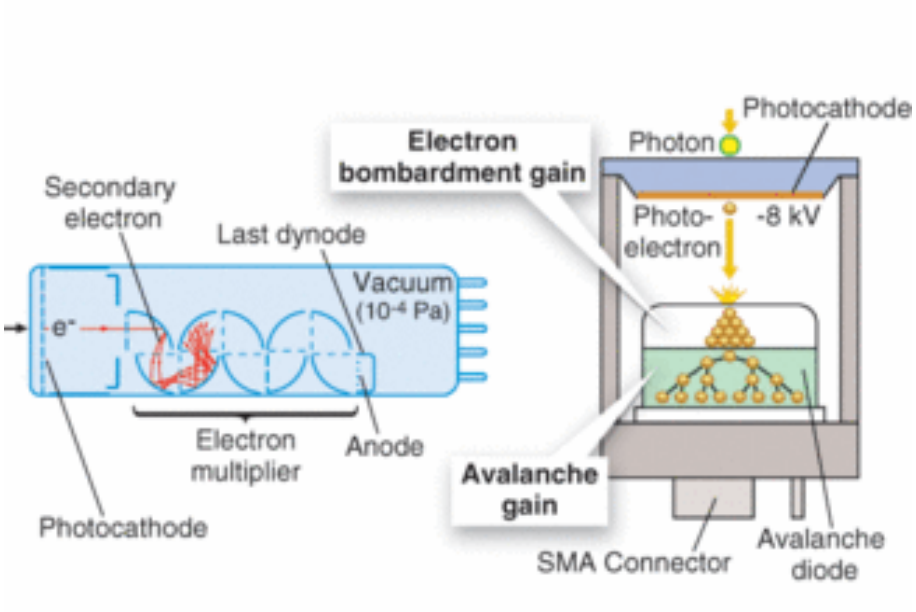
Day to day

- Protect the PMT from heavy oversaturation when in use – Some designs shut down to protect themselves
- Try to avoid very high gain levels, at least for prolonged periods
- Protect the PMT from light when not in use
- Keep room temperatures under 30 degrees C

Usually not a big concern:

- Keep away from strong magnetic fields (be wary of phones)
- Keep away from cosmic rays (deep in a building basement or underground)
- Keep away from loose helium

Detector variants

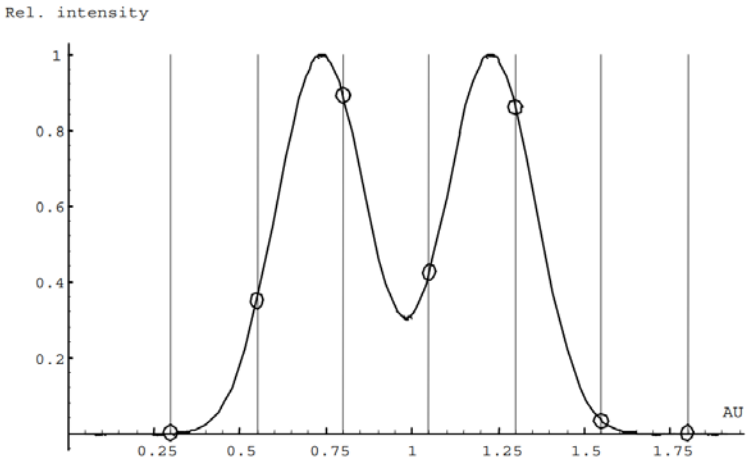


PMT (photomultiplier, based on anode/cathode technology)
APD (avalanche photodetector, based on avalanche diode)

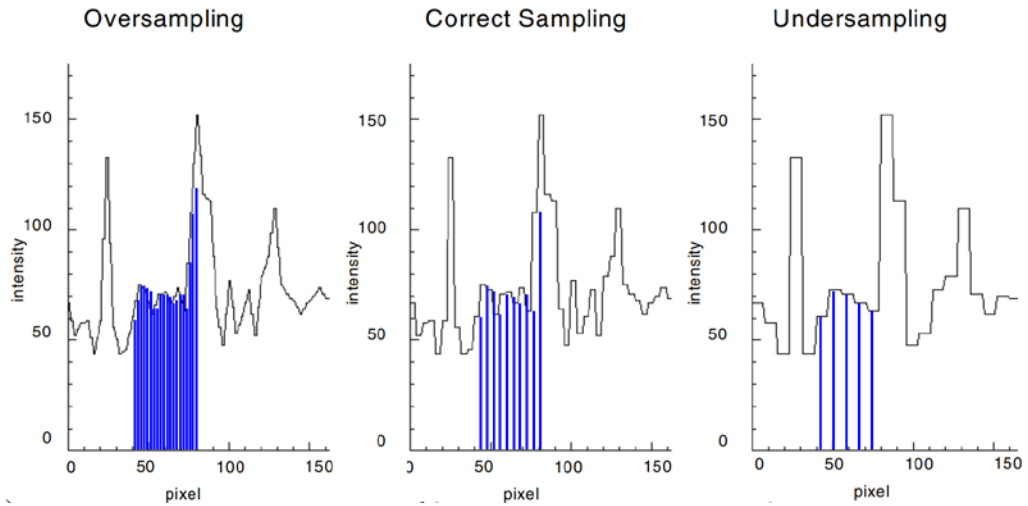
- PMT: PhotoMultiplier Tube
  - Most common used detector for confocal microscopy
  - Good light collection
  - Integration of photons -> low shot noise
  - Difficult to calibrate (though possible)
  - Broad spectrum coverage
  - Many different variants including GaAsP
  - Best dynamic range options
- APD: Avalanche PhotoDetector
  - Very fast responses
  - Typically used for photon counting
  - More narrow spectral coverage
  - Very high QE
  - Very limited count rate
- Hybrid detectors
  - Front end of a PMT, back end of an APD
  - The QE of a PMT – e.g. GaAsP
  - Very good time response for FLIM

Sampling

PMT's allow arbitrary pixel sizes – To Match Nyquist sampling



The graph illustrates the scanning of a two-point object with the minimum number of sampling points needed to avoid a loss of resolution (spacing of sampling points 0.25 AU).



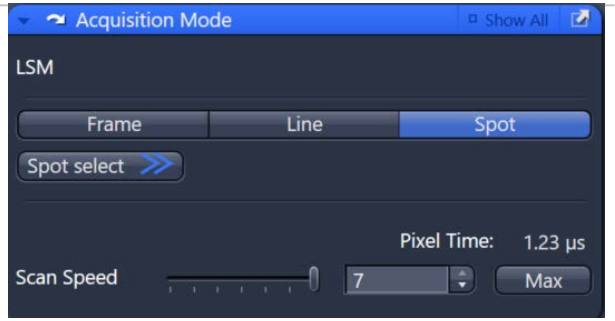
System can automatically recommend the correct pixel size knowing the magnification, field of view (Zoom) and wavelengths used.

Sampling

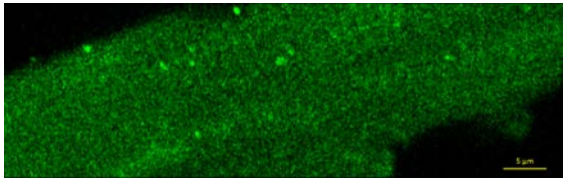
PMT's allow arbitrary pixel formats

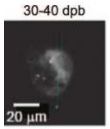
31x1 to 8192x9192 pixels
Up to 100's of fps

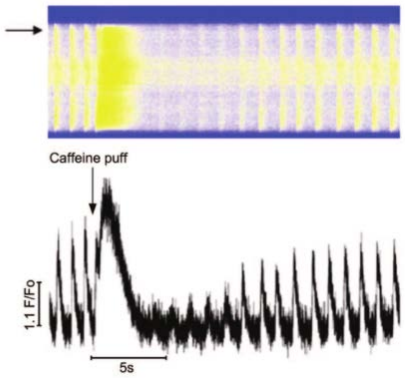
Maximum frequency of 6830 lines

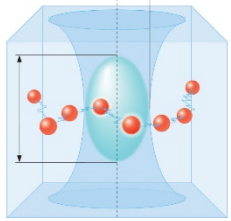


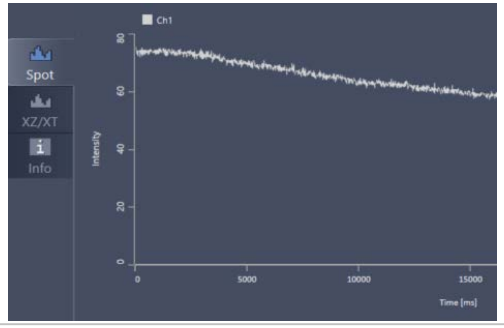
Maximum frequency of 813,008
(1.23 microseconds)











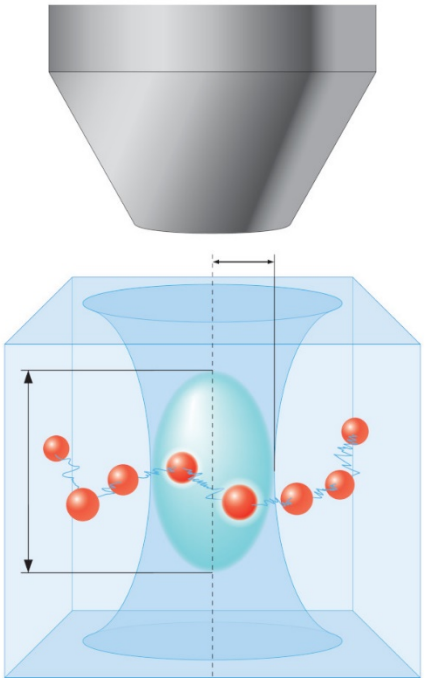
Cardiomyocyte Cells Loaded with Fluo4
Images and Samples Courtesy of
Ben Prosser, UPENN

Quantitative measurement of protein dynamics and interaction

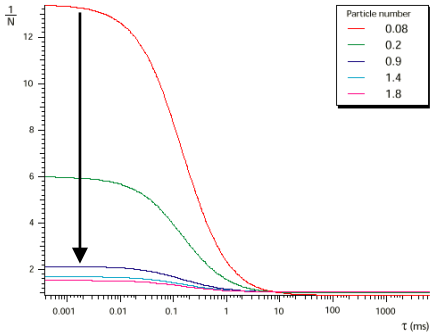
Fluorescence Correlation Spectroscopy

Objective Lens

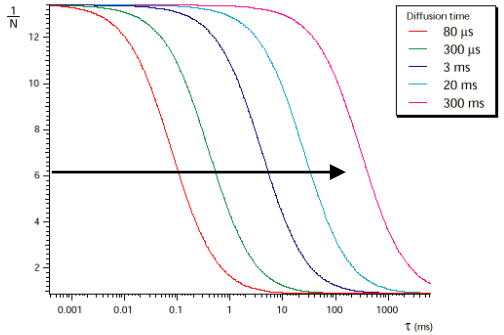
Excitation Volume



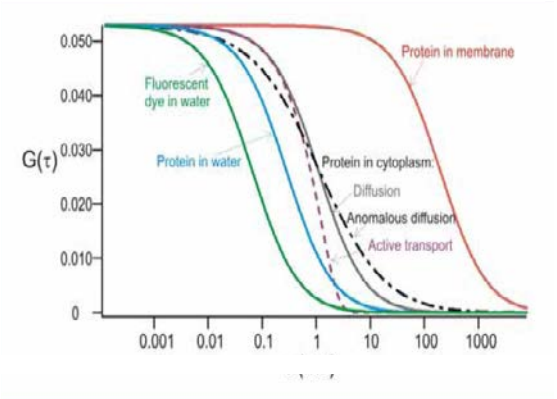
1. Concentration



2. Diffusivity

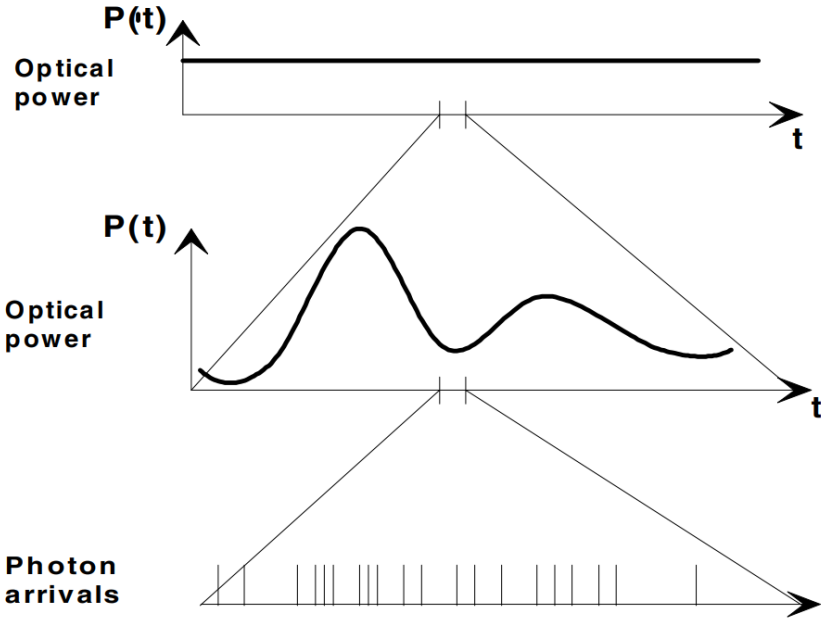


3. Type of Movement



FLIM

Time-Correlated Single Photon Counting



The quantum nature of light can be made visible in two ways:

a) by reducing the intensity down to the order of single photons

And

b) by shortening the observation time, despite high intensity.

The graph above illustrates case (b) – by cutting down the observation time, it is possible to resolve individual photons of the light flux in their irregular (statistical) succession.

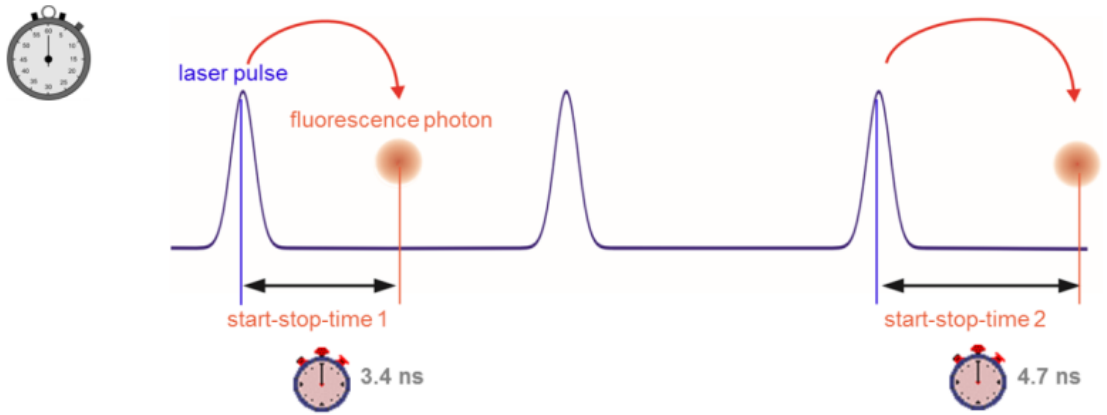
The ability of PMT's to count this quickly allows photon timings to be measured, just consider jitter, delays, after pulsing and count rate

Counting method -

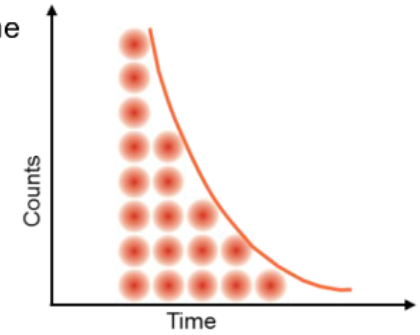
- Photon counting = Better for weak signals
- Analogue integration = Better for brighter signals
- Some detectors have both modes

FLIM

Time-Correlated Single Photon Counting



- Repeated measurement
  - Histogram with Counts/Time
  - Fluorescent decay curve



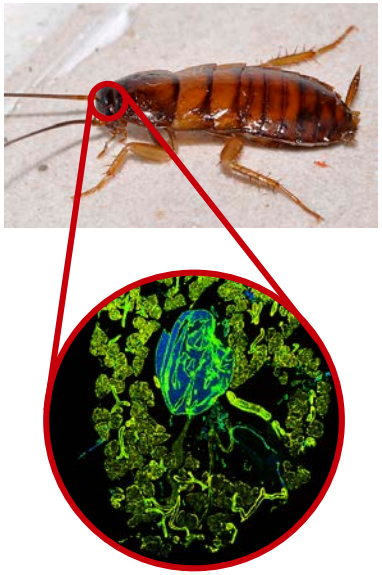
Requirements:

- Pulsed laser: single or multi-photon
- Single-photon detector (e.g. GaAsP PMT, HyD, APD)
- TCSPC electronics
- Software

FLIM

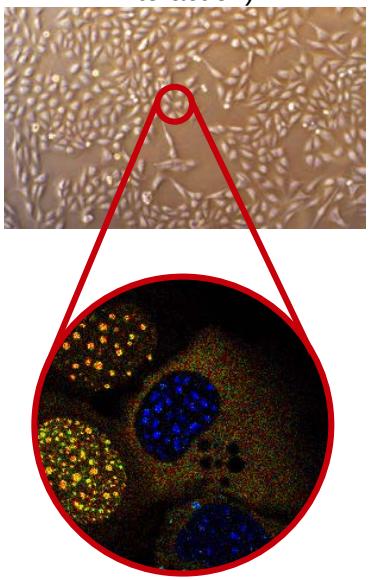
Application example: single wavelength probe

Environmental sensing
(pH, ion, oxygen, lipid)



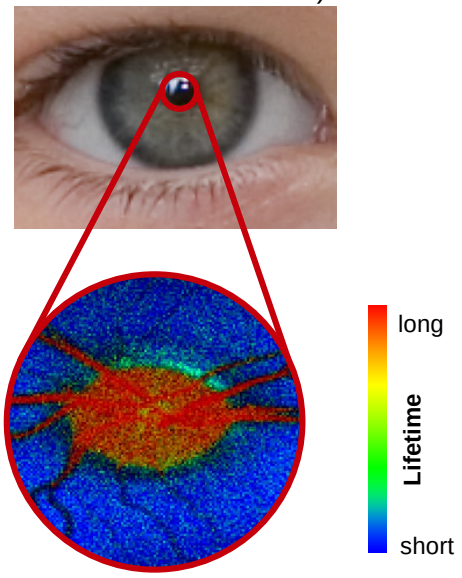
Carsten Hille, Carsten Dosche, Potsdam University, Germany

FLIM-FRET
(intra- and intermolecular interaction)



Courtesy of Sandra Orthaus, former member of Leibniz Institute for Age Research, Jena

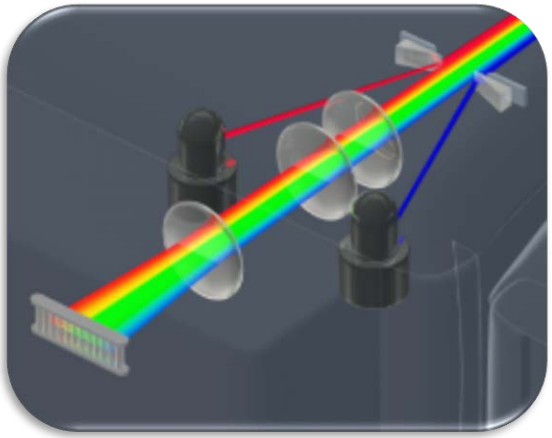
Medicine
(anatomical structures, disease-related markers)



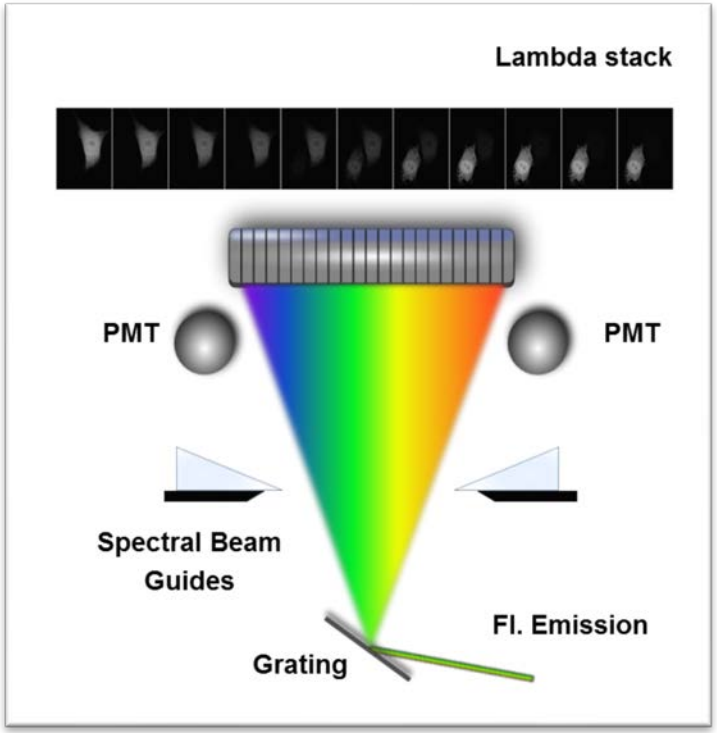
Courtesy of D. Schweitzer, Augenklinik FSU Jena

LSM 980 with Quasar detection

Versatile GaAsP Spectral Array Overcomes Crosstalk with a Single Scan



Spectral Unmixing to Overcome Crosstalk



LSM 980 with Quasar detection

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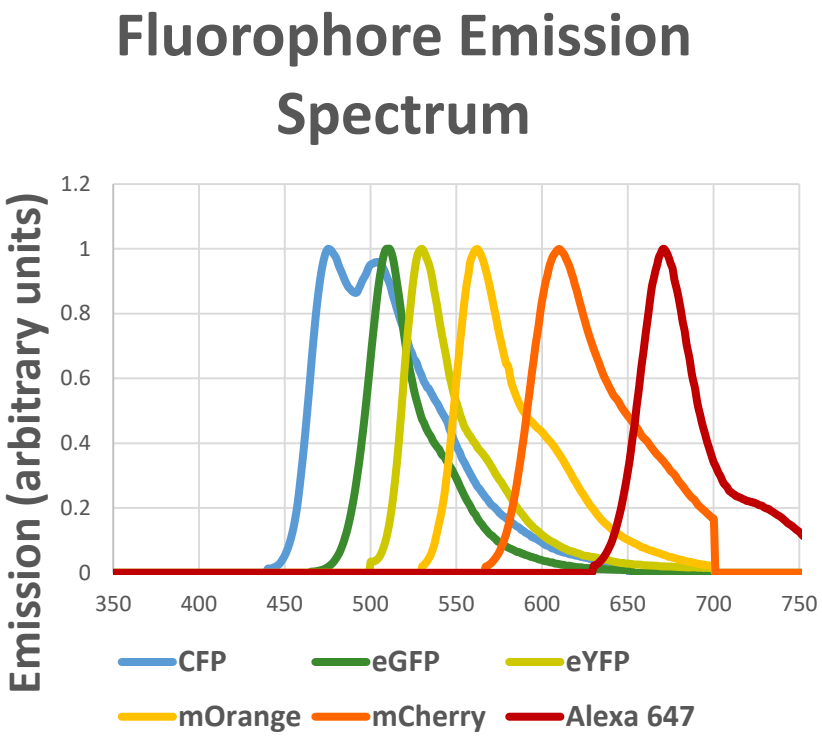

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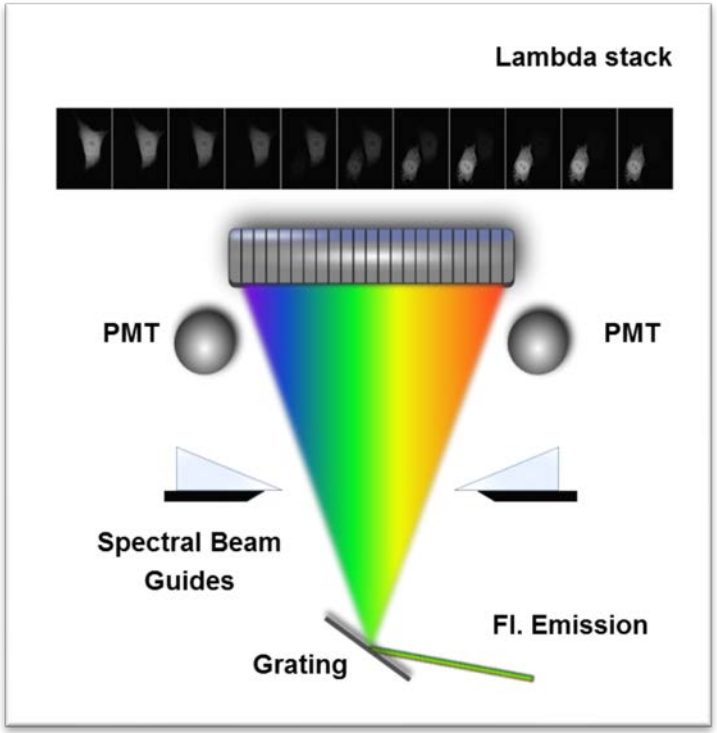
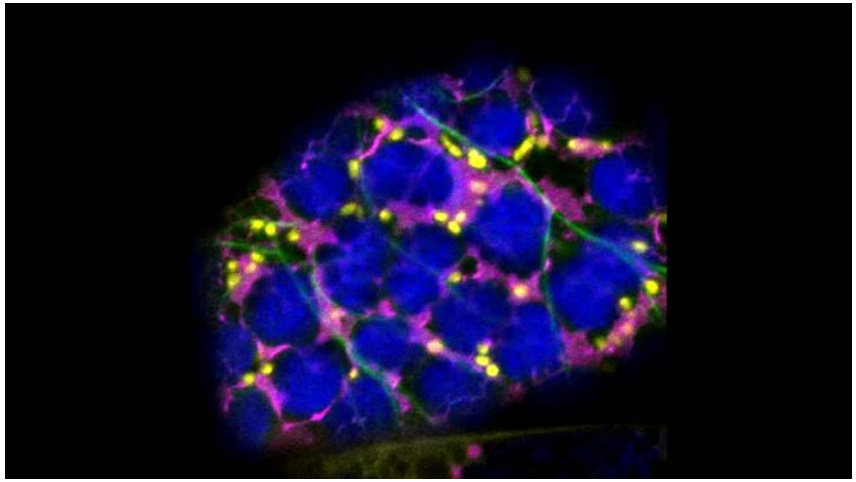
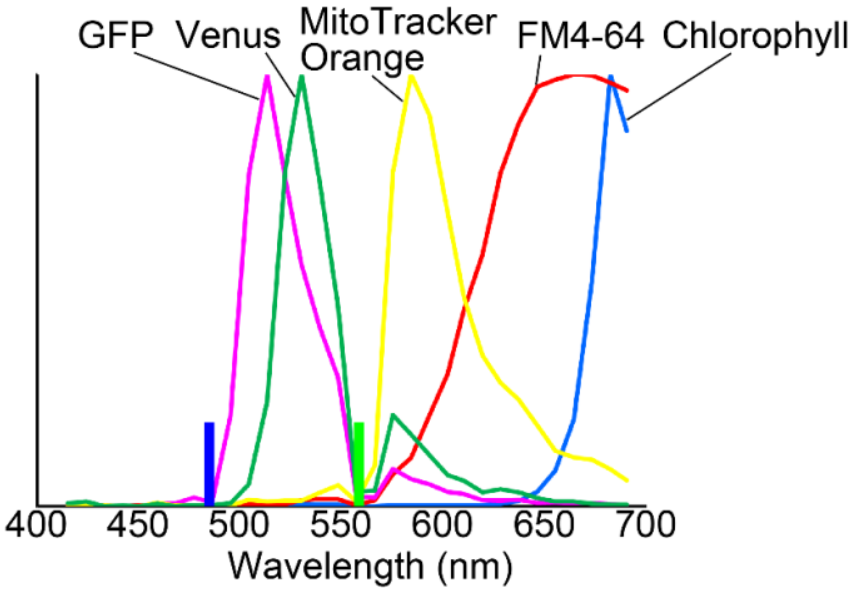


Simultaneous Spectral Imaging of Living Plant Cells

Realtime Separation of 4 Fluorophores in Presence of Chlorophyll





Application Note: Spectral Imaging: a Powerful Tool for Confocal Multicolor Imaging in Living Plant Cells: www.zeiss.com/microscopy

PMT’s versus Cameras

	Cameras	PMTs	APD
Quantum Efficiency	60-95%	20-56%	80%
Noise sources	Read Noise – noise of reading the signal - fixed	NA	
	Dark Current – noise from heat - exposure time and temperature dependent	Very low	
	Photon Shot Noise – square route of signal - signal dependent	.--> Same	
	Excess Noise Factor – EMCCD	NA	
	Clock Induced Charge – All but mainly observed in EMCCD	NA	
	Random Telegraph Noise - CMOS	NA	
Gain	1 to 10^3 for EMCCD	10^6 to 10^8	100 to 1000
Pixel formats	Fixed, typically 0.5 to 16 Megapixels	Freely configurable from 1 pixel to 64 Megapixel	
Frame rates large pixel format	Typically 10s to 100's	Single to 10's of frame per second	
Frame rates small pixel format	Up to Hundreds	Hundreds, thousands or more with line and pixel scanning	
Bandwidth	Mhz to Ghz	10s to 100s of Mhz	Low, 1Mhz
Dynamic range	Medium	High to very high depending on type	Low
Photon counting / FLIM?	Modulation only	TCSPC	
Fill factor	70-95%	95 to 100%	
Used in	Widefield, Spinnng Disk, Structured Illumination, TIRF, Lightsheet etc…	Confocal, Multi Photon	

PMT's versus Cameras

PMTs have much better SNR at the light levels in confocal than cameras, despite the QE difference

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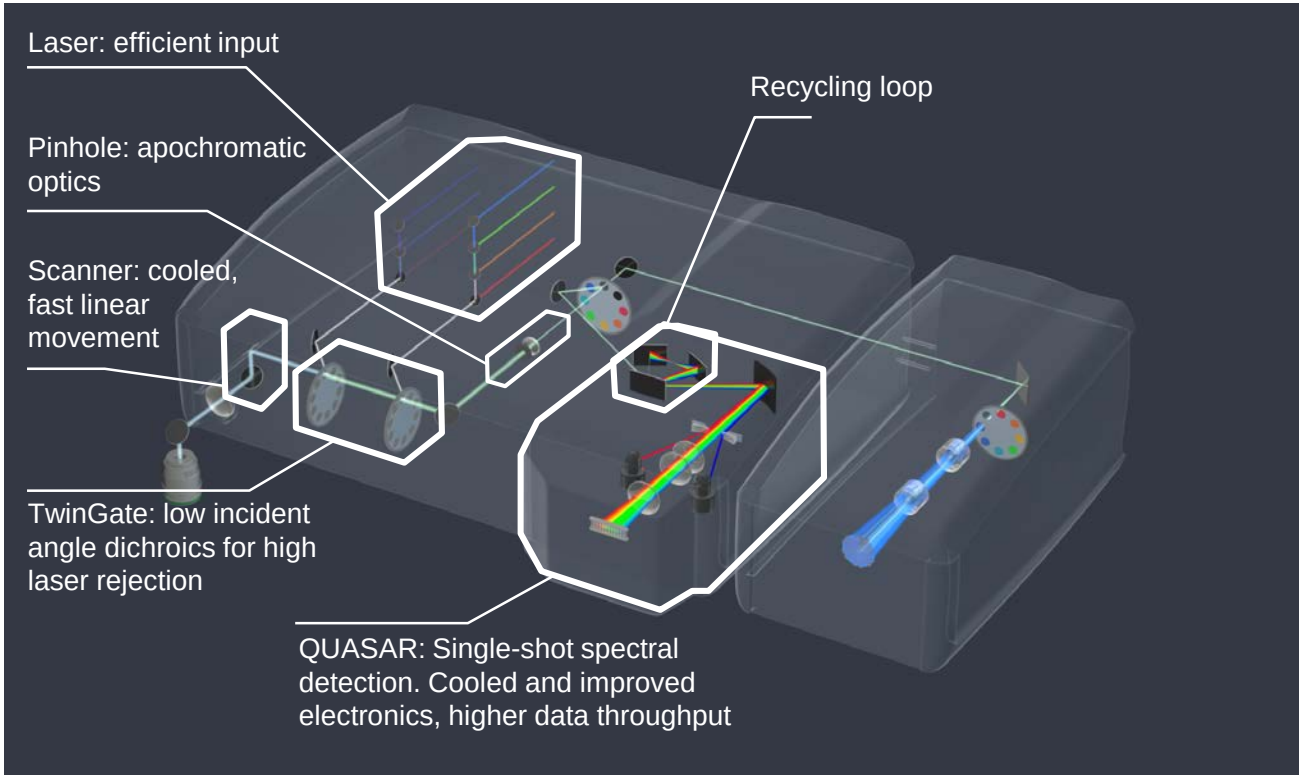
e.g. Image at 1024x1024 @ 1fps = 1,048,576 pixel per second

Camera read & dark noise = 1.6e^- per pixel* + 0.15*e^-/p/s = 1.75e^-* per pixel
1.75 x 1,048,576 = 1,835,008 false counts, roughly the same as shot noise of 3 photons per pixel

PMT read noise = Multi Alkali – Typically 3,000*e^-/p/s and GaAsP 800e^-/p/s*
The camera noise is 2,294x larger than a GaAsP

* = *This is true for full frame but is much higher for cameras run in the Mhz range and used as a point detector*

LSM 980
A scanhead build for efficiency



Carl Zeiss Microscopy, Chris Power, BioSciences

Importance of Gentle Imaging
Sensitive Imaging System

nature

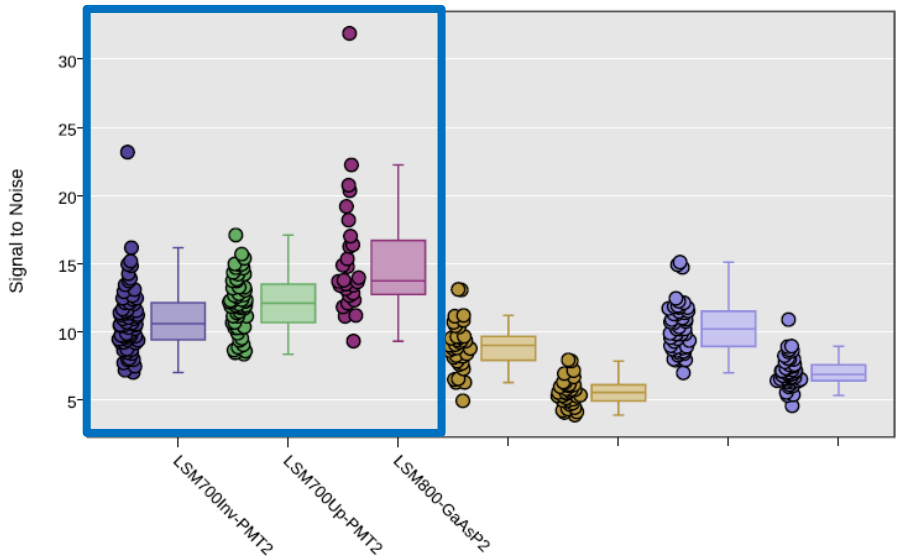
Using the NoiSee workflow to measure signal-to-noise ratios of confocal microscopes

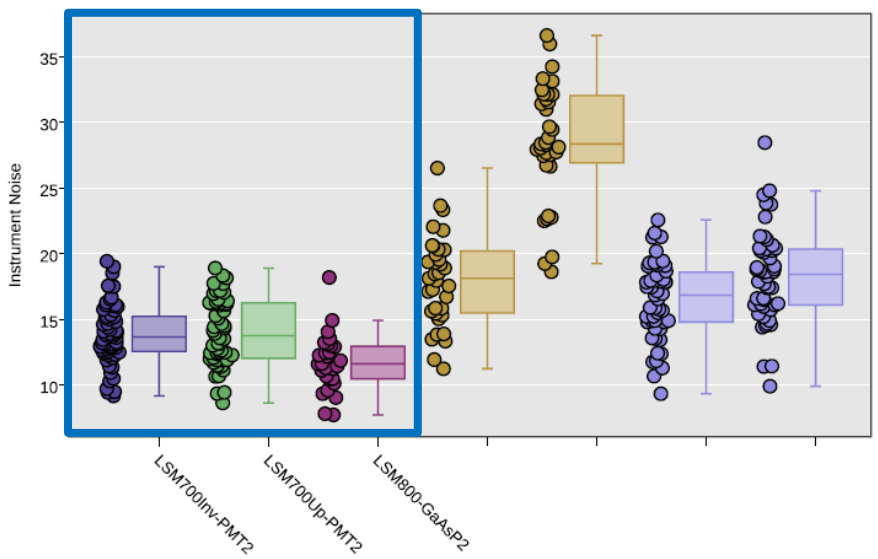
Alexia Ferrand, Kai D. Schleicher, Nikolaus Ehrenfeuchter, Wolf Heusermann & Oliver Biehlmaier

Sensitive Imaging

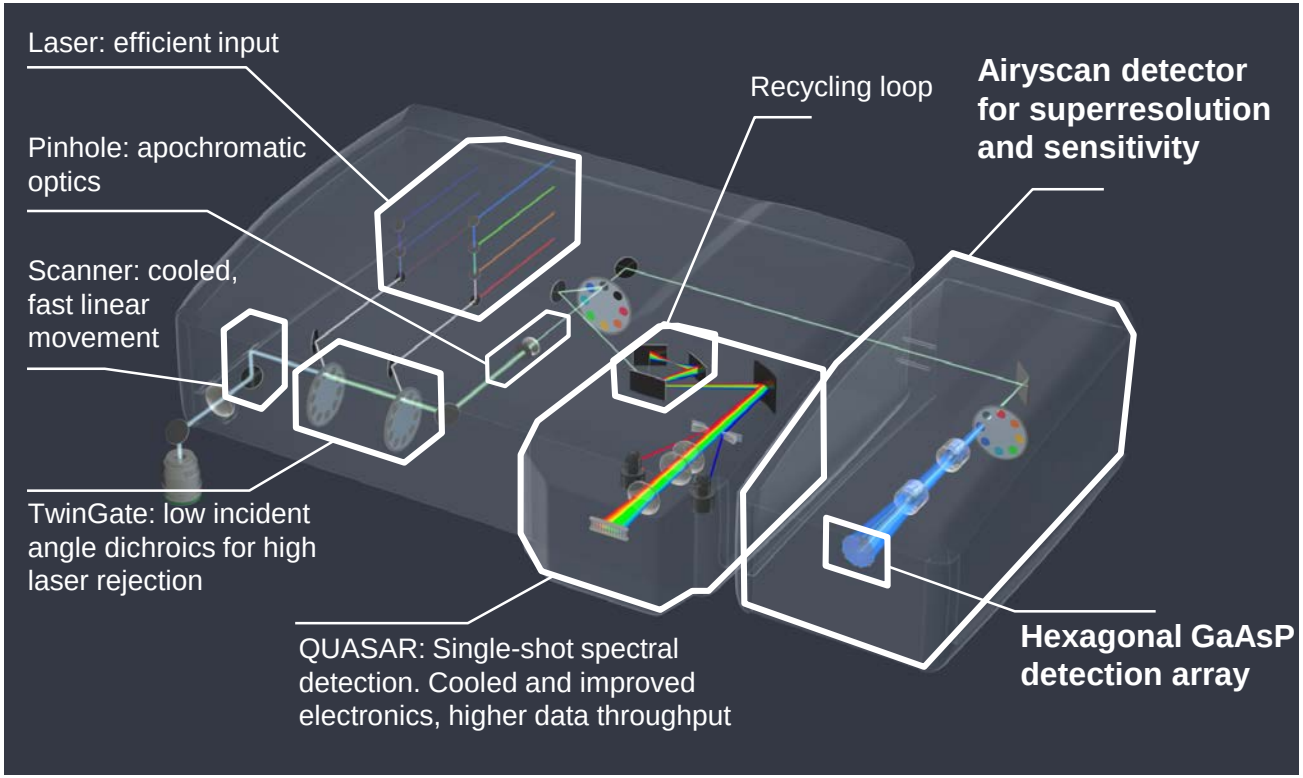
Highest Signal to Noise

Lowest Instrument Noise





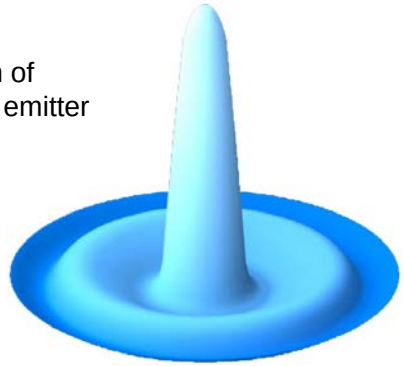
LSM 980 – Airyscan Detection
Superresolution, sensitivity and speed



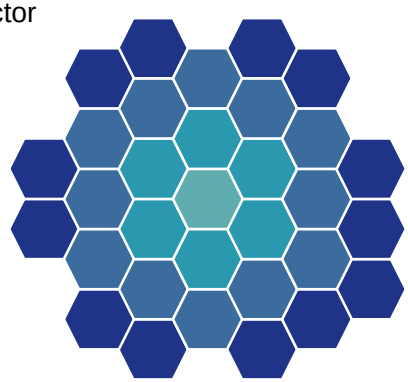
Carl Zeiss Microscopy, Chris Power, BioSciences

Airyscan
Take advantage of spatial information

Airy pattern of
a point-like emitter



Array detector
of Airyscan



The offset of individual detectors to the optical axis provides **additional spatial information** in Airyscan (detectors of a „conventional“ LSM just integrate all light passing through its pinhole).

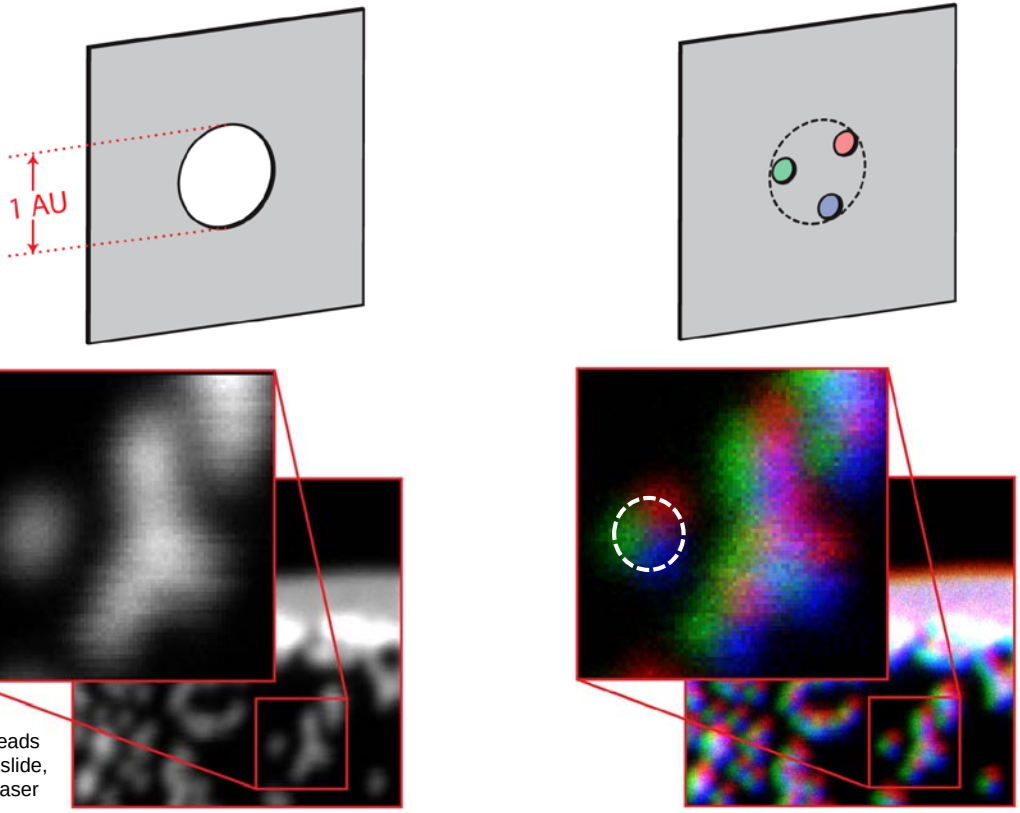
Linear deconvolution assigns all signals (and frequencies) recorded by individual detector elements to their appropriate locations.

Result:

Isotropic 1.7-fold increase in resolving power!

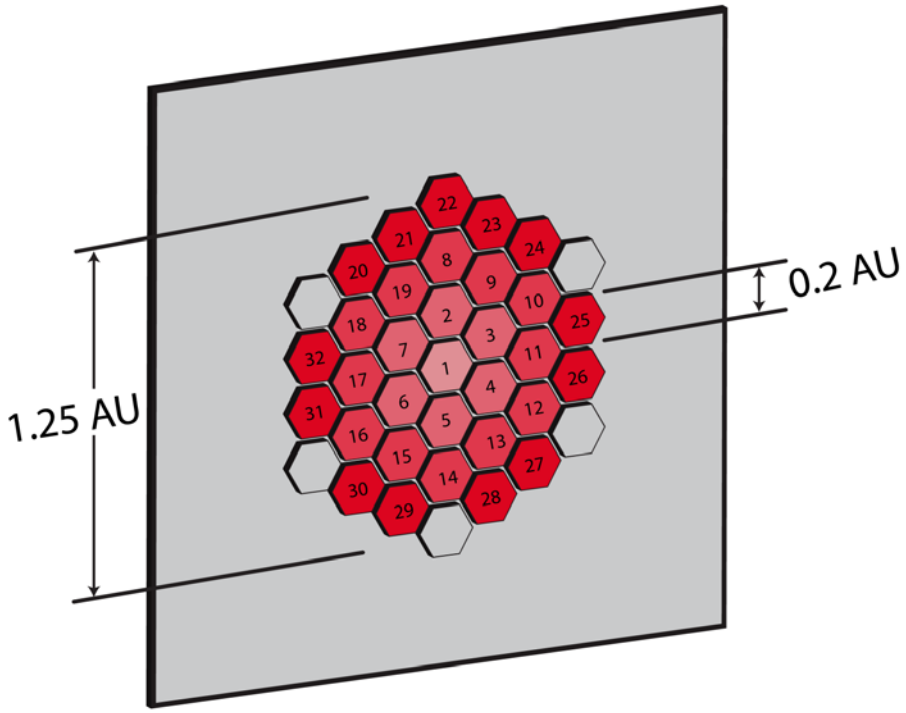
(Further reading: White paper on Airyscan)

Airyscan
Take advantage of spatial information



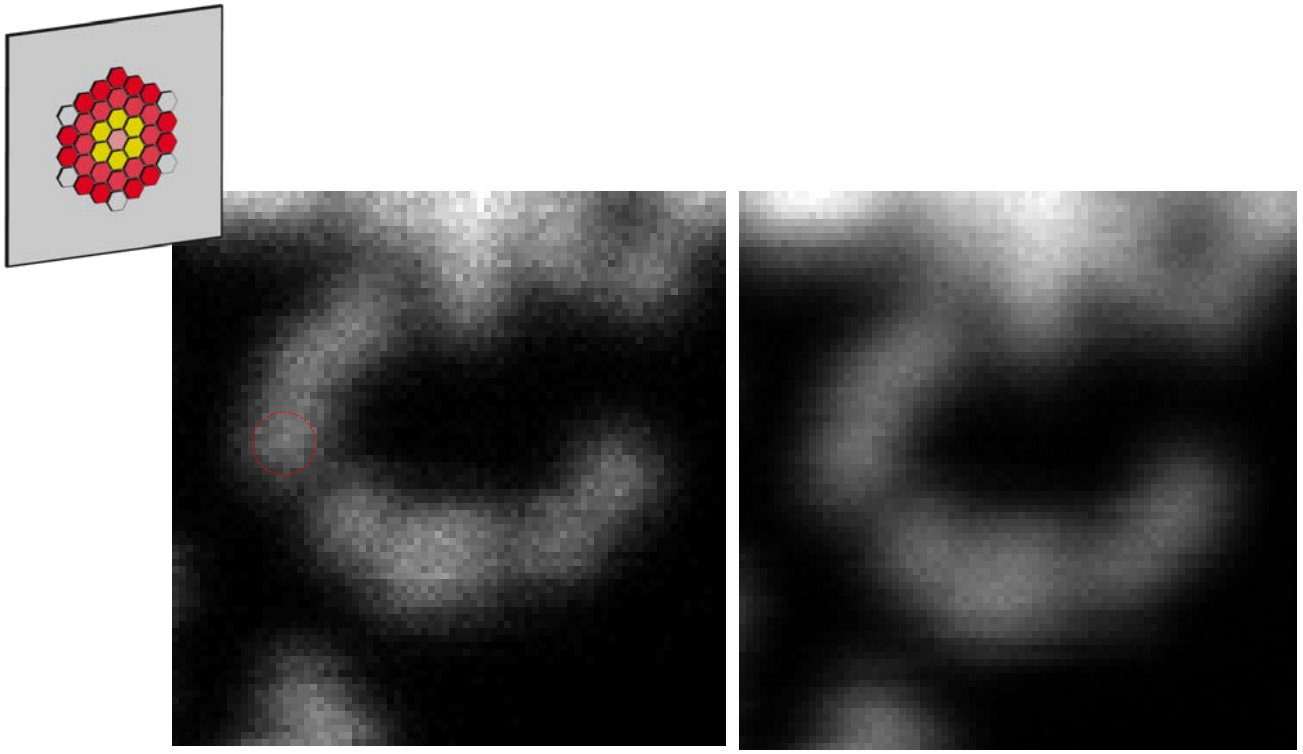
170nm fluorescent beads
adsorbed on a glass slide,
Imaged with 633nm laser

Airyscan
Take advantage of spatial information

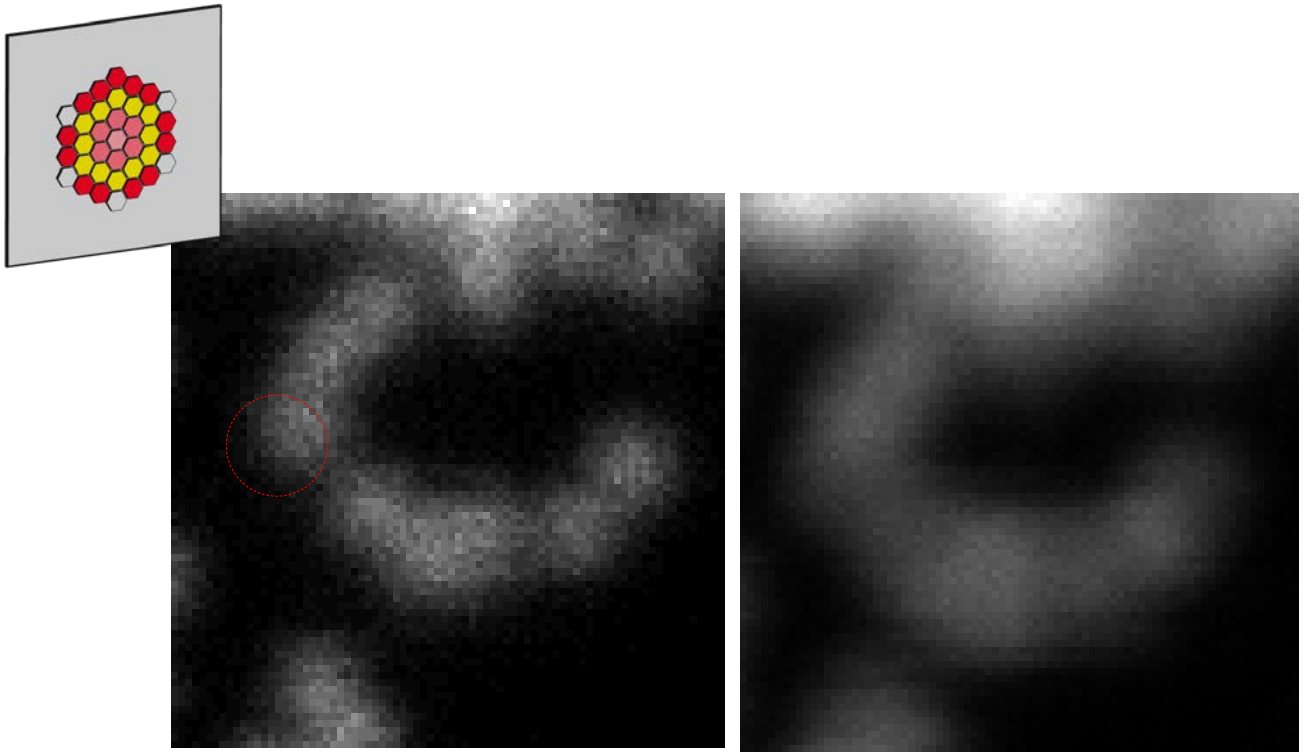


- 32 GaAsP detectors in hexagonal lattice
- Each detector approx. 0.2 AU in diameter
- Total detection area approx. 1.25 AU in diameter
- **Simultaneous improvement in resolution and signal!**

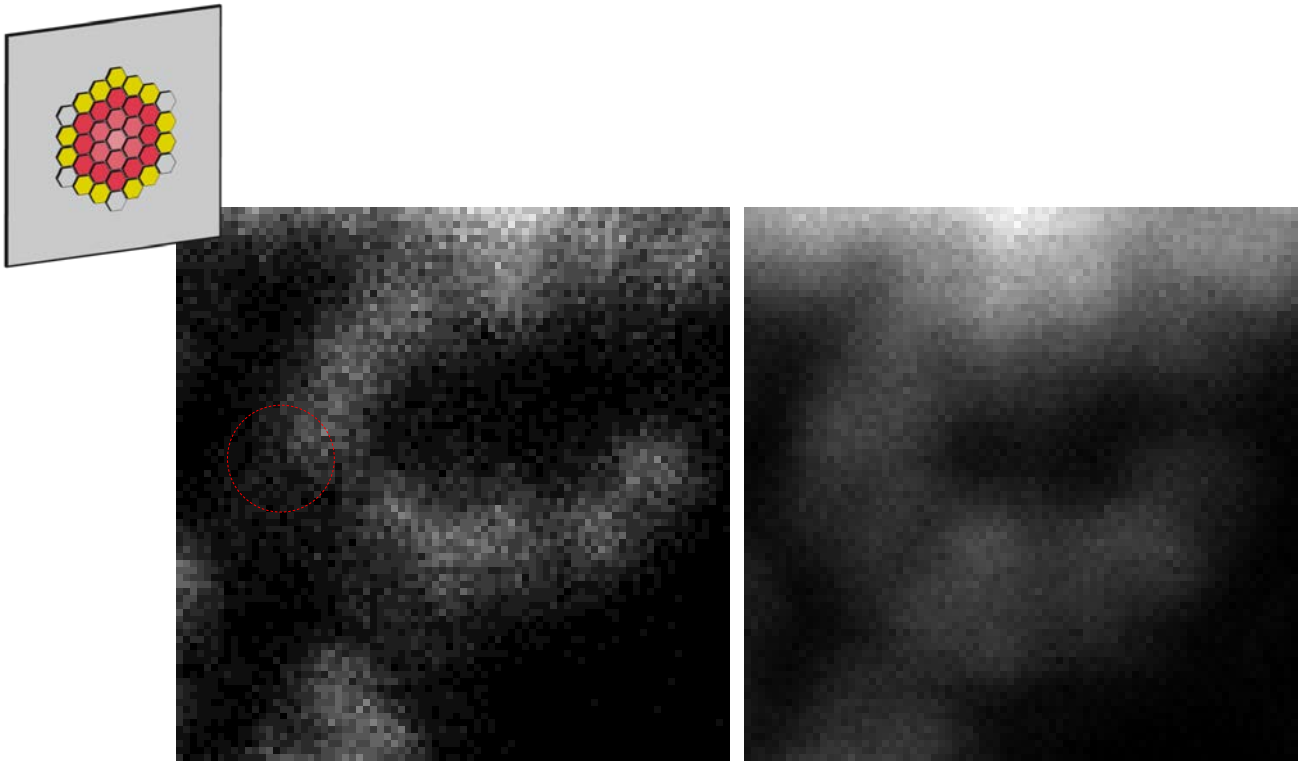
Airyscan detection
1st ring of detectors



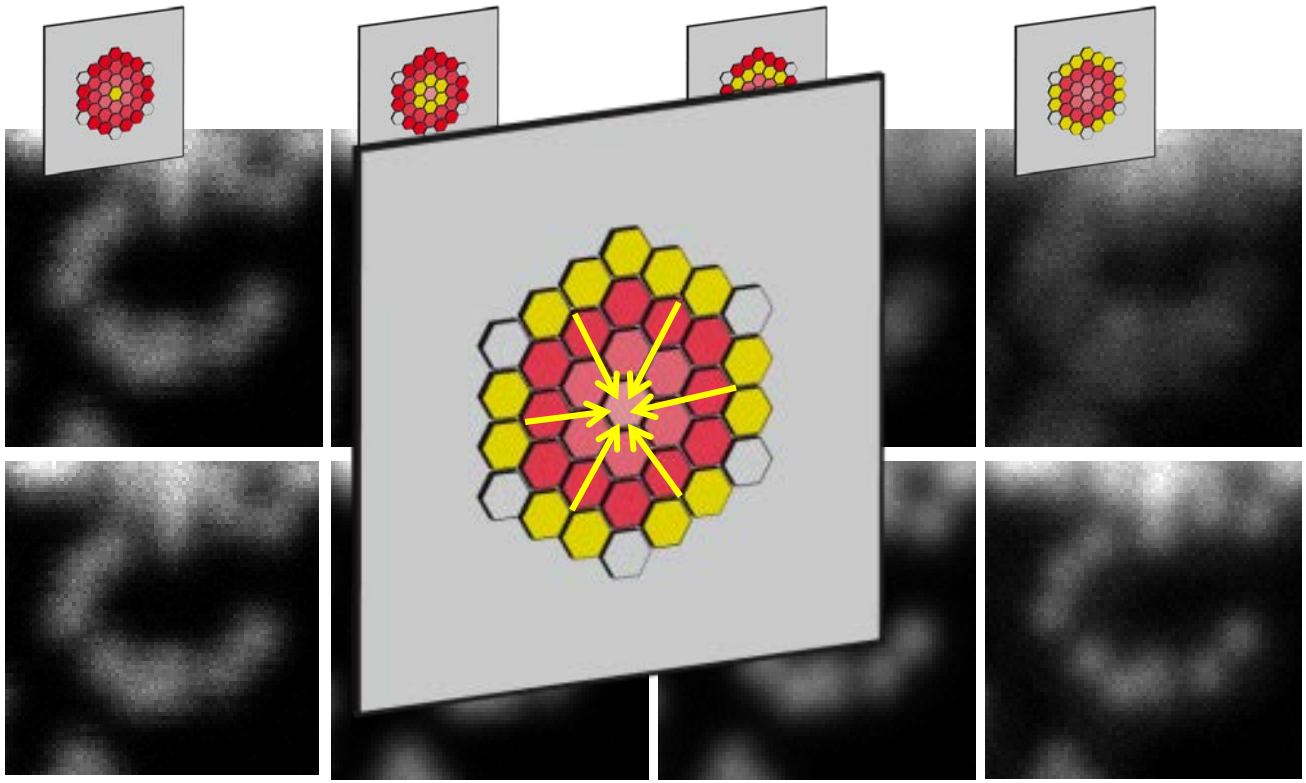
Airyscan detection
2nd ring of detectors



Airyscan detection
3rd ring of detectors

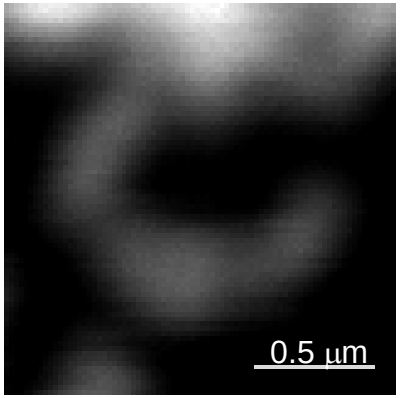


Pixel reassignment
Spatial reassignment of the signal



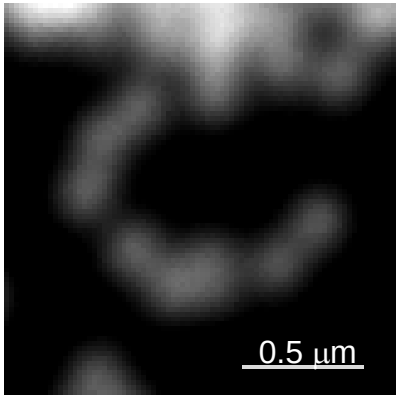
Airyscan processing
Isotropic resolution improvement

170nm fluorescent beads



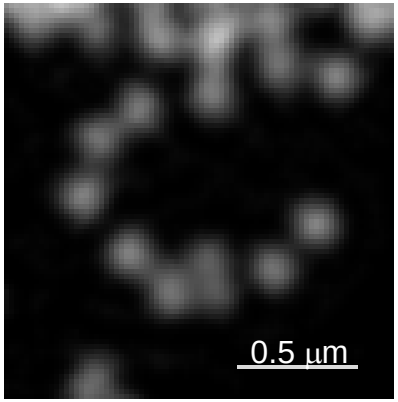
Confocal microscope
Plan-Apochromat 63x/1.4 Oil, 633nm illumination

Approx. resolution: 260nm



Pixel reassignment
1.4x improved resolution

Approx. resolution: 185nm

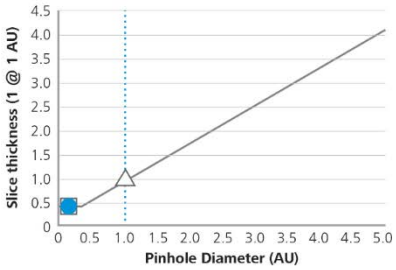


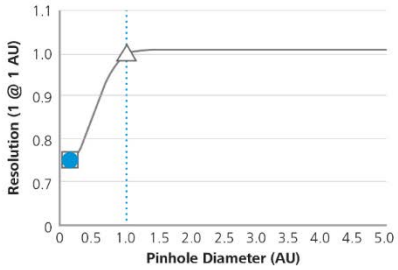
Airyscan processing
Up to 2 x improved resolution

Approx. resolution: 153nm

Airyscan 2 in Superresolution Mode

Maximum Signal-to-Noise with Simultaneous Superresolution

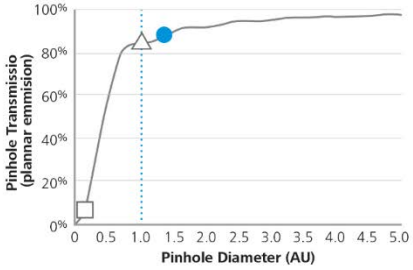


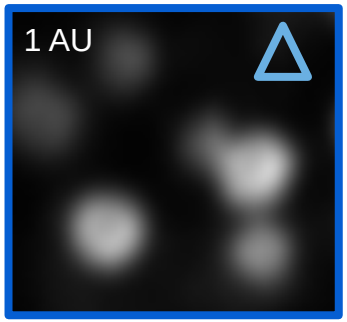


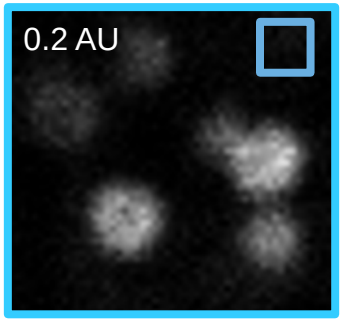
Traditional Confocal @ 0.2 AU

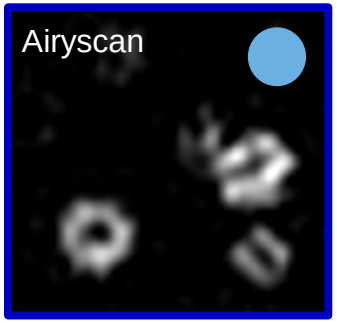
Traditional Confocal @ 1 AU

Airyscan



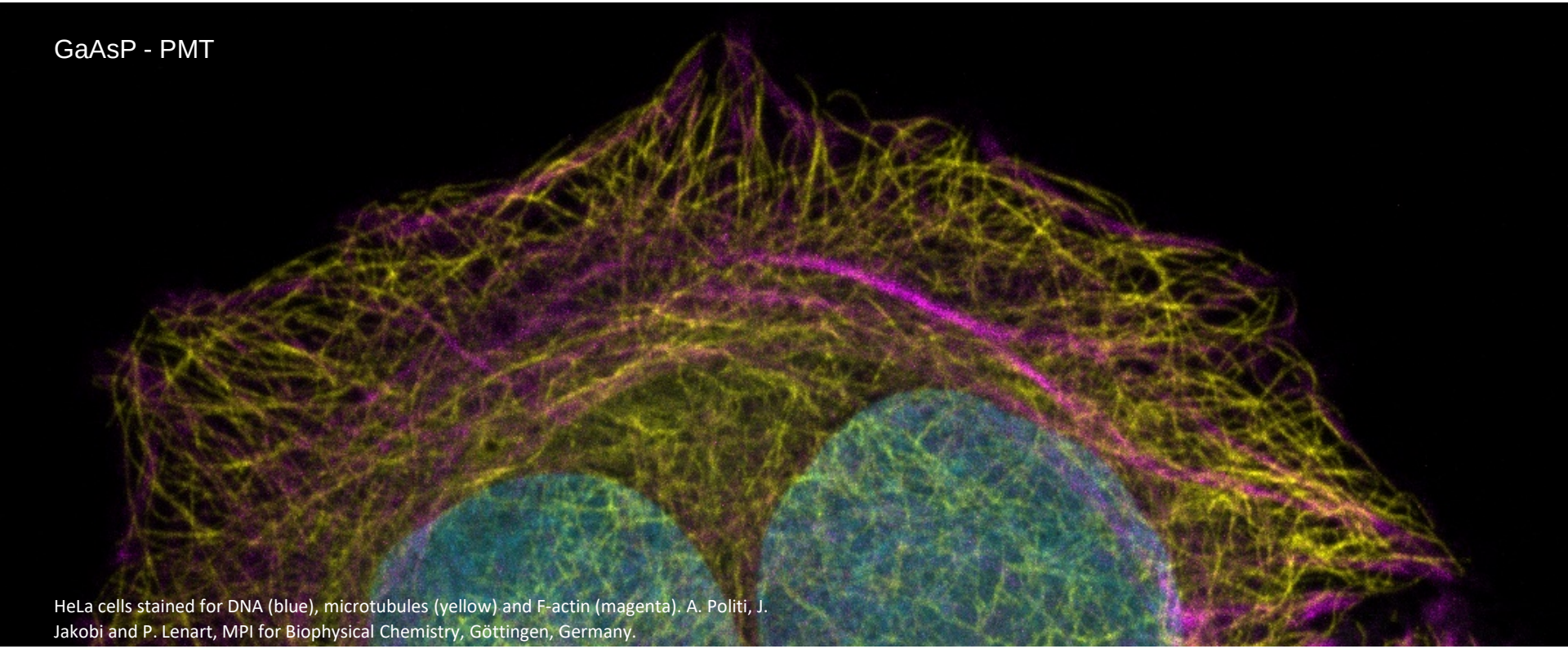






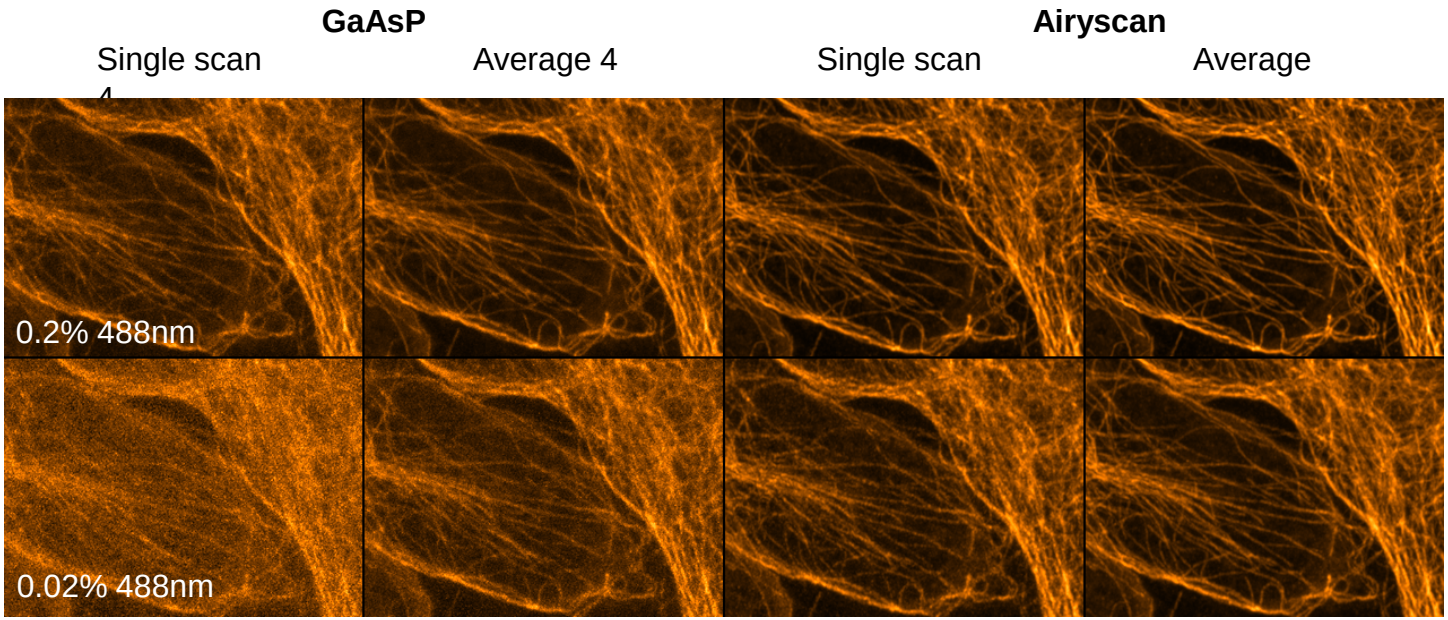
Neuromuscular junction, bruchpilot, *Drosophila melanogaster*, Sample courtesy of J. Pielage, Basel, Switzerland

Get better data faster
Your needs - our motivation



HeLa cells stained for DNA (blue), microtubules (yellow) and F-actin (magenta). A. Politi, J. Jakobi and P. Lenart, MPI for Biophysical Chemistry, Göttingen, Germany.

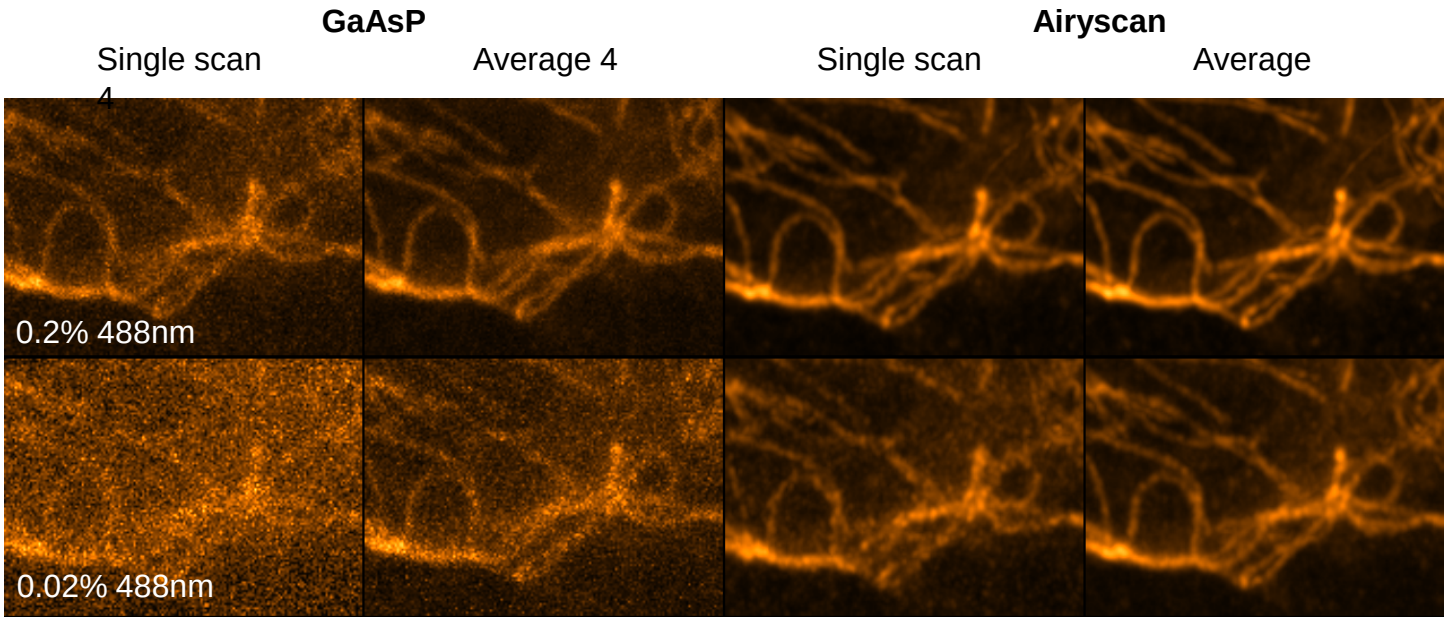
Airyscan
Up to 4x improvement in SNR over GaAsP PMT @1AU



- Same sample – Stable and hard to bleach
- Identical imaging parameters other than these stated above
- All images scaled with best fit (0.4% top and bottom)

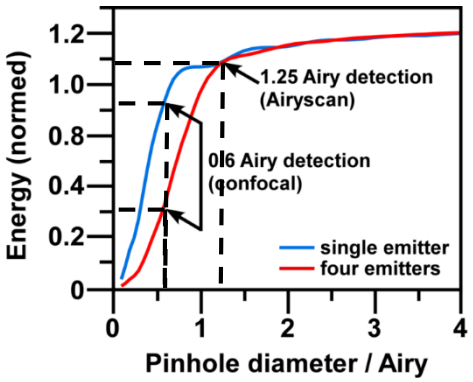
Carl Zeiss Microscopy, Chris Power, BioSciences

SNR Comparison on Microtubules



Carl Zeiss Microscopy, Chris Power, BioSciences

Other Options for Improving Resolution and SNR
Pitfalls of Closing the Pinhole & Deconvolution



THE ASTRONOMICAL JOURNAL VOLUME 104, NUMBER 3 SEPTEMBER 1992

RESOLUTION LIMITS FOR DECONVOLVED IMAGES

L. B. Lucy[1]

Space Telescope–European Coordinating Facility, Karl-Schwarzschild-Strasse 2, D-8046 Garching bei München, Germany

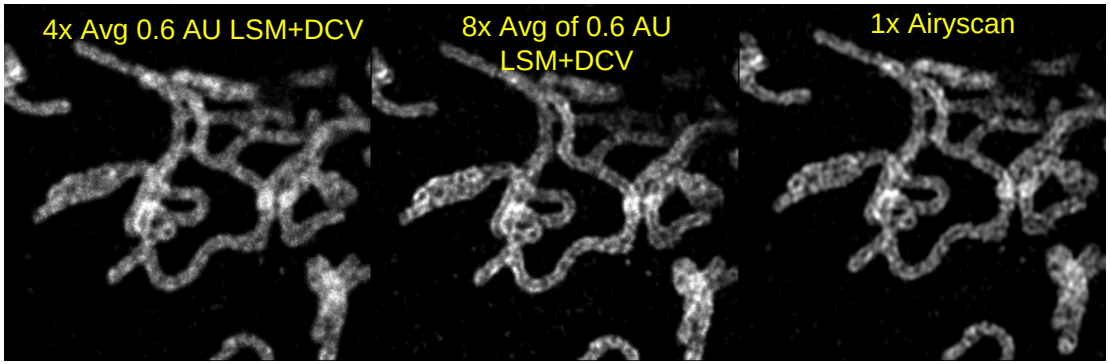
Received 17 October 1991; Revised 20 February 1992

ABSTRACT

The limits imposed by photon statistics on the resolution attainable by deconvolving seeing-broadened astronomical images are investigated. By analogy with the classical Rayleigh limit for diffraction-limited images, resolution is defined as the separation of the equal component double star that can be just resolved by deconvolution. An approximate analytic theory is given that predicts the dependence of resolution thus defined on the number of detected photons, and this theory is tested by deconvolution experiments on synthetic images. The disparity for restored images between the Rayleigh criterion of resolution and the effective seeing inferred from the angular diameters of deconvolved single stars is stressed.

I. INTRODUCTION

tional images, the classical Rayleigh test of resolution is well approximated by the more readily measured FWHMs

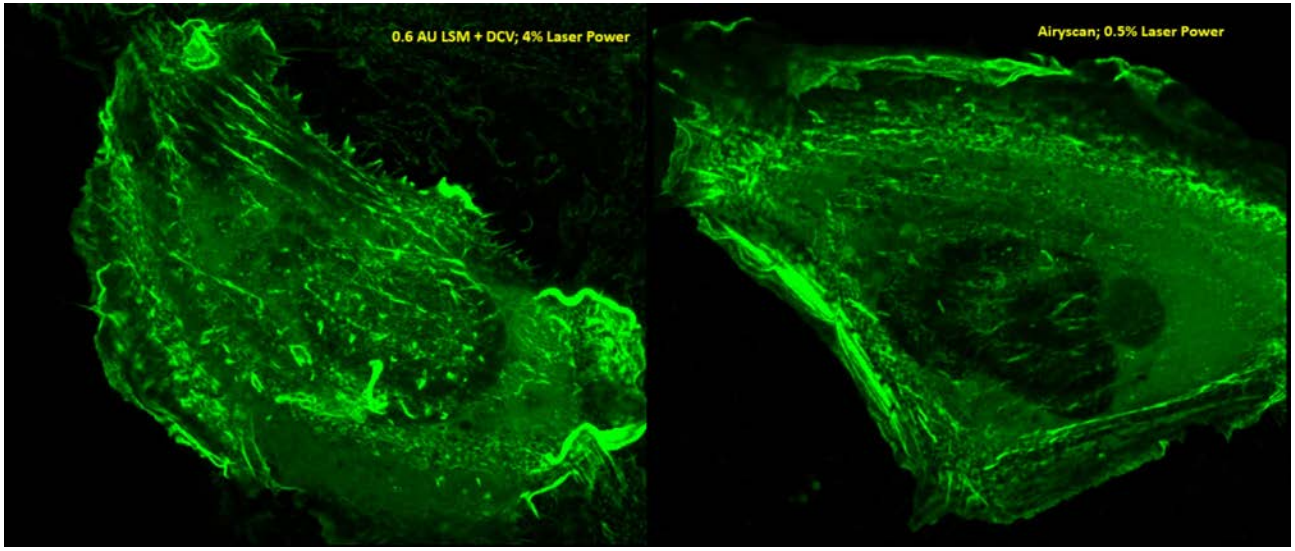


Carl Zeiss Microscopy, Chris Power, BioSciences

Airyscan for Gentle Imaging
Pitfalls of Closing the Pinhole & Deconvolution

4% Laser Power & DCV of 0.6 AU LSM

0.5% Laser Power & Airyscan



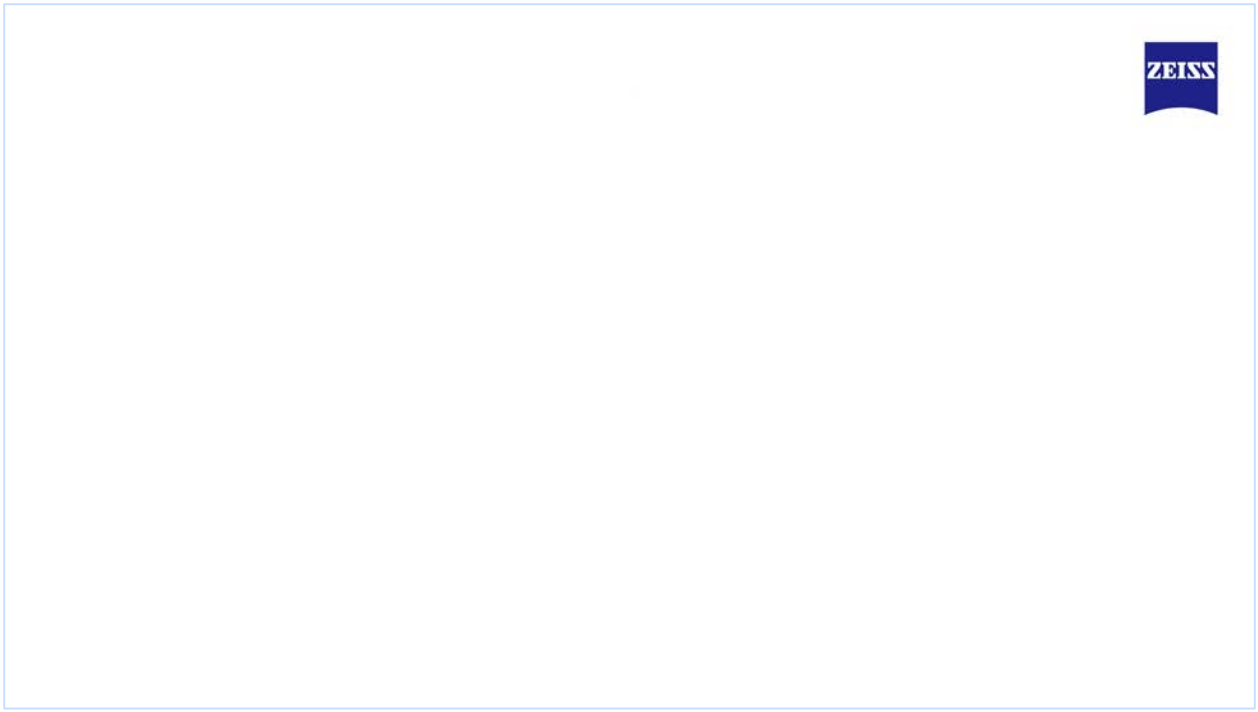
8x increase in laser power to match Airyscan SNR

Carl Zeiss Microscopy, Chris Power, BioSciences

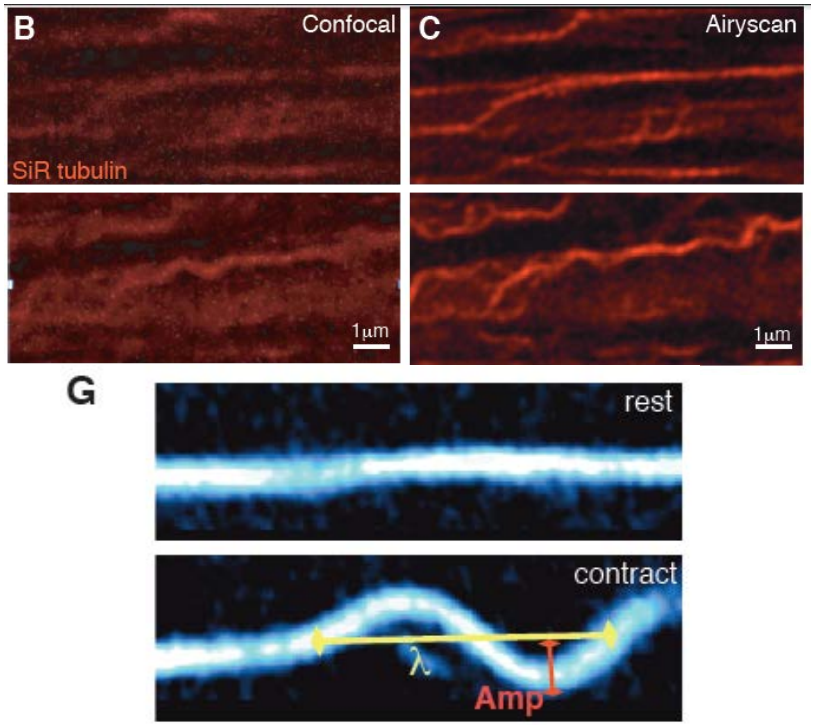
Scaling Airyscan 2 for Todays Model Systems with Multiplex Mode

Airyscan 2 with Multiplex 8Y Mode

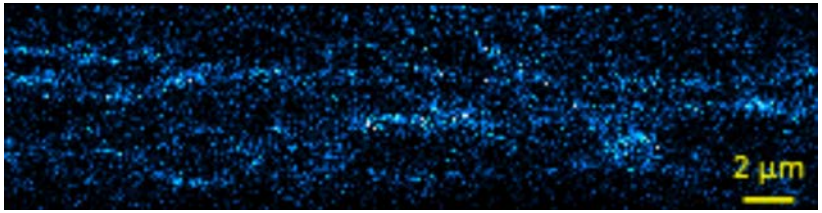
8x Parallelization for High Signal-to-Noise and Simultaneous Superresolution

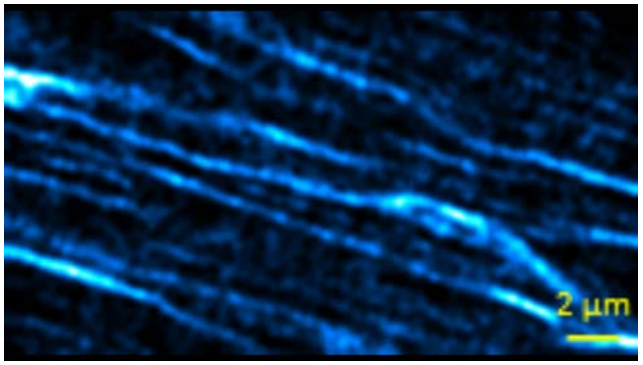


Recap LSM 880 with Airyscan Fast
In 2016 Fast Mode Provided Usable Speed



Resonance Scanner – **80 FPS**





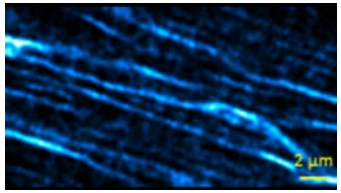
Airyscan Fast Mode – **96 FPS**

Cardiomyocyte Cells with tubulin-EMTB to measure microtubule buckling

Images and Samples Courtesy of Ben Prosser, UPENN – "Detyrosinated microtubules buckle and bear load in contracting cardiomyocytes", Science April 2016

Multiplex Mode for Airyscan 2 Provides Larger Fields-of-View

Maintain Resolution, SNR and Speed over Larger FOVs to Gain Context

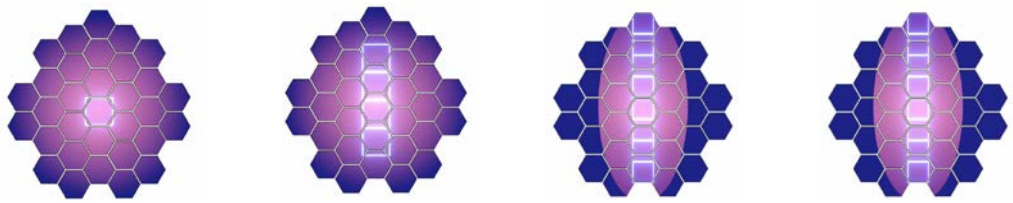


Cardiomyocyte Cells with tubulin-EMTB to measure microtubule buckling

Images and Samples Courtesy of Ben Prosser, UPENN – “Detyrosinated microtubules buckle and bear load in contracting cardiomyocytes”, Science April 2016

Multiplex mode for ZEISS LSM 980 with Airyscan 2

Fast and Gentle Confocal Superresolution Imaging of Large Model Systems

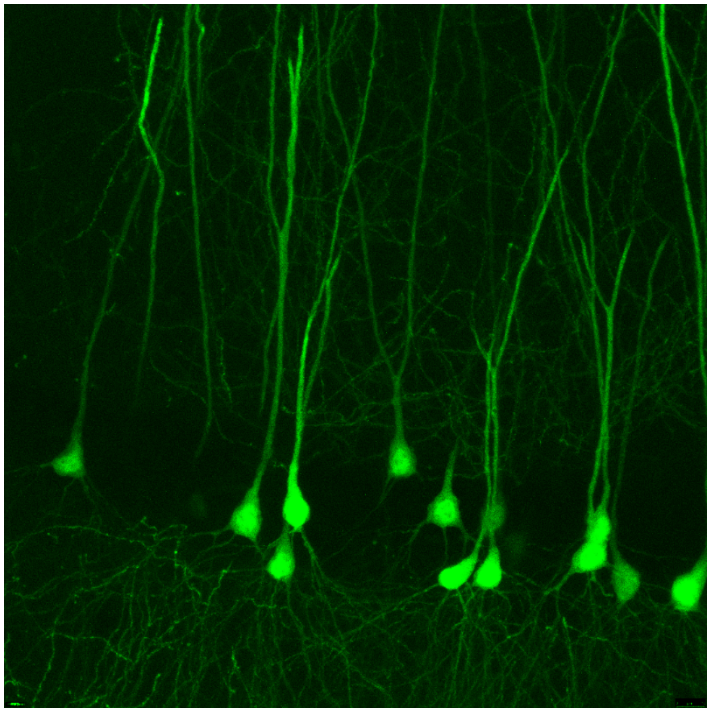


Mode	Confocal	Airyscan SR	Multiplex SR-4Y	Multiplex SR-8Y	Multiplex CO-8Y
Parallelization	1	1	4	8	8
Max. resolution X/Y nm	—	120/120	140/140	120/160	Confoc. or better
Max. resolution Z nm	—	350	450	450	Confoc. or better
Max. FPS @ 512x512	6.1	4.7	25.0	47.5	34.4
FPS @ max FOV (mm)	0.4 (@Zoom 0.6 / SF20)	0.2 (@Zoom 1.7 / SF7)	1.0 (@Zoom 1 / SF12)	2.0 (@Zoom 1 / SF12)	9.6 (@Zoom 1 / SF12)
Processing 1kx1k /150 slices	—	< 30s	< 30s	< 30s	< 30s
SNR vs convent. confocal	—	4-8x	4-8x + speed	4x + speed	—

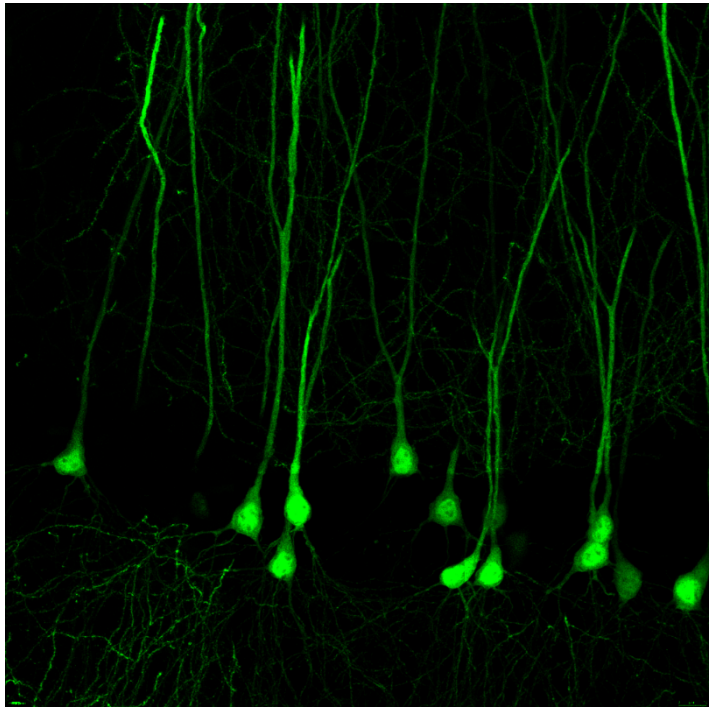
Fast Adaptive Deconvolution of Confocal Images with Hybrid Detectors

Lower Magnification Traditional Confocal with Closed PH Requires Slow Acquisition Rates

61 minute acquisition – 258μm x 258μm x 34μm



Automatic and adaptive deconvolution with decision masks

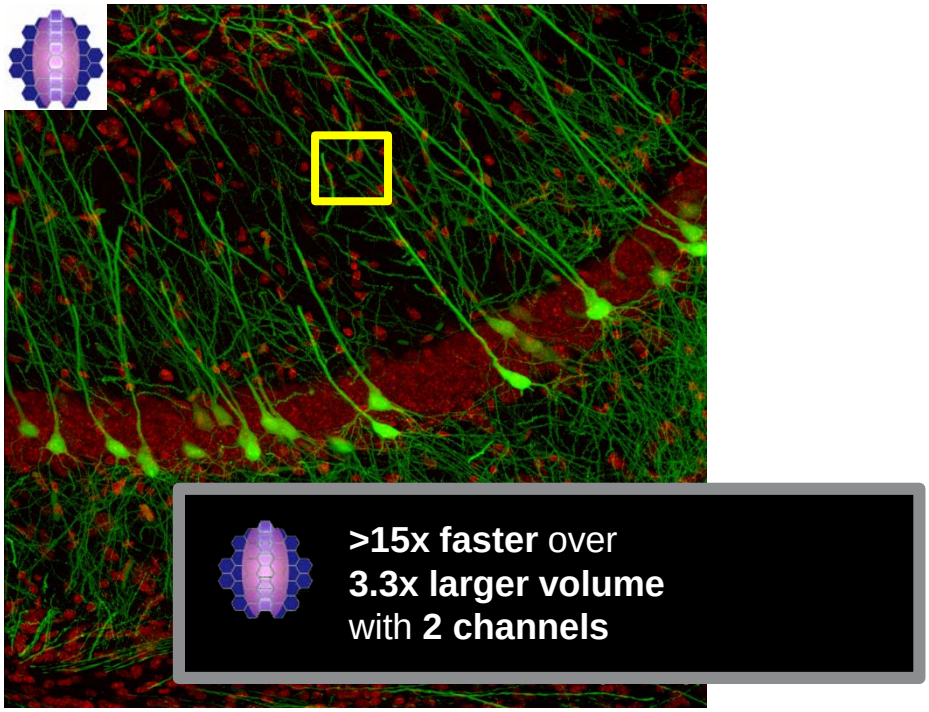


Mouse Brain Section PFA Fixation; DRAQ5 and cytoplasmic GFP

Airyscan 2 with Multiplex Mode

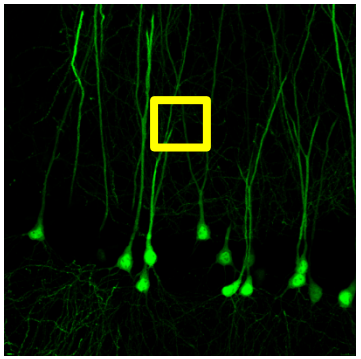
Gentlest and Fastest Confocal Superresolution Imaging over large volumes

4 minute acquisition – 467µm x 467µm x 34µm



8Y-SR Multiplex mode

61 minute acquisition – 258µm x 258µm x 34µm

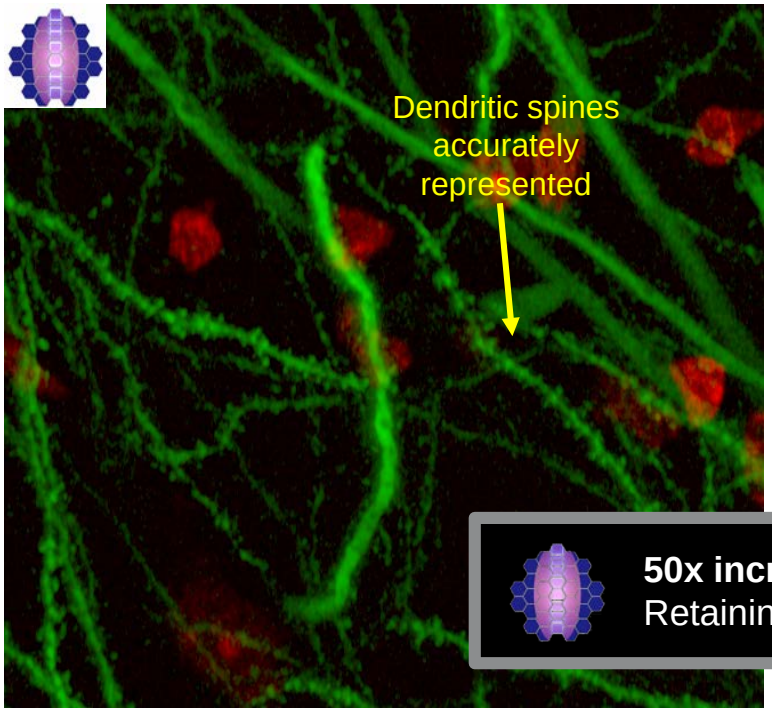


Traditional Confocal with automatic and adaptive deconvolution with decision masks

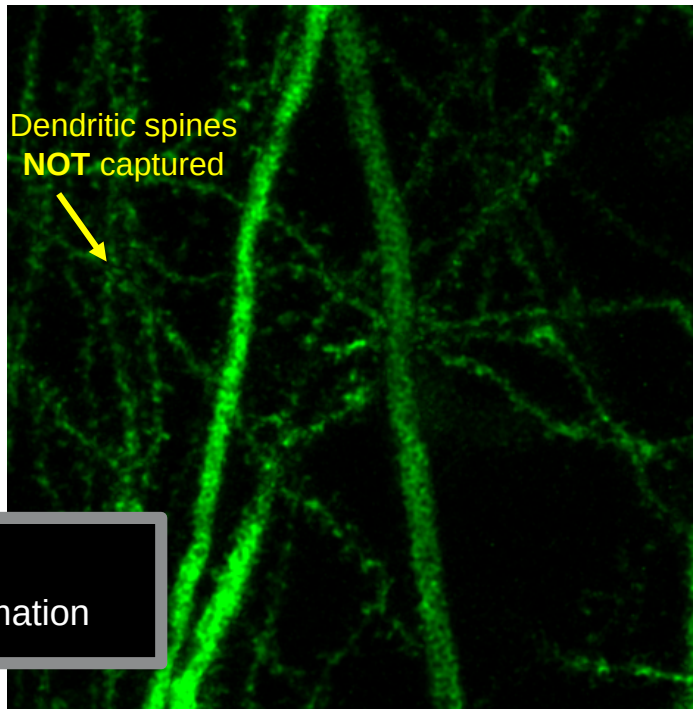
Airyscan 2 with Multiplex Mode

Gentlest and Fastest Confocal Superresolution Imaging over large volumes

4 minute acquisition – 467μm x 467μm x 34μm

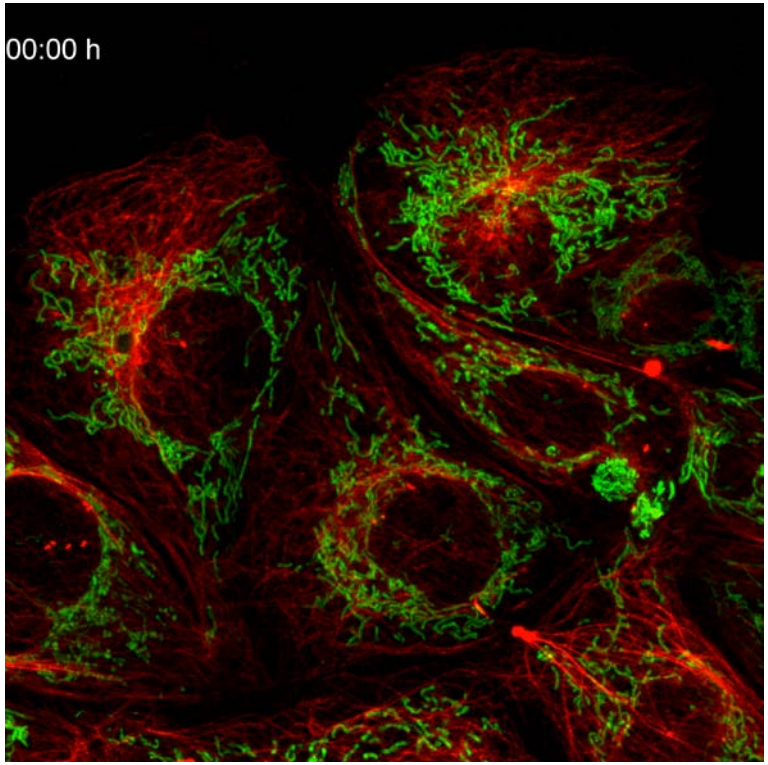


61 minute acquisition – 258μm x 258μm x 34μm



50x increase in μm³/sec
Retaining all spatial information

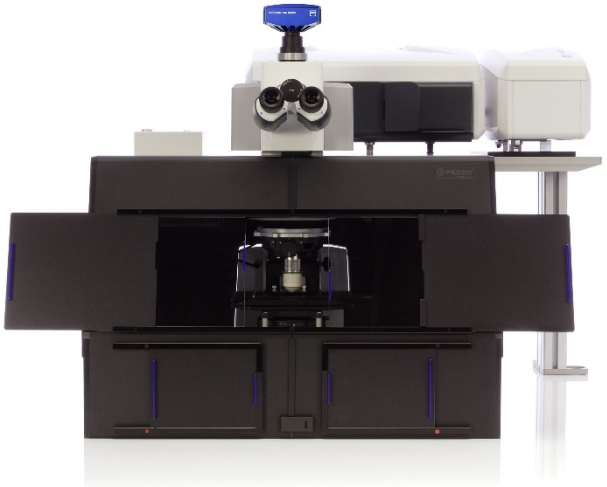
Airyscan 2 with Multiplex
Parallelization – Gentle and Fast imaging

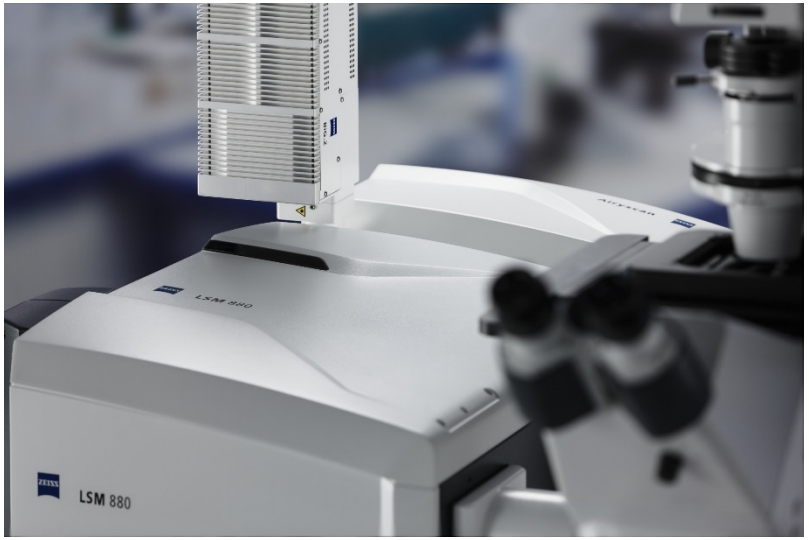


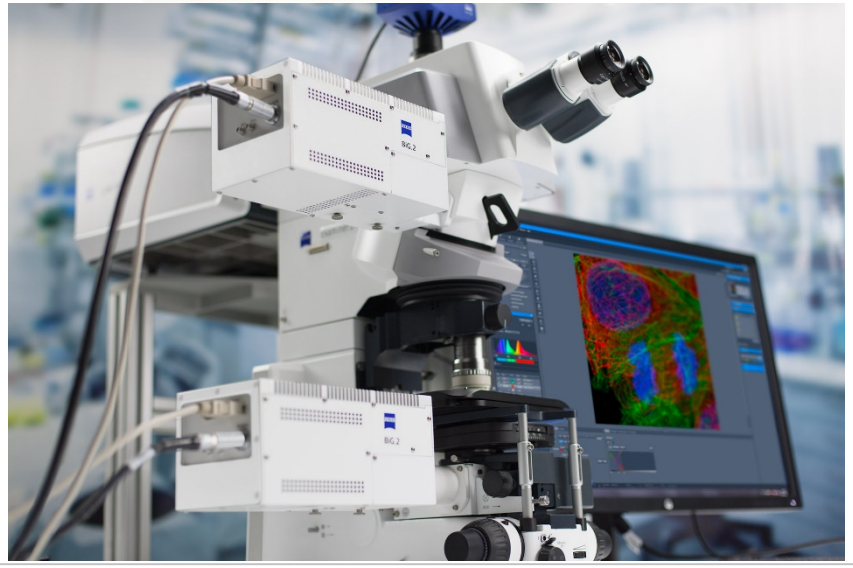
Courtesy of Sarita Patnaik, PhD, Univ. of Mainz

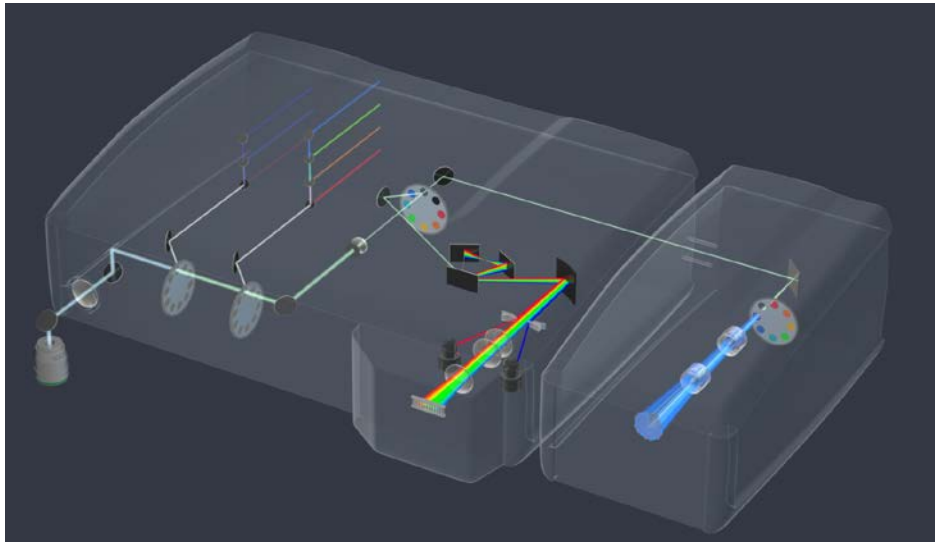
- Cells treated with cytotoxic drug
- Gentle superresolution imaging allows cells to recover after the noxious substance is removed
- Time lapse imaging for 20 hours every 10 min

Detectors used in the LSM 980









Detectors used in the LSM 980

Type	Where	Why	Confocal	NLO	FLIM	FCS
Diode detector	Transmitted light path	Cheap and very robust	X	x		
Traditional Multi Alkali	Transmission PMT & Quasar channel 1	Robust to high light, very high dynamic range, better blue sensitivity	X	x		
Multi alkali - Cooled	Quasar channels 3	Better sensitivity in red.	X	x		X
GaAsP	Quasar	Better general sensitivity	X	x		X
Multi Array Multi Alkali	Quasar	32 channels of simultaneous spectral detection, used in the 510, 710 and 880, now obsolete	X	x		X
Multi Array GaAsP	Quasar	Better general sensitivity, 6 or 32 channels of simultaneous spectral detection	X	x		X
Multi Array GaAsP	Airyscan	Super resolution & super sensitive imaging	X	x		
NDD Cooled Multi alkali	NDD Reflected light or Transmitted light	Better sensitivity in blue and red.		X		
Nosepiece GaAsP	NDD Reflected light	Best sensitivity in scattered light		X		
BiG (Binary GaAsP)	NDD Reflected light or Transmitted light	Better general sensitivity		X	X	
BiG (Binary GaAsP)	Confocal Direct couple port	Better general sensitivity	X	x	X	
GaAs or other Far red optimised PMT	Confocal Direct couple port	Best NIR detection	X		X	X
Hybrid detectors	Confocal Direct couple port	Fast FLIM response	x	x	X	x
Multi Channel Plate (MCP)	Confocal Direct couple port	Fastest FLIM response	x	x	X	
APD	Dedicated FCS Unit	Used to be used for sensitivity, now obsolete as GaAsP internal more cost effective and much higher count	X	x		X

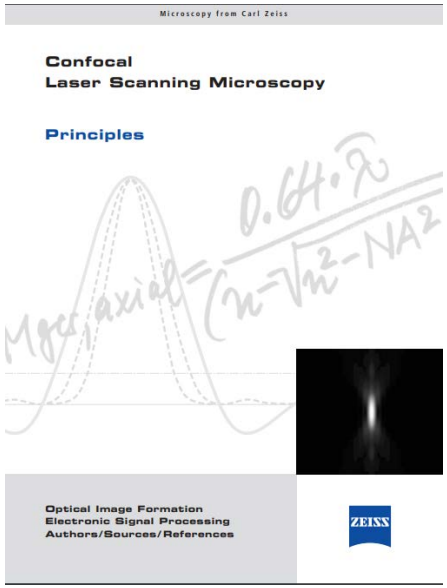
Further reading

A good basic introduction (15 pages) and the source of several diagrams used today
http://www.et-enterprises.com/files/file/Understanding-photomultipliers.pdf

Complete handbook (323 pages)
http://www.hamamatsu.com/resources/pdf/etd/PMT_handbook_v3aE.pdf

www.zeiss.com/airyscan
Whitepapers on airyscan and Multiplex modes

http://zeiss-campus.magnet.fsu.edu/referencelibrary/pdfs/ZeissConfocalPrinciples.pdf



We make it visible.