607SM - Techniche avanzate di indagine microscopica

Advanced microscopy techniques – 6CFU, 2024/25, 1st semester

Part1:

Dan COJOC , CNR-IOM Trieste

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Lecture 3

607SM - TECNICHE AVANZATE DI INDAGINE MICROSCOPICA - AA 2024/25

1. Optical Microscopy

1.1. Basics (Image formation, magnification, resolution, image quality) NO → Thalhammer

1.2. Digital camera (image acquisition, formats, properties) NO → Thalhammer

1.3. Phase imaging (qualitative and quantitative)

1.4. Dark field and Polarization microscopy

Lecture 1

Lecture 3

Lecture 2

1.5. Super resolution microscopy – STED, PALM/STORM, MINFLUX ....

1.6. Photobleaching-based Techniques for Assessing Cellular Dynamics

1.7. PhotoAcoustic Microscopy PAM

Lecture 4

1.8. Brilouin Microscopy

1.9. Non Linear Optical Microscpy

Lecture 5

!!! The order of the last three topics is slightly changed from the initial plan

Superresolution

separates features using (at least) 2 molecular ***states***

fluorescent	***non-fluorescent***
absorbing	*non-absorbing*
scattering	*non-scattering*
spin up	*spin down*
…	…

Super-resolution fluorescence microscopy = Nanoscopy

(continued from previous lecture with STED)

1. Increasing axial resolution – TIRF, 2 Photon microscopy

2. Increasing lateral and axial resolution –
   confocal, STED,

PALM Photo-Activated Localization Microscopy - principle example appl

MINFLUX microscopy - principle + example appl

SIM – Structured Illumination Microscopy – principle

BALM - Binding Activated Localization Microscopy

PALM (Photo-**A**ctivated **L**ocalization **M**icroscopy**)** Eric Betzig 2006

STORM (STochastic **O**ptical **R**econstruction **M**icroscopy) Sam Hess 2006

This technique takes advantage of the new generation of photo-activable and photo-switchable proteins that have been developed in the past few years.

Under irradiation by UV light, these proteins undergo a chemical conversion and switch from one particular state to another.

In the case of photo-activable proteins, they undergo a conversion from a dark 'off' state to a bright 'on' state.

For photo-switchable fluorophores, they will switch from one color to another color; this process may or may not be reversible, depending on the fluorophore.

The main difference between PALM and STORM is the fluorophores used for the experiment and the mechanism of switching between the bright and dark states:

- PALM uses photo switchable/convertible fluorescent proteins (FPs),
- STORM uses organic dyes as fluorescent probes for imaging

Internet **References**

Jennifer Lippincott-Schwartz (NIH) Part 3: Super Resolution Imaging
https://www.youtube.com/watch?v=Bom9d-Knz0w

Microscopy: Super-Resolution Microscopy (Xiaowei Zhuang)
https://www.youtube.com/watch?v=BmRRYPDq4bY
https://www.youtube.com/watch?v=w2Qo__sppcI

E. BETZIG Nobel Prize lecture 2014

http://www.nobelprize.org/mediaplayer/index.php?id=2407

W. MOERNER Nobel Prize lecture 2014

http://www.nobelprize.org/mediaplayer/index.php?id=2411

PALM (Photo-**A**ctivated **L**ocalization **M**icroscopy**)** Eric Betzig 2006

STORM (STochastic **O**ptical **R**econstruction **M**icroscopy) Sam Hess 2006

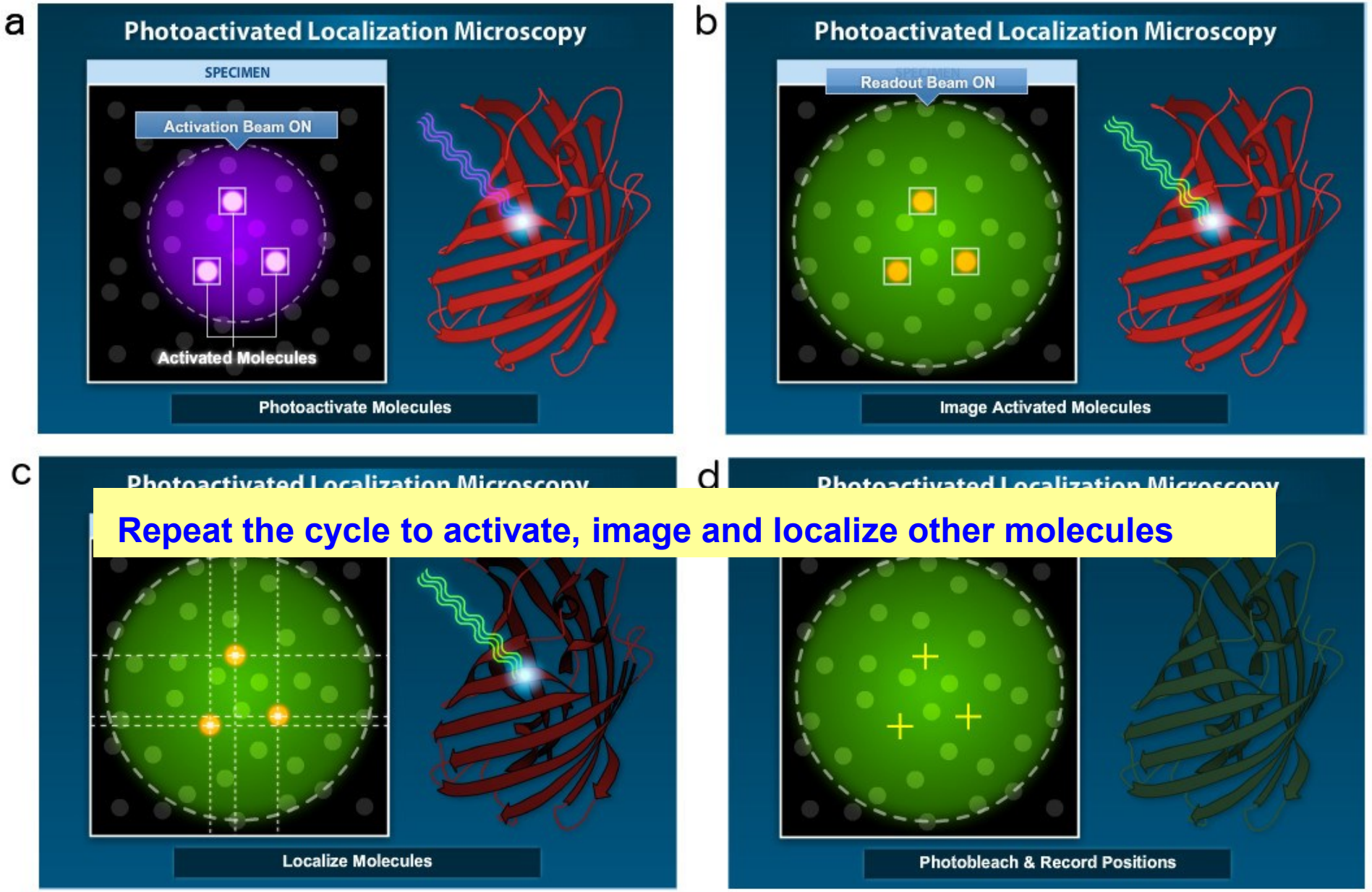
Single molecule detection/localization – Dickson,..., and Moerner, Nature (1997)

WORKING PINCIPLE

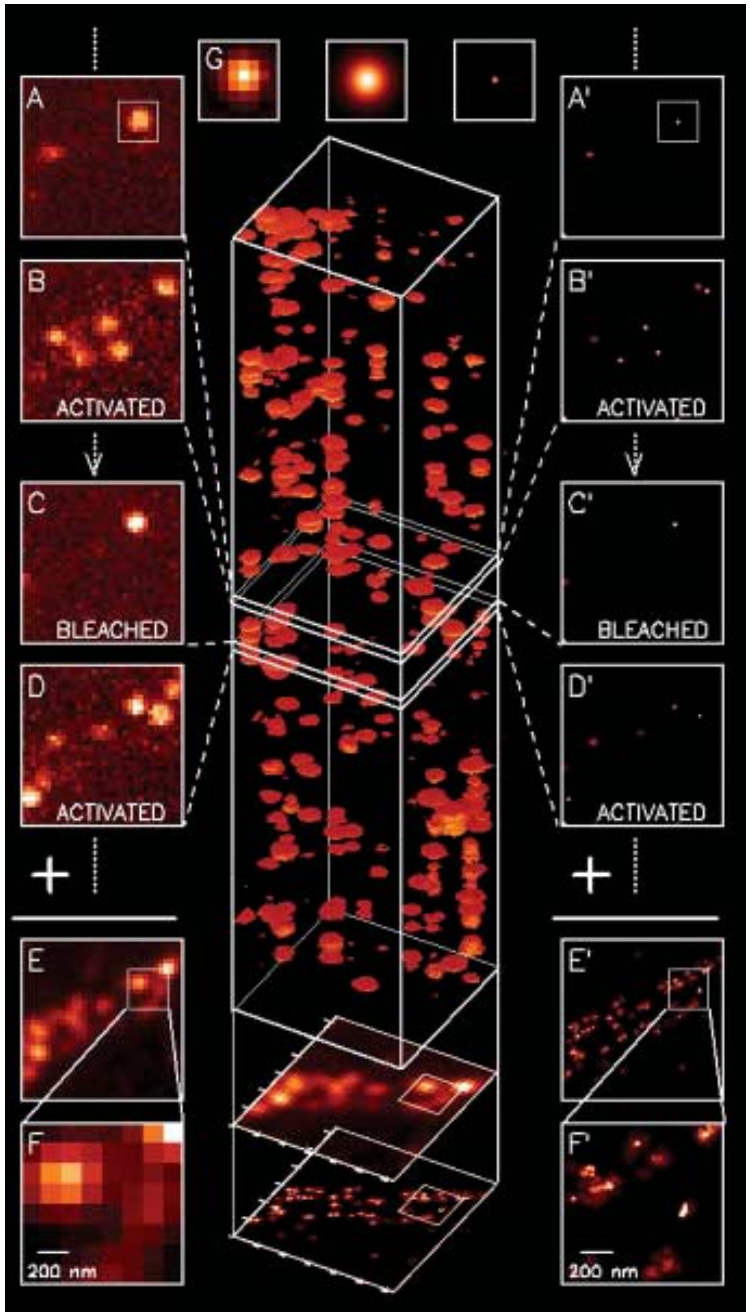
In the examples below, the fluorophore EOS will undergo a conversion from a green state to a red state.

EOS FP is a photoactivatable green to red fluorescent protein.

Its green fluorescence (516 nm) switches to red (581 nm) upon UV irradiation of ~390 nm

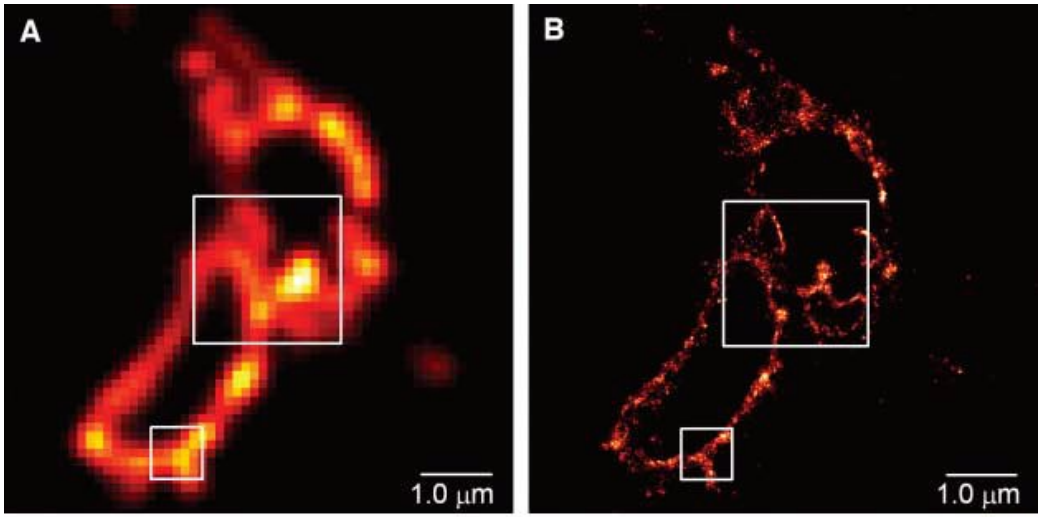


http://advanced-microscopy.utah.edu/education/super-res/



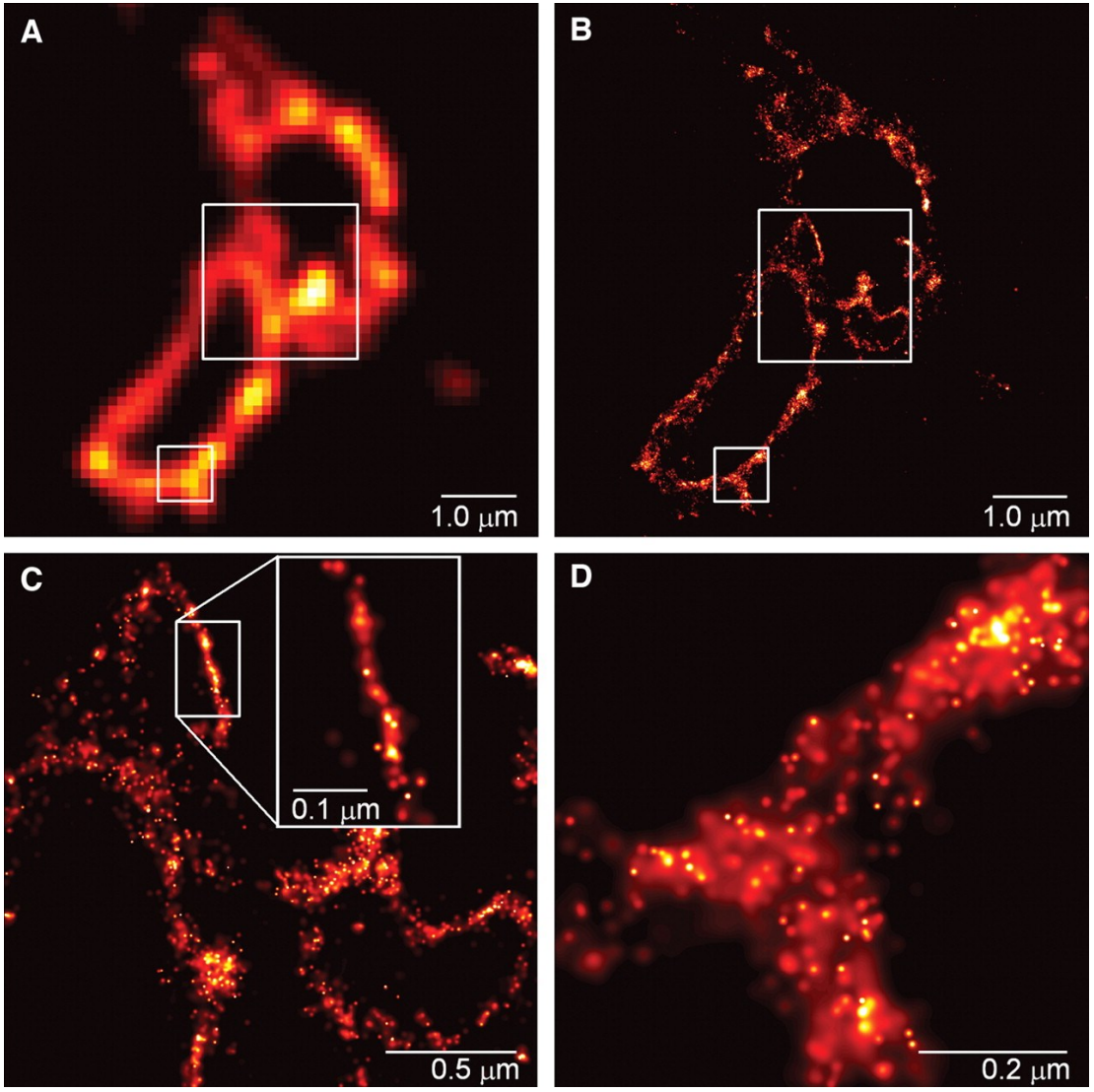
Cycles sequence involved in creating a PALM image - Betzig *et al*, Science, (2006)

TIRF PALM



N.B. : The molecules activated in one cycle should be separated by distances bigger than the diffraction limit!

TIRF (**A**) and PALM (**B**) images of the same region within a cryo-prepared thin section from a COS-7 cell expressing the lysosomal transmembrane protein CD63 tagged with the PA-FP Kaede.



The larger boxed region in (B), when viewed at higher magnification (**C**) reveals smaller associated membranes that may represent interacting lysosomes or late endosomes that are not resolvable by TIRF. In a region where the section is nearly orthogonal to the lysosomal membrane, the most highly localized molecules fall on a line of width ~10 nm (inset). In an obliquely cut region [(**D**), from the smaller boxed region in (B)], the distribution of CD63 within the membrane plane can be discerned.

Betzig *et al*, Science, (2006)

Molecule localization

Central to the performance of PALM is the **precise localization** of single fluorescent molecules. When such localization is performed by a least-squares fit of an assumed 2D gaussian point spread function (PSF) to each single molecule image, the **mean-squared position error** is:

$$\sigma_{x,y} \sim s / (N^{1/2})$$

where s is the standard deviation of the PSF and N is the total number of photons measured from the molecule. (PSF – the image of a molecule). $N^{1/2}$ Poissonian noise

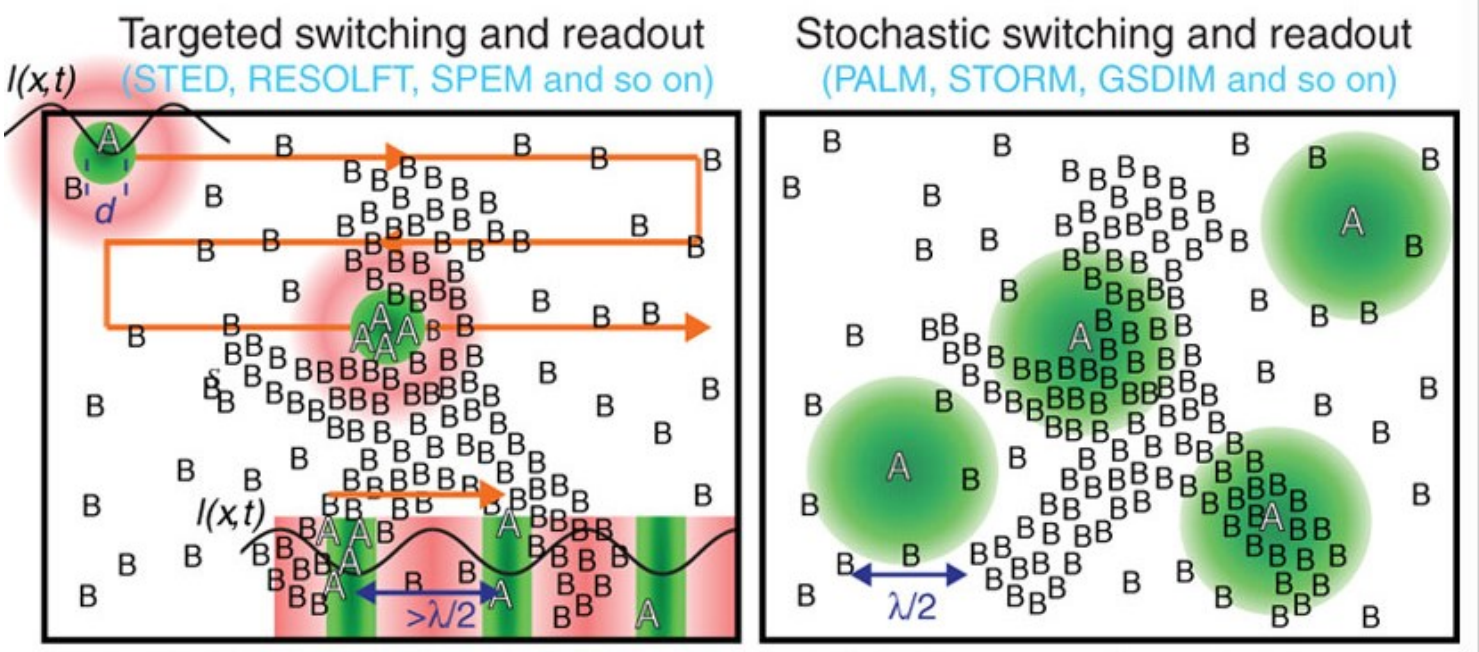
Ex: If s ~ 200 nm, and N > 10^4 → **1-2 nm resolution can be achieved**

Betzig, Science 2006:

Here, we developed a method for isolation of single molecules at high densities (up to ~ $10^5/\mu m^2$) based on the serial photoactivation and subsequent bleaching of numerous sparse subsets of photoactivatable fluorescent protein (PA-FP) molecules within a sample.

PALM pro and cons

- Very high lateral resolution (2 nm) on fixed samples and many photons
- Slow because it requires iterative steps activation – exposure
- Special fluorophores required
- Works for fixed and live cell images, with limitations
- Sample damage – phototoxicity is an issue



In the **targeted** mode, a spatial light intensity distribution $I(x,t)$ having a zero intensity point in space switches the molecules such that one of the states—here A—is confined to sub-diffraction dimensions d. E.g. in STED microscopy the zero-intensity point is realized by a doughnut-shaped beam $I(x,t)$ for molecular de-excitation (upper left corner) switching off all molecules that are not located at the zero, thus sharply confining a region with diameter $d << \lambda/(2n \sin \alpha)$ in which the molecules are on (in state A). The image is assembled by shifting the pattern $I(x,t)$ over the sample (scanning) and recording adjacent features sequentially in time. Several molecules can reside in the same sub-diffraction-sized region. To parallelize the recording procedure (lower right corner),$I(x,t)$ can also feature an array of zero lines or points with pitch $> \lambda/(2n \sin \alpha)$ and implement camera recording. To super-resolve in all directions, the line pattern must be tilted and scanned an appropriate number of times.

In the **stochastic** switching mode, such as in PALM and STORM, individual molecules are switched on (to state A) randomly in space, emitting $m >> 1$ photons in a row, while the surrounding molecules remain in the dark state. The distance between the 'on' molecules should be $> \lambda/(2n \sin \alpha)$ to facilitate recognition of individual molecules. Their coordinates are gained by calculation of their centroids. In the variant GSDIM, the

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The Nobel Prize in Chemistry 2014

Photo: A. Mahmoud
Eric Betzig
Prize share: 1/3

Photo: A. Mahmoud
Stefan W. Hell
Prize share: 1/3

Photo: A. Mahmoud
William E. Moerner
Prize share: 1/3

The Nobel Prize in Chemistry 2014 was awarded jointly to Eric Betzig, Stefan W. Hell and William E. Moerner *"for the development of super-resolved fluorescence microscopy"*.

https://www.nobelprize.org/nobel_prizes/chemistry/laureates/2014/

Suggestion: Read the Nobel lectures !!!

(Replacing the) **Conclusions** with recommendation to read the review:

Microscopy and its focal switch

Stefan W Hell

Until not very long ago, it was widely accepted that lens-based (far-field) optical microscopes cannot visualize details much finer than about half the wavelength of light. The advent of viable physical concepts for overcoming the limiting role of diffraction in the early 1990s set off a quest that has led to readily applicable and widely accessible fluorescence microscopes with nanoscale spatial resolution. Here I discuss the principles of these methods together with their differences in implementation and operation. Finally, I outline potential developments.

Most textbooks still assert that a light microscope cannot resolve objects that are closer than about a quarter of a micrometer. However, as this issue of *Nature Methods* highlights, fluorescence microscopy has clearly turned into nanoscopy. And, as with many other leaps in sci-

$$\Delta x \approx \frac{\lambda}{2n\sin\alpha}$$

24 | VOL.6 NO.1 | JANUARY 2009 | NATURE METHODS

Super-Resolution (Nanoscopy)

$$\Delta x \approx \frac{\lambda}{2n\sin\alpha\sqrt{1+\frac{I}{I_s}}}$$

Stefan Hell

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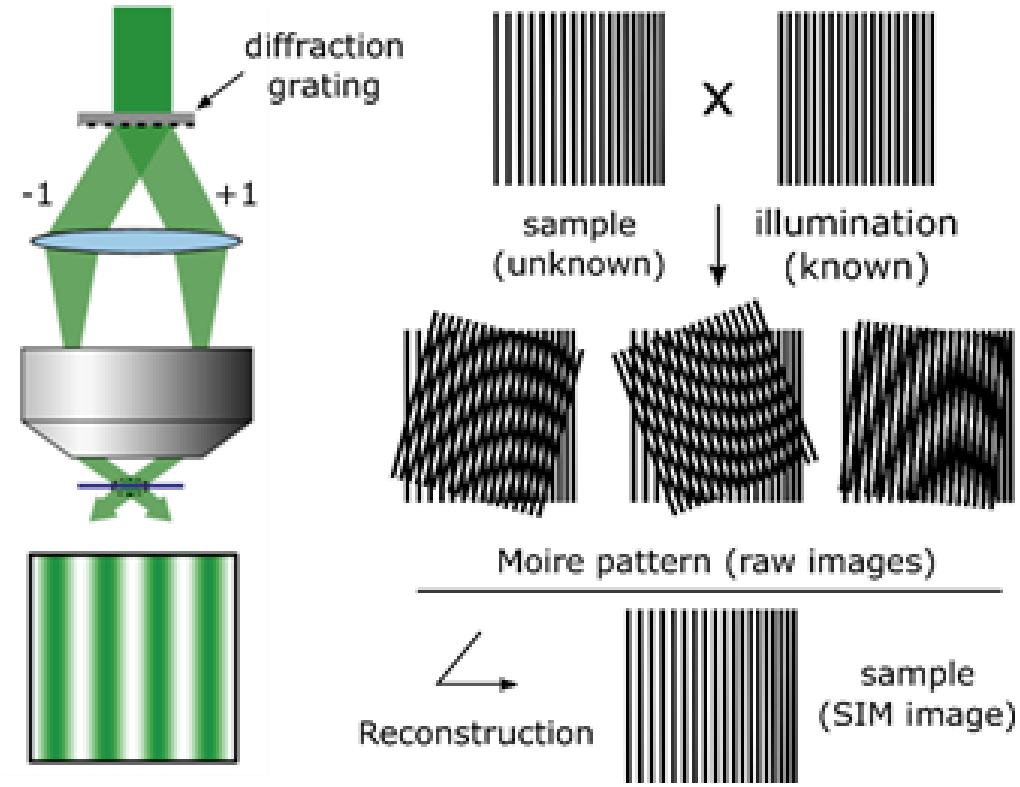
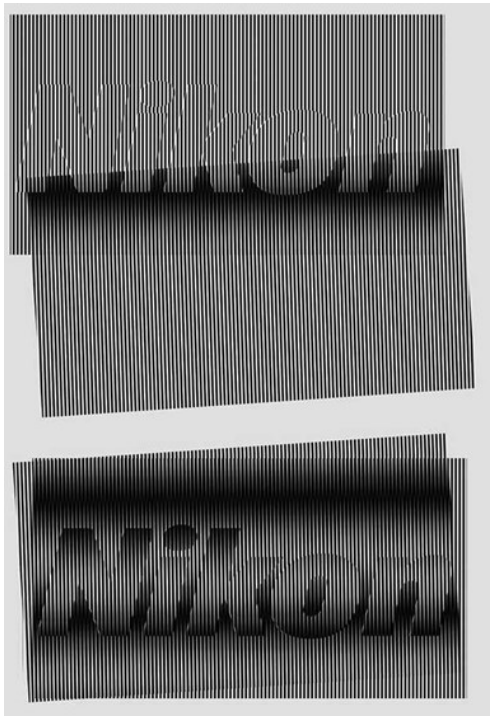
SIM Structured Illumination Microscopy - Principle

SIM relies on a laser-based wide-field microscopy set-up to which a movable diffraction grating has been inserted into the excitation beam path. Zero order and first order diffracted laser beams are allowed to pass through the objective. MAX gain only 2-3 X, but it is fast. See LAB

These laser beams interfere with each other at the focal plane of the objective and create an illumination in stripes (intensity following sinusoidal wave). This stripe pattern of light by its superimposition with the sample generates a so-called Moiré effect. The super resolution in the sample is recovered from the Moire image by frequency reconstruction .



Andor

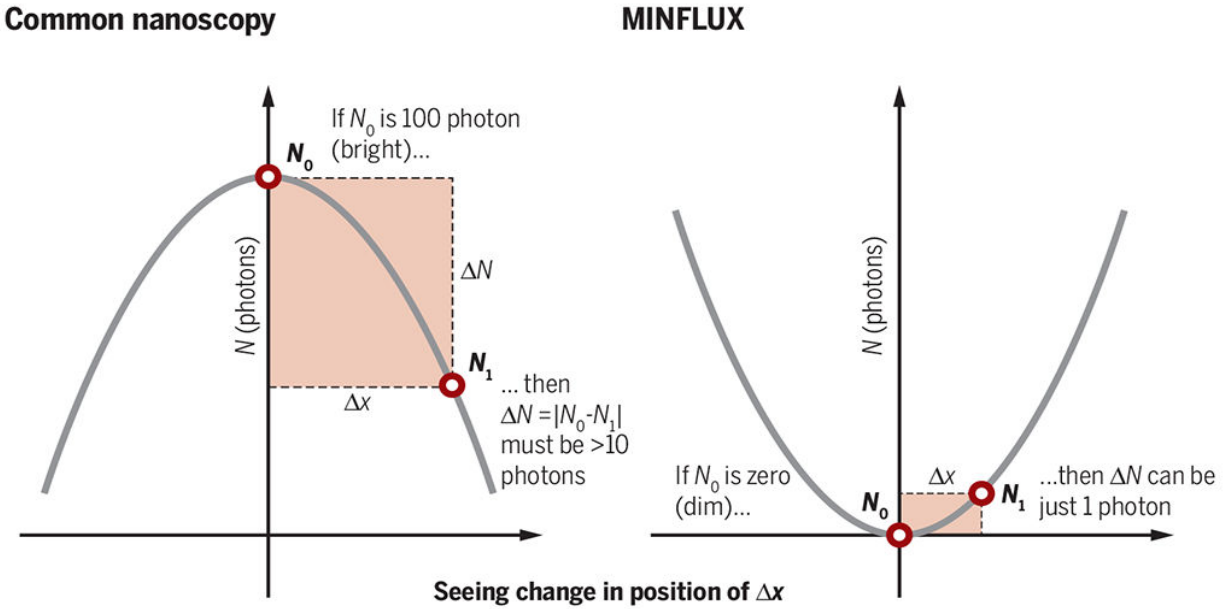


Nikon sites

MINFLUX MINimal photon FLUXes

IDEA: localize a molecule near the signal → requires less photons

Maximum *Minimum*



In order to confidently declare that the molecule has moved by Δx to a new nearby position x_1 (N_1 photons), the associated signal change $\Delta N = N_0 - N_1$ should be larger than the Poissonian noise: $\Delta N > \sqrt{N_0}$

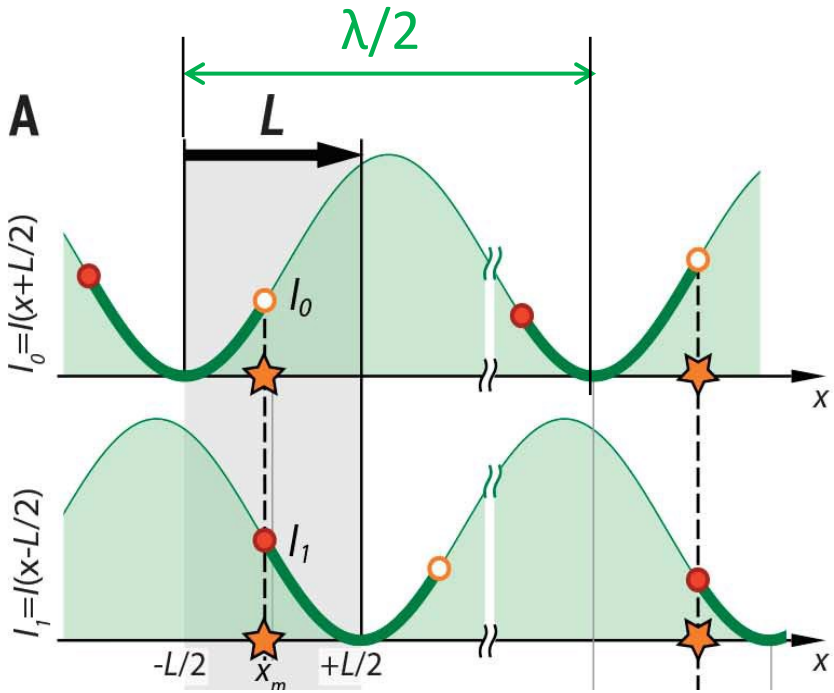
zero signal at position x_0 (signal $N_0 = 0$), much smaller Δx can be detected as long as $N_1 > 1$

Francisco Balzarotti, ..., Stefan W. Hell, Nanometer resolution imaging and tracking of fluorescent molecules with minimal photon fluxes, SCIENCE, 2017
Doi: 10.1126/science.aak9913

MINFLUX microscope - Commercially available since 2020: https://www.abberior.com/

MINFLUX MINimal photon FLUXes

How it works – explanation for 1D



x_m→ position of the molecule

1D intensity pattern for illumination / fluo excitation:

I(x) with *I(0)* = 0

(standing wave in the figure but it can be any pattern)

The unknown position x_m of a fluorescent molecule is determined by translating the illumination pattern such that one of its intensity zeros travels from x= *-L/2* to x=*L/2*, where $L < \lambda / 4$.

The presence of the fluorescent molecule (approximative position) in this interval is determined first by the mediated fluorescence signal.

Intensity profiles I_0 and I_1 of the illumination pattern at times 0 and 1:

$$I_0(x) = I(x+ L/2) \qquad I_1(x) = I(x- L/2)$$

Intensity values of the illumination pattern at the position of the molecule:

$$I_0(x_m) = I(x_m+ L/2) \qquad I_1(x_m)= I(x_m- L/2)$$

The molecular fluorescence when the illumination pattern moves

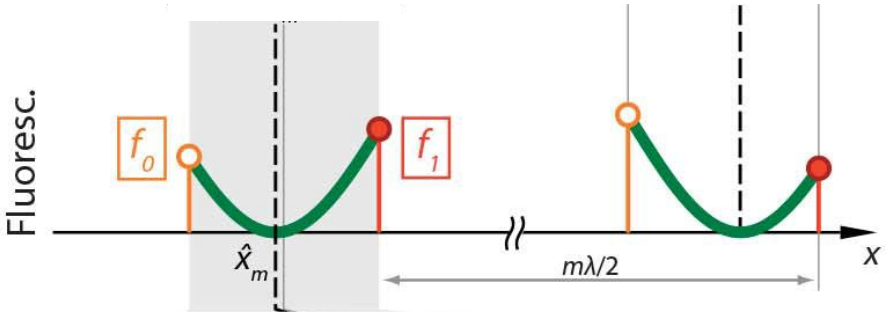
$$f(x)=C\ I(x_m- x)$$

C – constant related to the molecular brightness and detection sensitivity

Balzarotti, …, Hell, Science 2017

MINFLUX MINimal photon FLUXes

How it works in 1D



Balzarotti, …, Hell, Science 2017

Since the molecular fluorescence *f(x)* becomes zero at x_m solving $f(x_m)= 0$ yelds the molecular position x_m. The molecule can be located exposing the sample to only two intensity values belonging to functions $I_0(x)$ and $I_1(x)$ that have zeros at x= – L/2 and x= L/2 respectivelly.

More molecules can be located in parallel if they are separated by a distance bigger than λ/2

$f(x)=C\, I(x_m- x)$

The molecular fluorescence when the illumination pattern moves

$f_0=C\, I(x_m+ L/2)= C\, I_0(x_m)$

$f_1=C\, I(x_m- L/2)= C\, I_1(x_m)$

Signal (fluo intensity) values at the two end points of the probing range

$f(x_m)=C(x_m-x^2)= 0$

For small L, f(x) can be approximated quadratically around x_m

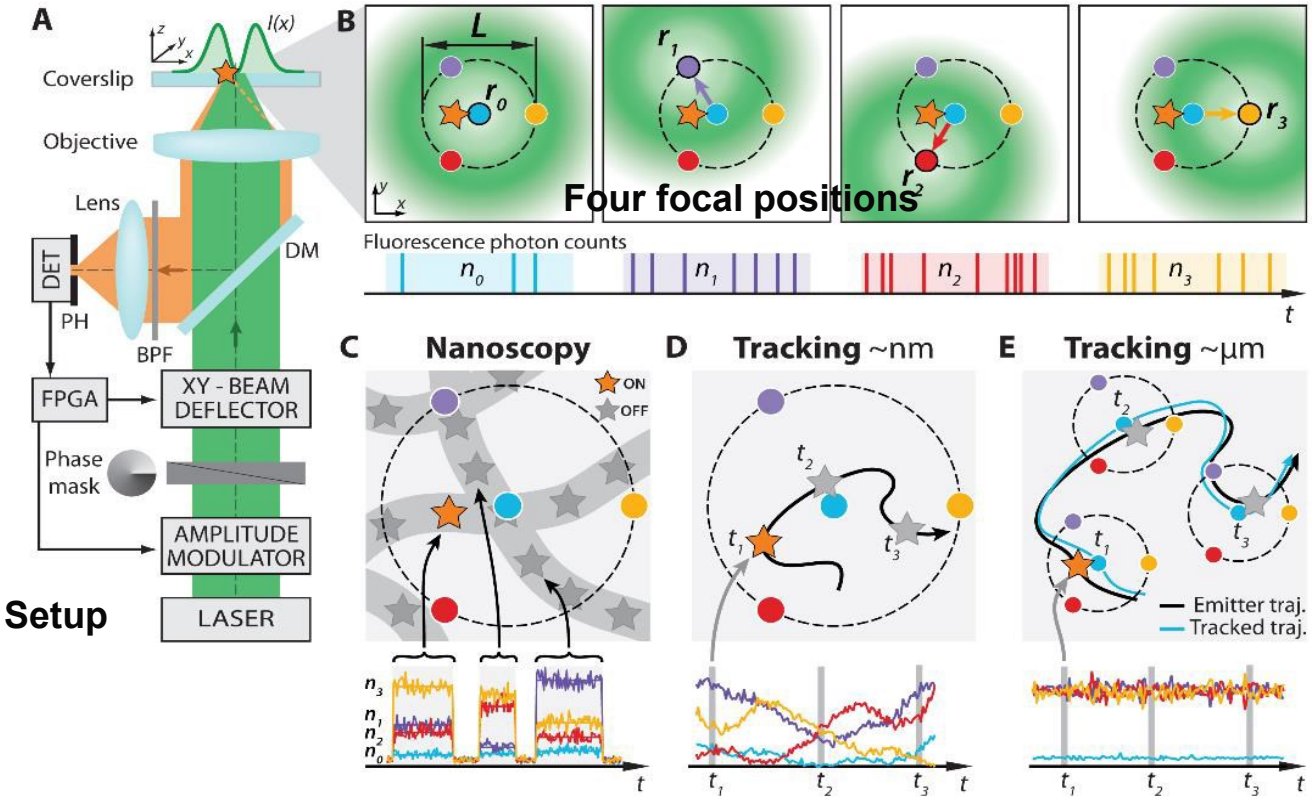
$$x_m = L[1/(1+\sqrt{f_1/f_0})-1/2]$$

The solution for the localization of the molecule with nm precision

The localization precision depends on *L*: smaller *L* higher localization precision

The localization precision does not depend on the wavelength !

2D illumination pattern - donut



The fluorescence photons $n_{0,1,2,3}$ counted for each doughnut (focal) position $r_{0,1,2,3}$ by the detector (DET) are used to extract the molecular location

Applications

(C) Nanoscopy: A nanoscale object features molecules whose fluorescence can be switched on and off, such that only one of the molecules is on within the detection range. They are distinguished by abrupt changes in the ratios between the different $n_{0,1,2,3}$.

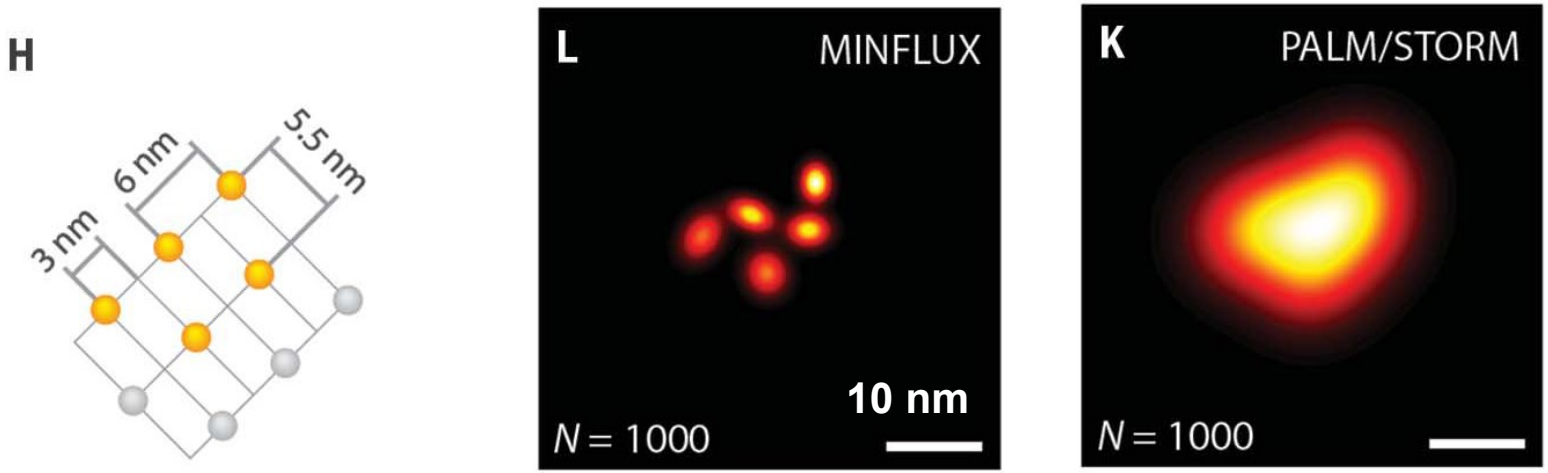
(D) Nanometer-scale (short-range) tracking: The same procedure can be applied to a single emitter that moves within the localization region of size L. As the emitter moves, different fluorescence ratios are observed that allow the localization.

(E) Micron-scale (long-range) tracking: If the emitter leaves the initial L-sized field of view, the triangular set of positions of the doughnut zeros is (iteratively) displaced to the last estimated position of the molecule.

2D ilumination pattern - donut

MINFLUX nanoscopy of labeled DNA origamis

resolved fluorescent molecules spaced only 6 nm apart from each other on a DNA origami structure, with only 1000 photons per molecule in ~2 min imaging time
(Alexa Fluor 647, 405 nm activation, 642 nm excitation, L= 50-70 nm)
Localization precision 1.2 nm



Balzarotti, …, Hell, Science 2017

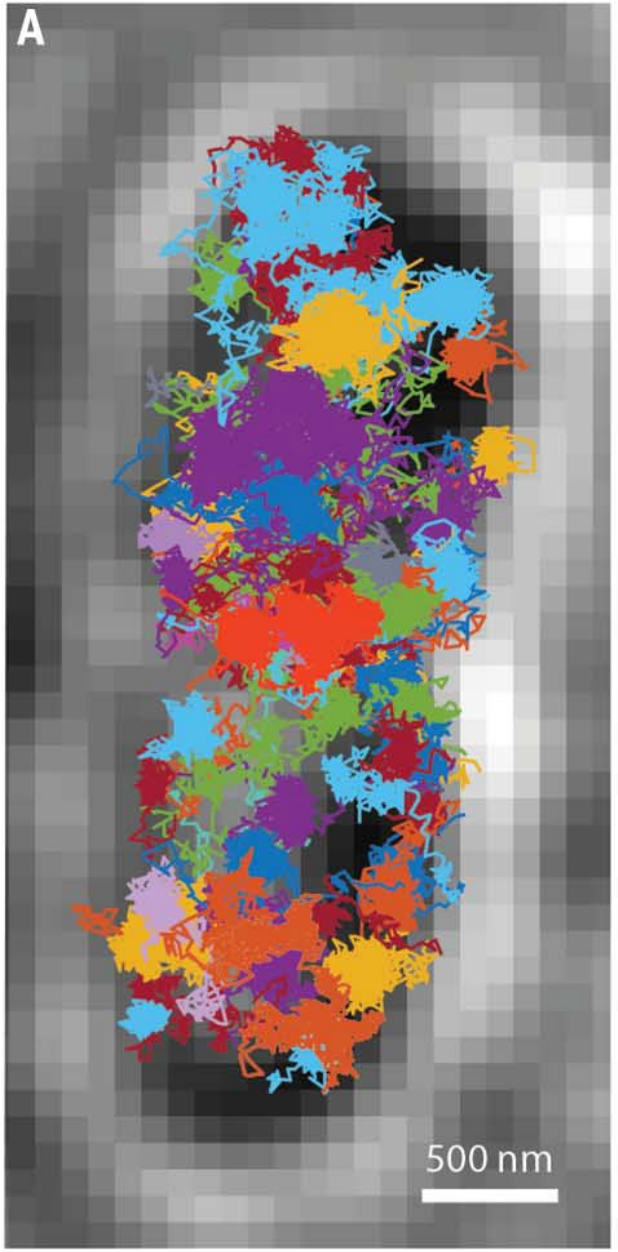
Similar resolution was achieved of DNA origami previously by using a method called DNA PAINT, but with 50,000 photons and an image acquisition time of 2 hours
Dai M, Jungmann R, Yin P, Nat. Nanotechnol 11, 798 (2016). [PubMed: 27376244]

Single-molecule MINFLUX tracking in living E. coli bacteria

The minimal photon flux feature of MINFLUX is particularly advantageous for single-molecule tracking experiments that are often limited by rapid photobleaching of fluorescent proteins.

Individual 30S ribosomal protein subunits labeled with photoactivatable fluorescence protein mEos2 diffusing in live bacterial cells were followed for orders-of-magnitude more time points.

The average length reached ~750 time points per trajectory compared with ~5 to 10 in standard tracking experiments (with camera), making it possible to detect temporal changes in diffusion coefficient.



Transmission image of a bacterium overlaid with 77 independent tracks

Single-molecule MINFLUX tracking in living E. coli bacteria

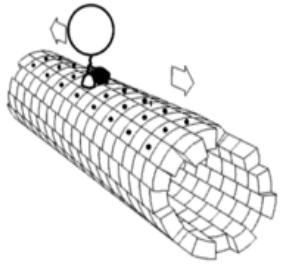
Recent (revolutionary) application:

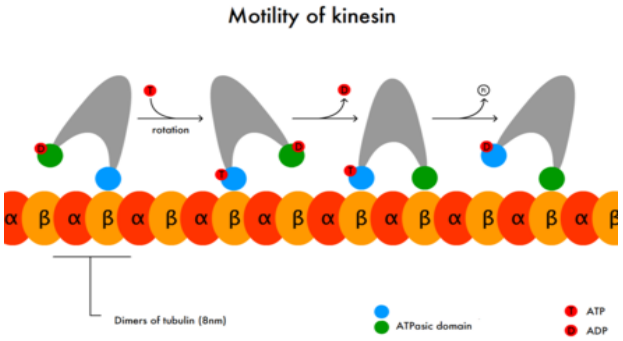
J.O. Wirth, …, S.W. Hell, MINFLUX dissects the unimpeded walking of kinesin-1, SCIENCE 2023, 379, 1004-1010

MINFLUX microscope that records protein movements with up to 1.7 nanometer per millisecond spatiotemporal precision.

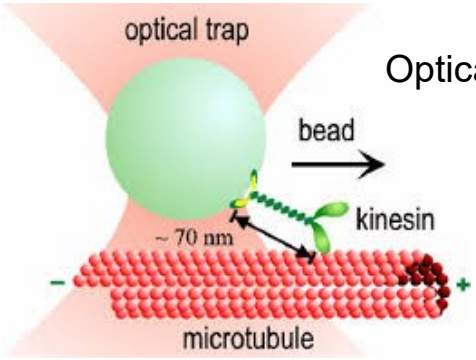
Study the stepping of the motor protein kinesin-1 on microtubules at up to physiological ATP concentrations.

Quantifies (sub)millisecond conformational changes of proteins with minimal disturbance.

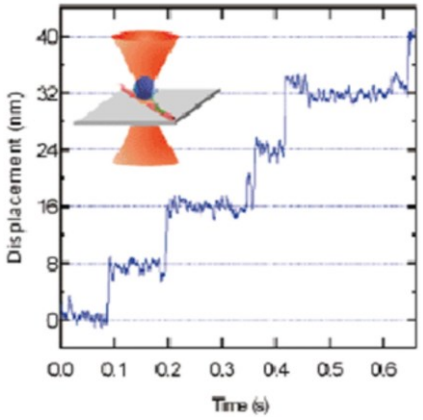




Other techniques to characterize the motor protein kinetics as e.g. optical tweezers, provide more information related to the force and energy states but the protein is somehow impeded by constructs.



Optical tweezers assay



Single steps of kinesin-1 measured with optical tweezers

BALM - Binding Activation Localization Microscopy

Nucleic acid stains show a strong enhancement of their fluorescence upon binding to double-stranded DNA.

This property can be exploited to provide super-resolution imaging based on the localization of individual binding events.

By adjusting the chemical environment:
- the properties of both the dye and DNA (in DNA-binding dyes) can be properly controlled and modified, which in turn controls the fluorescence signal.
- DNA hybridization and melting becomes reversible, meaning that the process itself becomes more dynamic.

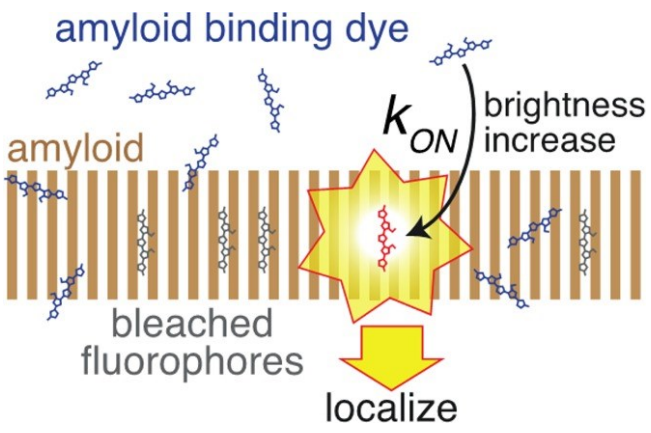
The principle of BALM can be applied to other targets (for example, proteins), and even other types of dye.

Examples of applications:

1. 2011, NanoLetters, Schoen et al: capturing images of the organization of the E. Coli bacterial chromosome. Using BALM, they visualized structures with a resolution of ~14 nm and a spatial sampling of 1 nm. This was possible due to the dynamic labeling technique and optimization of fluorophore brightness. Doi:10.1021/nl2025954
2. 2013, ACS Chem Neurosci, Reis et al: image α-synuclein amyloid fibrils throwing light on the structures and processes involved with amyloid structure formation, which plays a role in various neurodegenerative conditions, including Alzheimer's and Parkinson's disease. It could also help in the design of therapeutic drugs that perturb amyloid structure formation.

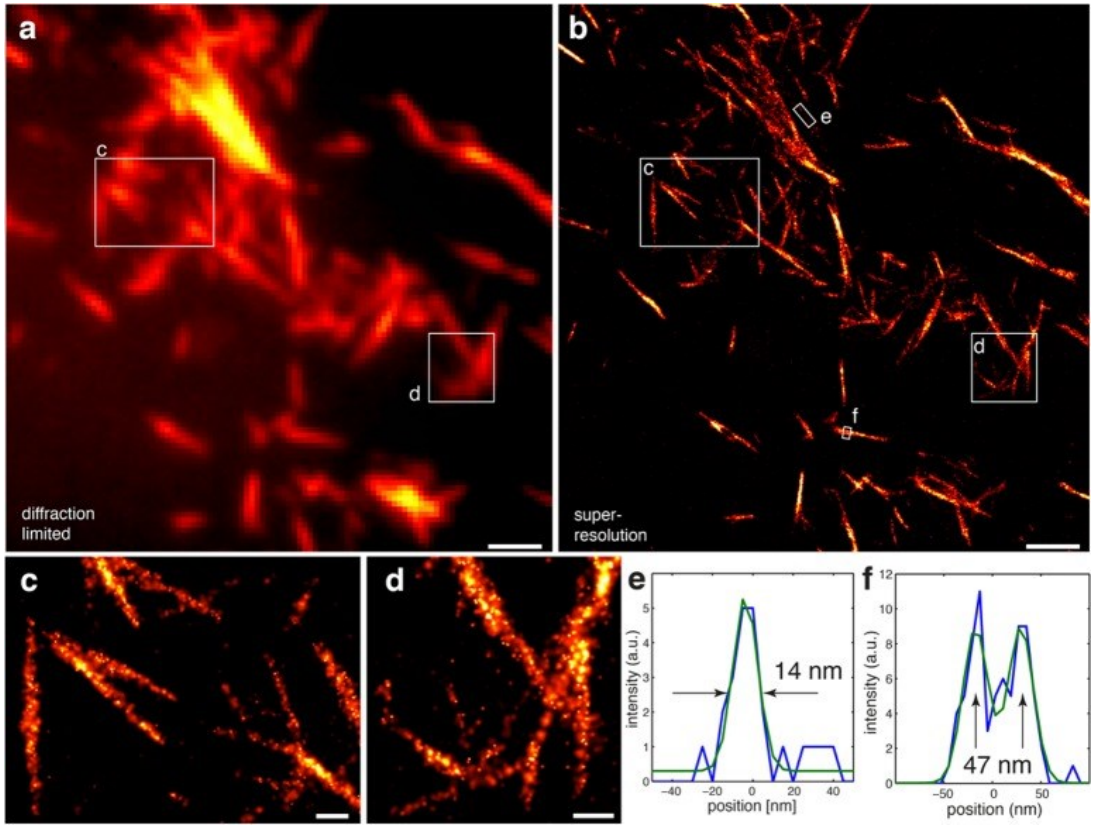
Principle of BALM on amyloids

Amyloid binding dyes at low concentration in solution undergo a strong fluorescence enhancement upon binding. Bright bound fluorophores can be imaged and localized before they are bleached.



2013, ACS Chem Neurosci, Ries et al.

doi: 10.1021/cn400091m

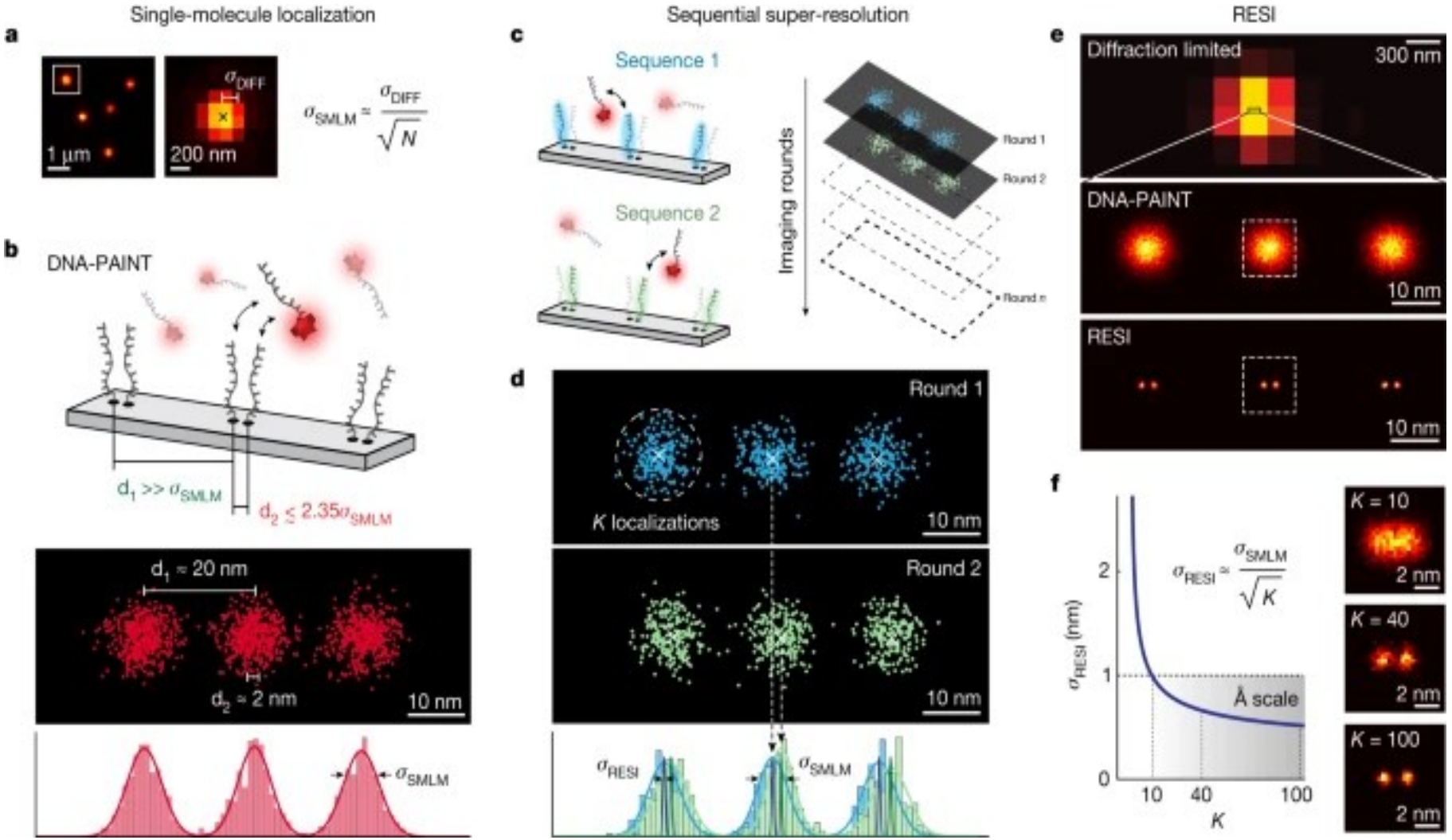


Scale bars 1 μm (a,b) and 200 nm (c,d).

BALM imaging of α-synuclein fibrils

(a) Diffraction limited image and (b) superresolution image of α-synuclein fibrils obtained with BALM using LCO (luminescent conjugated oligothiophenes) (c,d) Magnifications of (b). (e) profile of a thin structure (blue) and fit to Gaussian (green) to determine the resolution of this approach. The apparent thickness of the structure is 14 nm. (f) Profile across two neighboring fibrils with a distance of 47 nm that can be distinguished.

Resolution Enhancement by Sequential Imaging (RESI)



Ångström-resolution fluorescence microscopy

Reinhardt, S.C.M., Masullo, L.A., Baudrexel, I. et al.
Nature 617, 711–716 (2023). https://doi.org/10.1038/s41586-023-05925-9

Fig. 1 | RESI concept. a, In SMLM, σ_{SMLM} of a single dye scales with $\frac{\sigma_{DIFF}}{\sqrt{N}}$, ultimately limiting the achievable spatial resolution. **b**, SMLM approaches such as DNA-PAINT feature approximately 10 nm spatial resolution (resolution approximated as full-width at half-maximum $\approx 2.35\,\sigma_{SMLM}$). Whereas targets separated by 20 nm (d_1) can thus be routinely resolved, objects spaced 2 nm apart (d_2) are unresolvable because the resulting distributions of localizations overlap. **c**, Using orthogonal DNA sequences (blue and green) and sequential acquisition as in Exchange-PAINT, localizations from targets spaced more closely than the SMLM resolution limit can be unambiguously assigned for each target. **d**, Combining all localizations per target (K) for each imaging round improves localization precision from s.d. (σ_{SMLM}) to s.e.m. (σ_{RESI}). **e**, As super-resolution revolutionized fluorescence microscopy, RESI results in another paradigm shift by reapplying the concept of localization to super-resolution data. **f**, Localization precision in RESI scales with $\frac{1}{\sqrt{K}}$, and thus resolution improvement in RESI is independent of σ_{SMLM}, reaching localization precision on the Ångström scale.

Standard Error of the Mean (SEM) is calculated by taking the standard deviation (SD) and dividing it by the square root of the sample size.

SEM gives the accuracy of a sample mean by measuring the sample-to-sample variability of the sample means.

SD shows how dispersed the data is in relation to the mean

$$SE = \frac{\sigma}{\sqrt{n}}$$

← Standard deviation

← Number of samples

DNA-PAINT

PAINT : Point Accumulation for Imaging in Nanoscale Topography

PAINT is a single molecule localization microscopy (SMLM) technique that can reach a spatial resolution of of 10-30 nm.

The method is based on transient binding events between a pair of short complementary DNA sequences:

1. docking DNA strand that is immobilized on the target molecules, usually an antibody, nanobody or aptamer;

2. imager strand, a complementary ssDNA that is covalently bound to a fluorophore and suspended in the solution.

A series of quickly binding and unbinding events create a blinking effect that allows the localization of single molecules with high precision.

Exchange-PAINT

As imager strands only transiently bind to the docking strands, DNA-PAINT allows for a new multiplexing approach wherein orthogonal imager strands are sequentially applied to the same sample.

Initially, different target species are labeled with orthogonal docking strands. Once all components are labeled, the first imager strand species P1* (complementary to docking strands P1) is introduced and a DNA-PAINT image is acquired only for the targets labeled with P1.

In a subsequent washing step, imager strands P1* are removed and imager strands P2* are introduced. Another image for only P2 is then acquired. In each imaging step, the respective docking sites are super-resolved and a unique pseudocolor is assigned. Washing and imaging steps are repeated until all desired targets are imaged. These images are then aligned and combined to produce the final multicolor image for the entire sample.

RESI

RESI – Resolution Enhancement by Sequential Imaging

Similar with DNA Exchange Paint and DNA – Paint as idea.

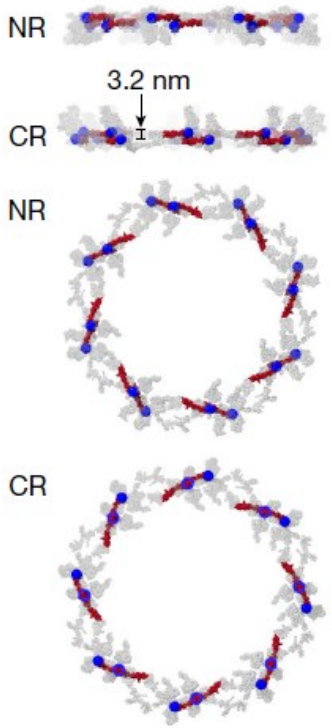
The implementation is different: a single target species is ‘multiplexed’ by separating it into multiple, sparser subsets.

By imaging them sequentially, sufficiently spaced and isolated groups of localizations are measured.

Determining the center of each group of localizations yields a resolution enhancement.

RESI resolves single nuclear pore complex (NPC) proteins

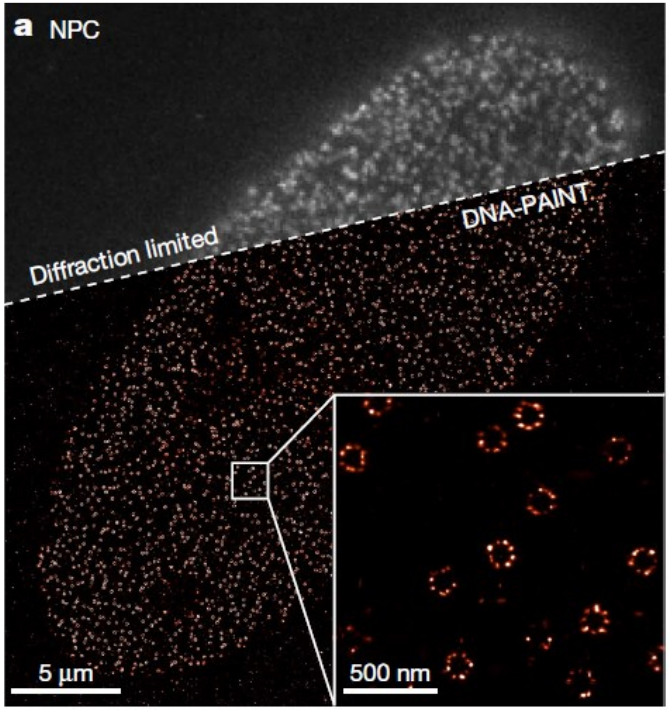
NPC structure



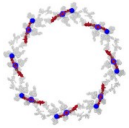
NUP96 is a structural NPC protein (part of the so-called Y-complex) present in eight pairs exhibiting an eight-fold symmetry on both cytoplasmic and nuclear rings, totaling 32 copies per NPC. Individual pairs of NUP96 proteins are spaced approx. 10 nm laterally and 3 nm axially

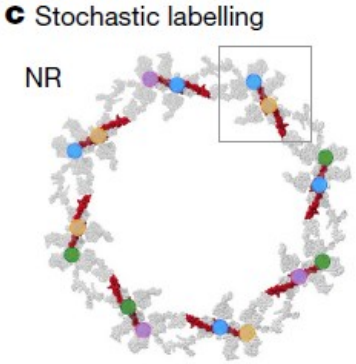
Reinhardt et al, Nature 2023

RESI resolves single nuclear pore complex (NPC) proteins

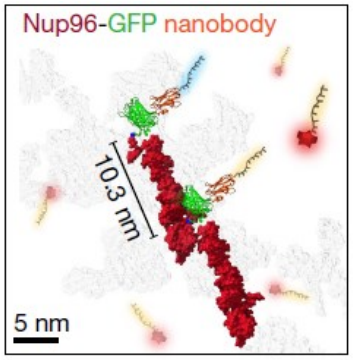


Diffraction limited and DNA-PAINT image of Nup96-mEGP labbeled with DNA-conjugated andti-GFP nanobodies. DNA – PAINT indicates the eight fold simmetry



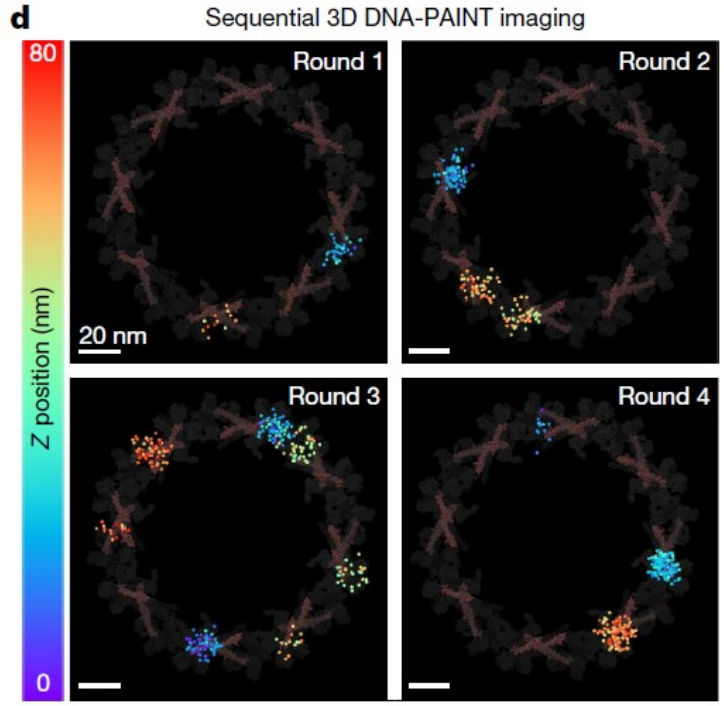


To enable RESI neighbouring copies of Nup96 proteins must be labelled with orthogonal DNA sequences. Sample is incubated with anti-GFP nanobodies, each conjugated with one out of four orthogonal sequences (represented by blue yellow magenta and green dots)



Reinhardt et al, Nature 2023

RESI resolves single nuclear pore complex (NPC) proteins

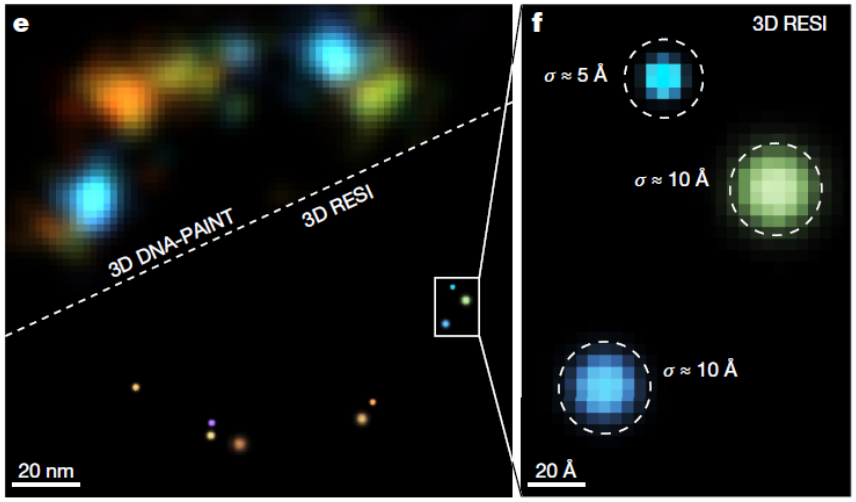


Sequential 3D DNA-PAINT imaging

Background represents cryo-EM structure

The average number of localizations K_{av}= 38

Color code: Z position

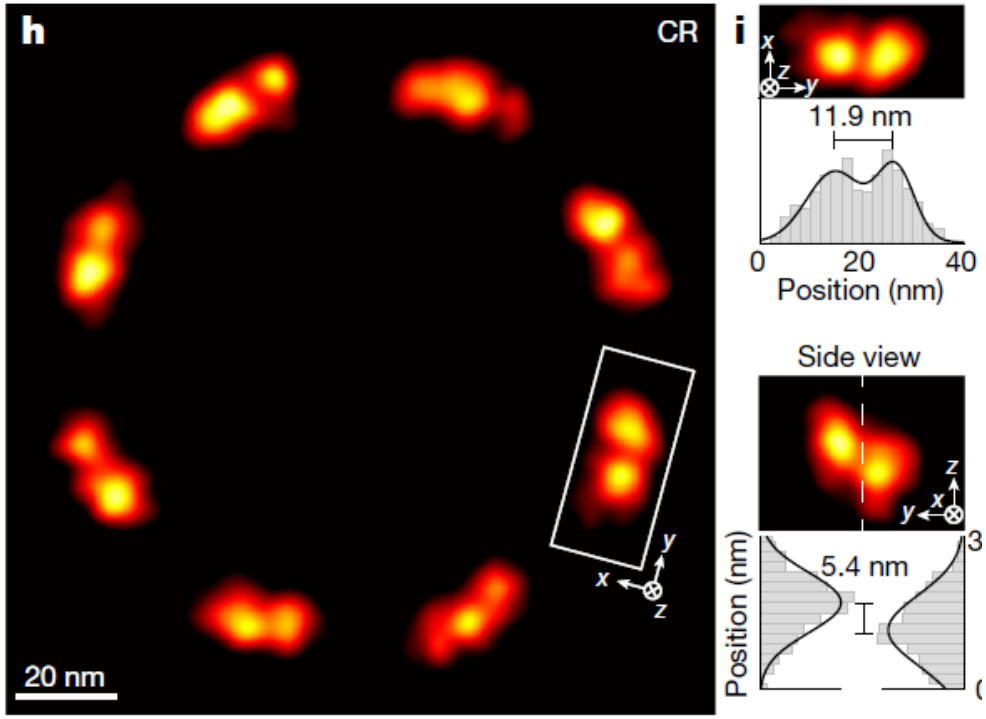


3D DNA-PAINT vs 3D RESI

Localization precision as good as 5 Angstrom !

Reinhardt et al, Nature 2023

RESI resolves single nuclear pore complex (NPC) proteins



RESI resolved adjacent Nup96 in a structural average by optical microscopy is consistent with the cryo-EM revealed structure !!!

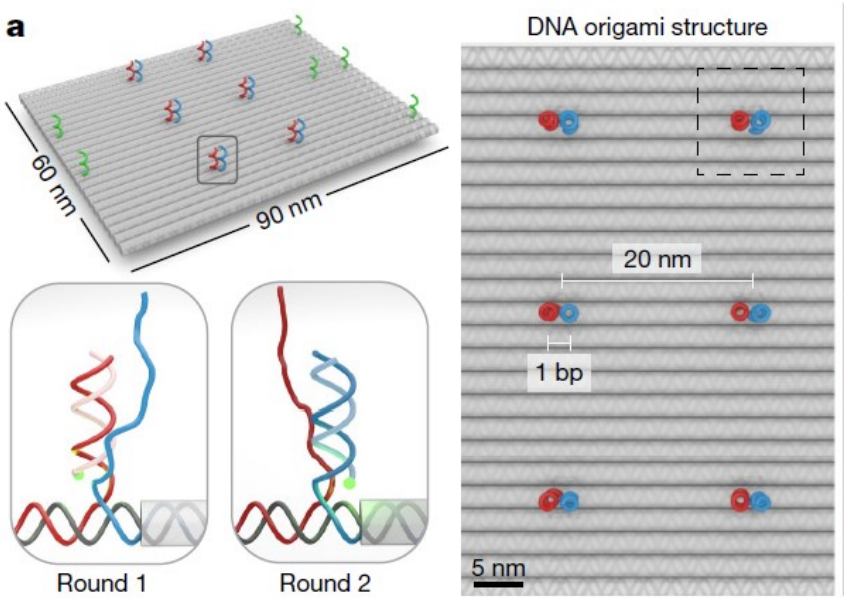
It shows that adjacent Nup96 proteins are spaced about 12 nm laterally and 5.4 nm axially !!

The differences come from the linkage error due to the label size

Full field of view access – 70x70 um with more than 1000 NPC imaged in parallel !

Reinhardt et al, Nature 2023

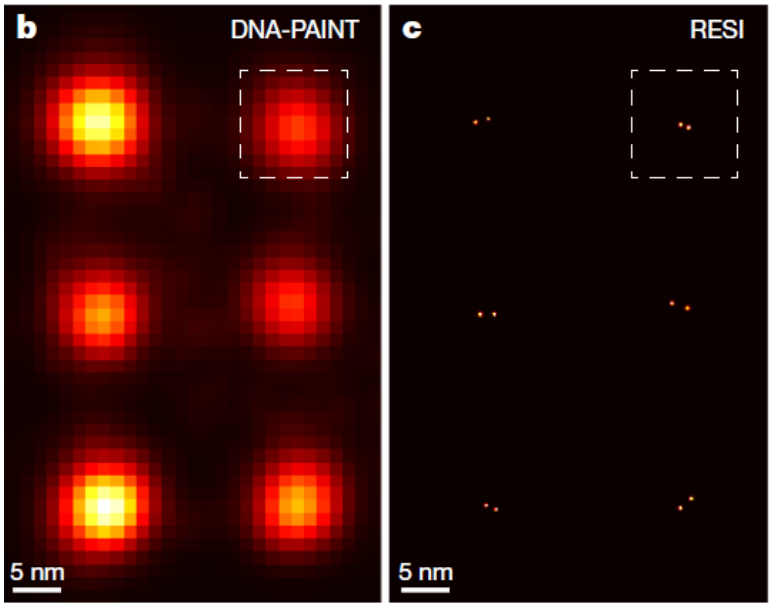
RESI provides imaging of DNA Base at Angstrom resolution



DNA origami with docking strands spaced by a single base pair bp (red and blue strands.)

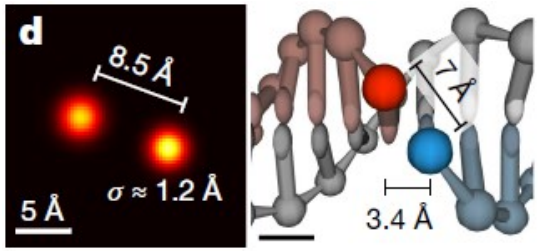
Alignment markers in green

!!! 1 bp = 0.34 nm = 3.4 A



DNA PAINT resolved 20 nm spacing;

RESI resolves the adjacent docking strands



Measurement vs model

Reinhardt et al, Nature 2023

SUPER RESOLUTION - FOR EXAM:

From the super-resolution microscopy part you have four research papers that can be presented at the exam. Allocated time for presentation 15 min: principle, main characteristics, one application specifying why superresolution is important in the bio application presented from the respective paper.

STED
Elisa D'Este et al, STED Nanoscopy Reveals the Ubiquity of Subcortical Cytoskeleton Periodicity in Living Neurons, CELL REPORTS 2015, Doi: 10.1016/j.celrep.2015.02.007

PALM
Eric Betzig et al, Imaging Intracellular Fluorescent Proteins at Nanometer Resolution, SCIENCE 2006 DOI: 10.1126/science.1127344

MINFLUX
Francisco Balzarotti, ..., Stefan W. Hell, Nanometer resolution imaging and tracking of fluorescent molecules with minimal photon fluxes, SCIENCE 2017 Doi: 10.1126/science.aak9913

RESI
Reinhardt, S.C.M., Masullo, L.A., Baudrexel, I. et al.
Ångström resolution fluorescence microscopy
NATURE 617, 711-716 (2023). https://doi.org/10.1038/s41586-023-05925-9

Photobleaching-based Techniques for Assessing Cellular Dynamics

- Fluorescence Recovery after Photobleaching (FRAP) → Thalhammer
- Fluorescence Loss in Photobleaching (FLIP)
- Fluorescence Localization after Photobleaching (FLAP)
- Photo-Activation (PA)

REVIEW :
H. C. Ishikawa-Ankerhold et al,
Advanced Fluorescence Microscopy Techniques—FRAP, FLIP, FLAP, FRET and FLIM ,
Molecules 2012, 17, 4047-4132; doi:10.3390/molecules17044047

Fluorescence Recovery after Photobleaching (FRAP)

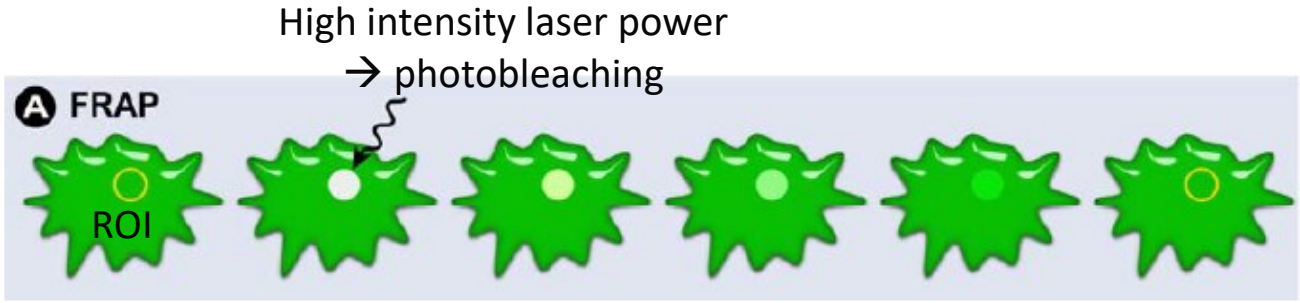
In 1970 FRAP was developed as a technique to study protein mobility in living cells by measuring **the rate of fluorescence recovery at a previously bleached site**. Originally it was used to measure the diffusion in cellular membranes and later also in cell interior.

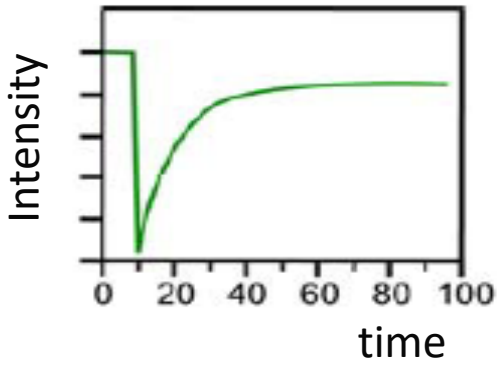
FRAP is a versatile method, and it has become a common **technique for studying dynamics** in almost all aspects of cell biology, including:

cytoskeletal dynamics, vesicle transport, cell adhesion, mitosis, chromatin structure, transcription, mRNA mobility and DNA-interacting molecules, protein recycling and signal transduction.

FRAP working principle

In FRAP, fluorescent molecules are irreversibly photobleached in a small area (ROI) of the cell by high intensity illumination with a focused laser beam.
Subsequently, diffusion of the surrounding non-bleached fluorescent molecules into the bleached area leads to recovery of fluorescence with a particular velocity, which is recorded at low laser power.
!! During FRAP, the high light intensity in the presence of molecular oxygen causes irreversible damage to the fluorochrome.





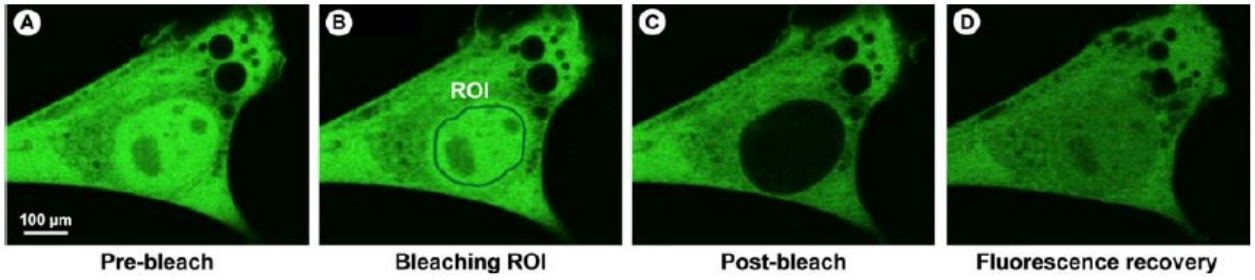
FRAP example

monomeric GFP-Myosin III can easily traverse the nuclear envelope membrane.

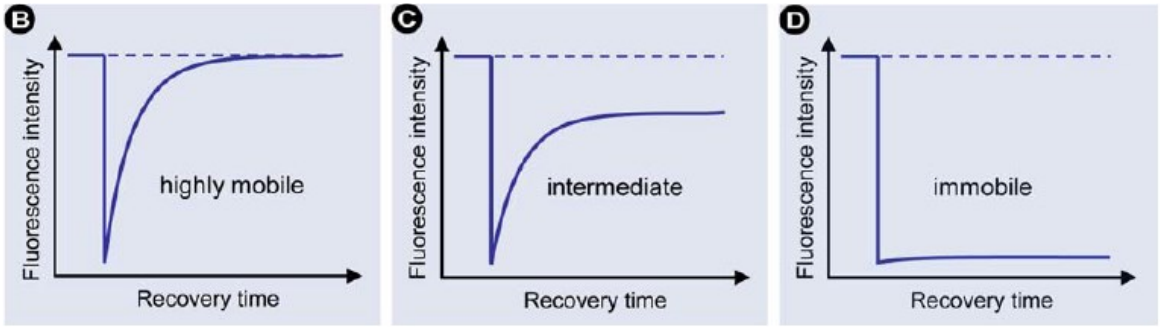
A nuclear region (ROI) is bleached with high intensity (~500 ms; >30 mW) with a 488 nm laser.

Subsequently, the nucleus is devoid of green fluorescence.

Over time the fluorescence recovers and reaches a plateau.



Notice by comparing A,D that the total fluorescence intensity decreases, because a significant number of fluorochromes were irreversibly bleached.



Kambara, T.; Human myosin III is a motor having an extremely high affinity for actin. J. Biol. Chem. 2006,

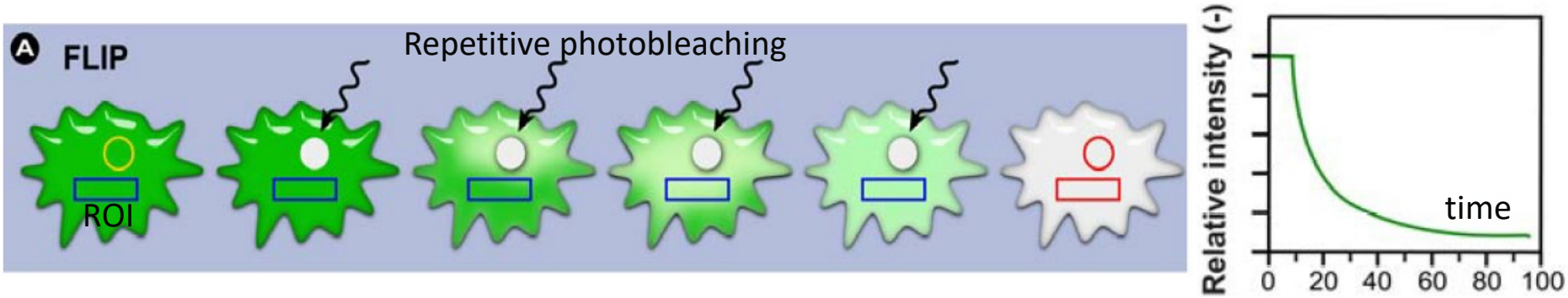
Fluorescence Loss in Photobleaching (FLIP)

In FLIP experiments the repetitive bleaching occurs adjacent to the unbleached ROI

The loss in fluorescence in the ROI defines the mobile fraction of the fluorescently labeled protein.

Conversely, the incomplete loss in fluorescence defines the immobile fraction of fluorescently-labeled protein that does not move into the continuously photo-bleached area.

The observation that molecules do not become bleached suggests that they are isolated (immobilized) in distinct cellular compartments.



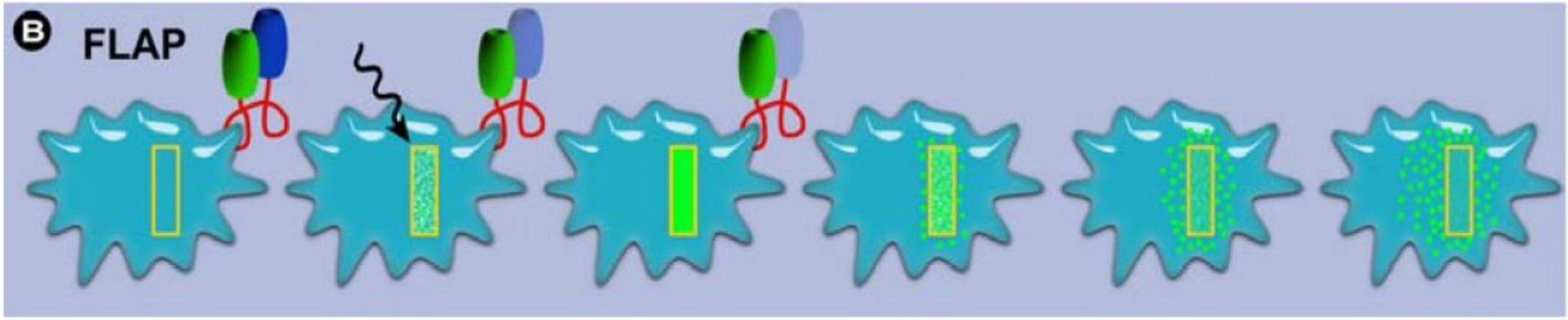
FLIP experiments are very useful to demonstrate the **connectivity and fluxes between different regions of the cell** and thus is an ideal and direct method for studying **the exchange of molecules between two compartments** (e.g., compartments that are separated by lipid bilayers).

The continuity of cellular structures, such as the Golgi apparatus, the endoplasmic reticulum, the protein traffic between the nucleus and cytoplasm, the nucleolus and splicing factor compartments, and the nucleolus and nucleoplasm have all been studied using FLIP .

FLIP is often used in combination with FRAP experiments to obtain combined information regarding active or passive transport. In fact, FLIP can be used as a control for FRAP experiments.

Fluorescence Localization after Photobleaching (FLAP)

In FLAP, a protein is tagged with two fluorescent labels: one is photobleached and the other acts as a reference. The use of a reference fluorochrome allows the tracking of the distribution of the labeled molecules by simple image differencing (I) and thus enables measurement of fast relocation dynamics.



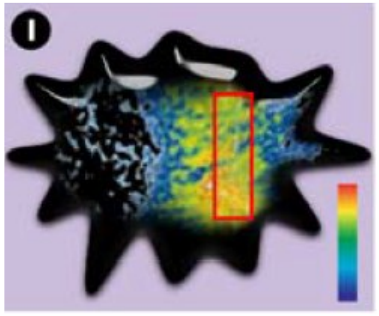


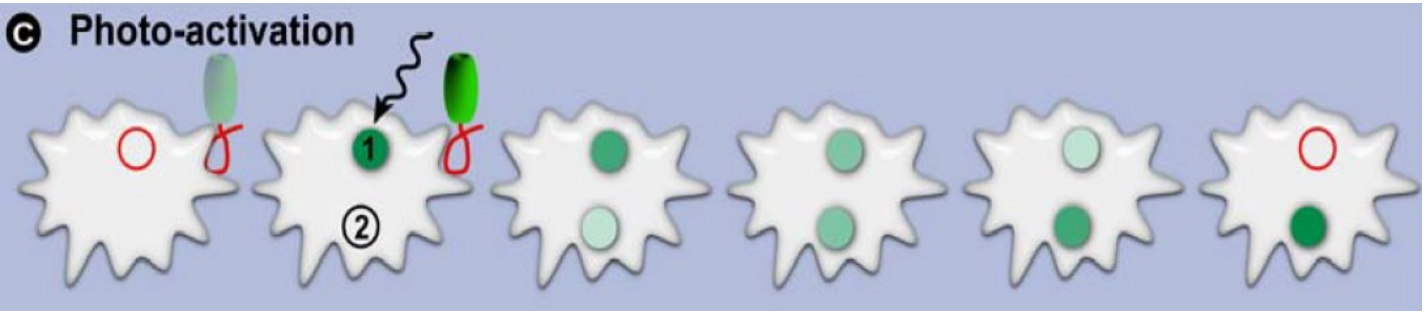
Photo-Activation (PA)

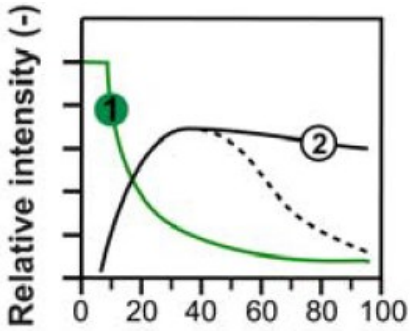
In photo-activation (PA), a fluorescent label, often a fluorescent protein, is irreversibly activated from a low fluorescent (dark) state to a bright fluorescent one by irradiating the sample with light of a specific wavelength, intensity and for a particular duration.

The change in fluorescence intensity is monitored in both the compartment in which the probes are activated (compartment 1) and the destination compartment (compartment 2). The loss in fluorescence in compartment 1 and gain in 2 are monitored simultaneously, which provides information on protein dynamics and compartment interconnectivity.

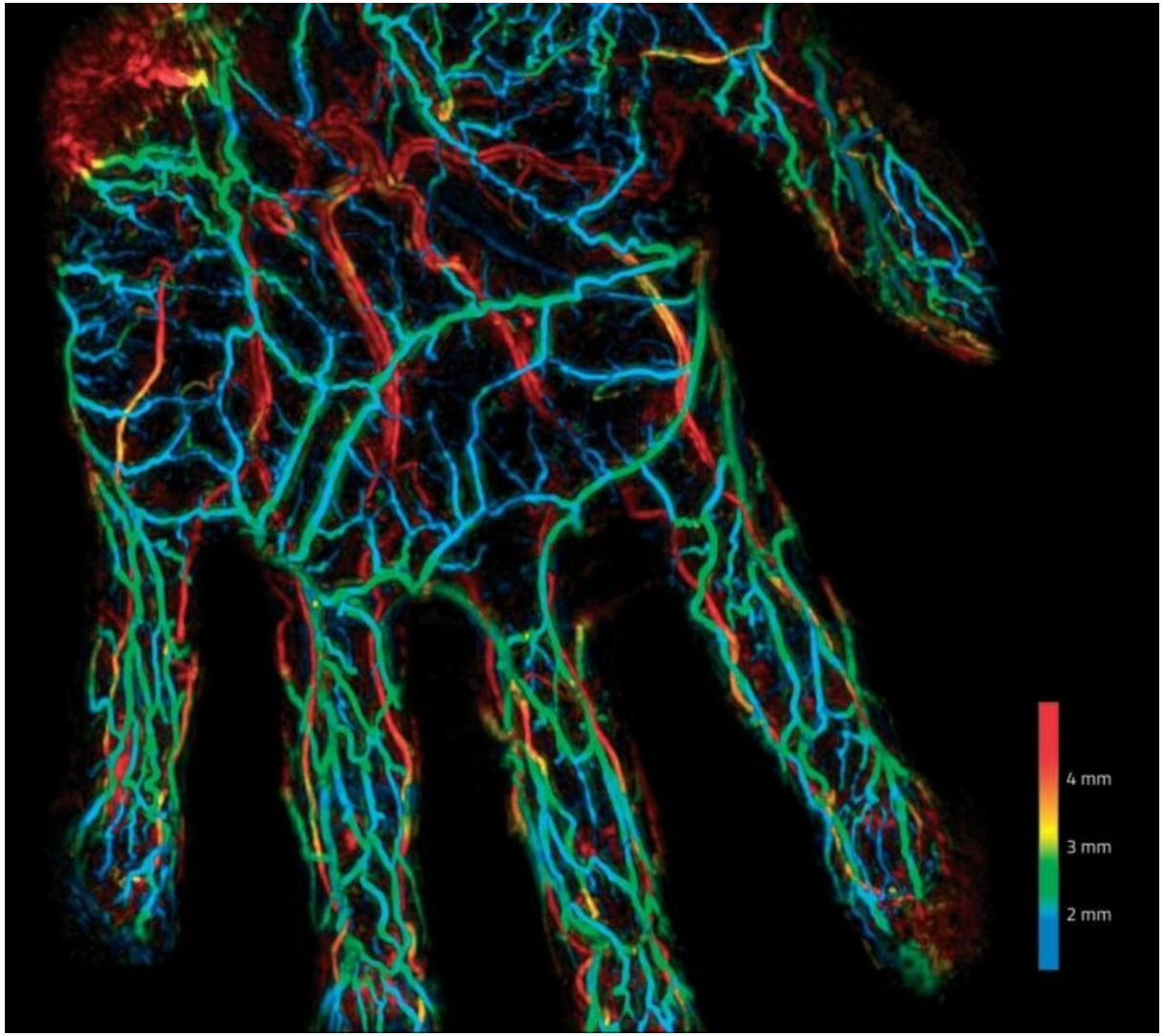
Note that when the fluorochrome moves from 1 to 2 and subsequently diffuses out of that compartment, the curve reaches a maximum and decreases again (dotted black line).

Even though these procedures are similar to iFRAP, PA offers the advantage that the entire cell does not need to be bleached and consequently requires less energy and time to start the experiment.



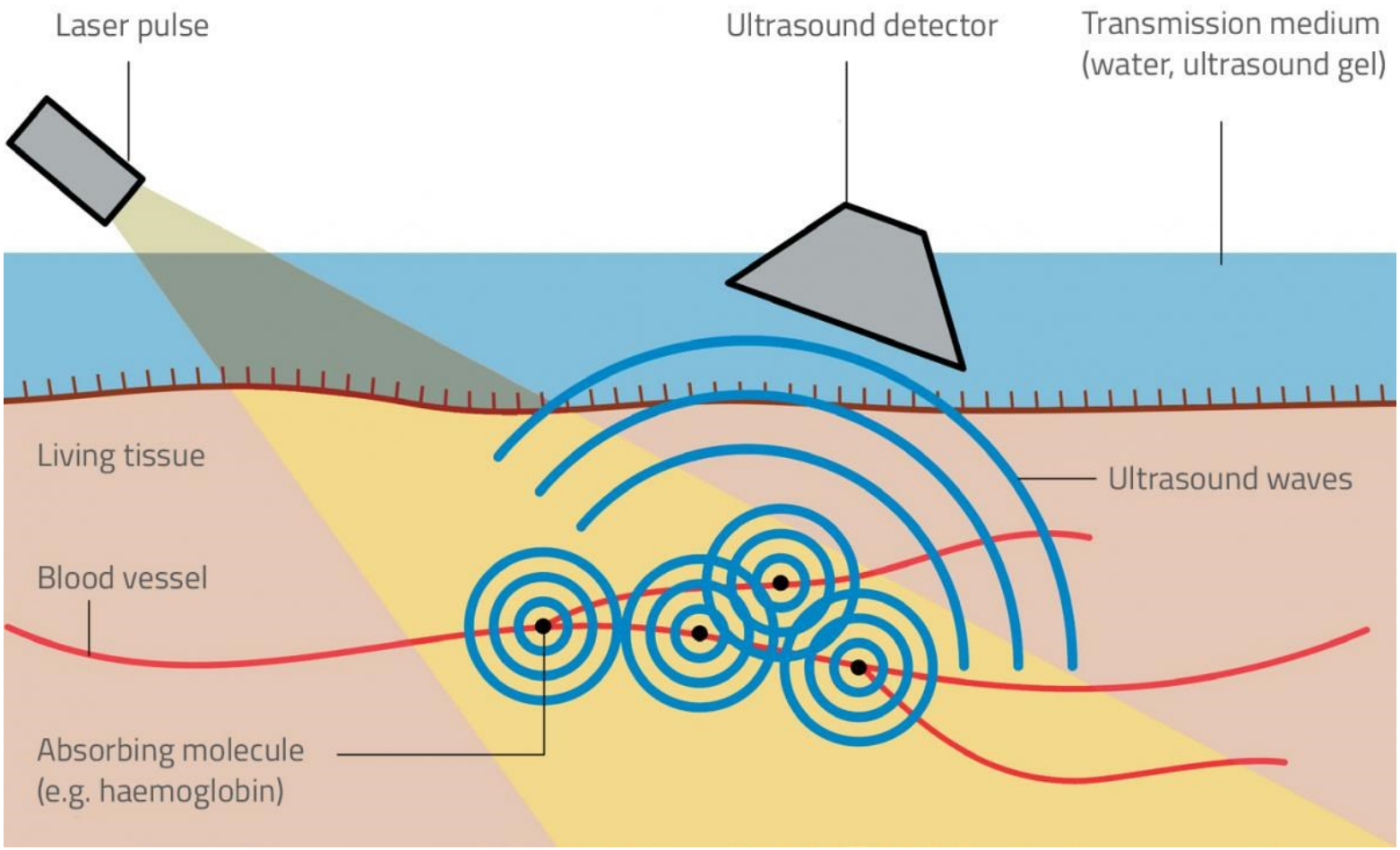


What does this image represent ?



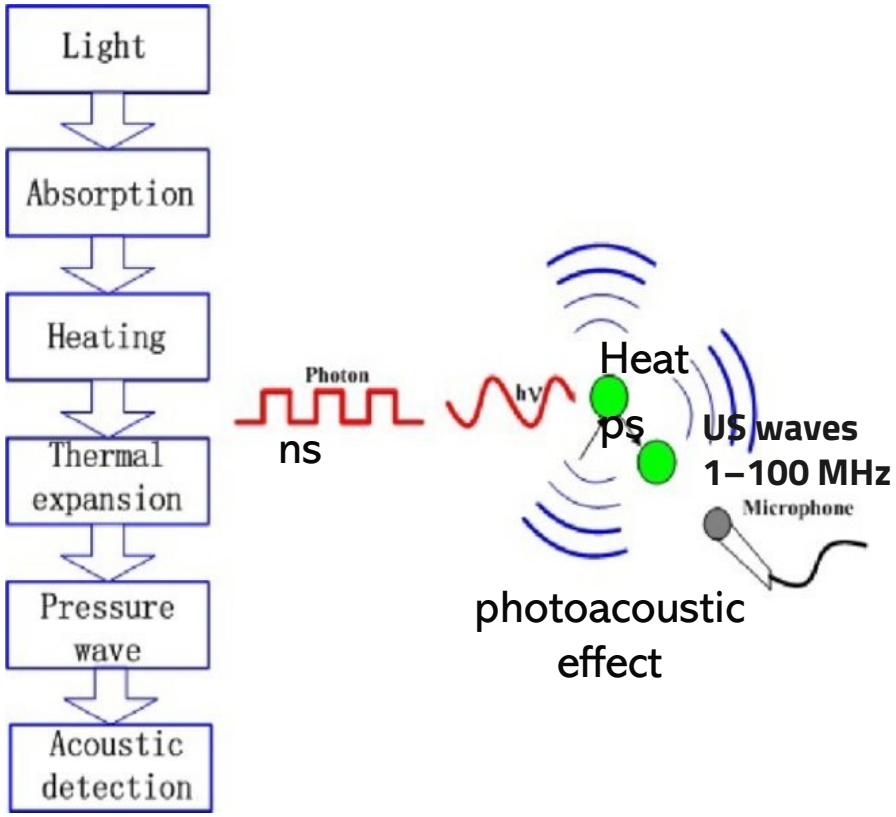
Photoacoustic image of blood vessels in a human palm.
Matsumoto et al. (2018) *Scientific Reports*

How photoacoustic imaging works



PhotoAcustic Microscopy (PAM)

To see living tissues



Principle:

Converts photon energy into ultrasound energy on the basis of the photoacoustic effect (PAE) (Alexander Graham Bell in 1880)

PAE: acoustic waves are generated as a result of light incidence on a material with specific properties.

The incidence of light on the material raises its temperature, and as a result, it thermally expands.

The continuous thermal expansion and retraction will then generate ultrasound (US) waves which are detected by an US transducer to form an image which maps the original optical energy deposition in the tissue.

https://www.scienceinschool.org/article/2019/photoacoustics-seeing-sound/

US scattering coefficient (at 5 MHz) by tissue in human skin is $\mu_s \sim 1.2 * 10^{-3}$ mm^{-1} while the optical scattering coefficient is $\mu_o \sim 10$ mm^{-1} at 700 nm (much bigger).

Since the amplitude of the PA signal is proportional to the optical energy deposition → PAM is sensitive to the rich optical absorption contrast of tissue.

PAM employs raster-scanning of optical and acoustic foci and forms images directly from acquired depth-resolved signals.

PAM maximizes its detection sensitivity by confocally aligning its optical illumination and acoustic detection.

While the axial resolution of PAM is primarily determined by the imaging depth and the frequency response of the ultrasonic transducer, its lateral resolution is determined by the combined point spread function of the dual foci. Optical Resolution OR-PAM vs Acoustic Resolution AR-PAM

Reflection, transmission or double illumination mode – depending on the application

Refs

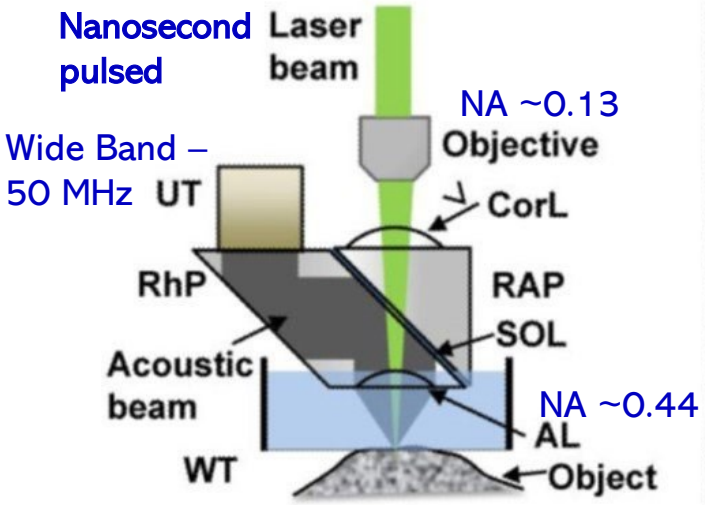
J. Yao ad Lihong V. Wang, Photoacoustic Microscopy, Laser Photon Rev. 2013 1; 7(5)

L. V. Wang and Song Hu, Photoacoustic Tomography: In vivo from organelles to organs, Science 2012, 23: 335(6075)

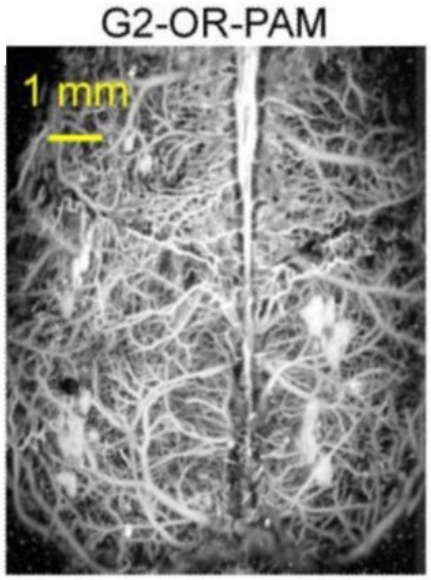
Optical Resolution OR-PAM implementation

(higher lateral resolution lower depth)

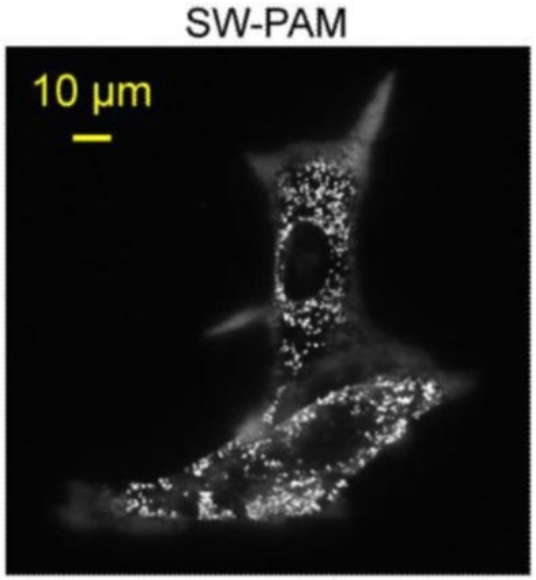
$$R_{L,OR}=0.51\frac{\lambda_0}{NA_0} \sim 2.5\ \mu m$$



AL, acoustic lens; Corl, correction lens;
RAP, right angled prism;
RhP, rhomboid prism;
SOL, silicone oil layer;
UT, ultrasonic transducer; WT, water tank.



cortical vasculature in a living mouse with the scalp removed but the skull intact

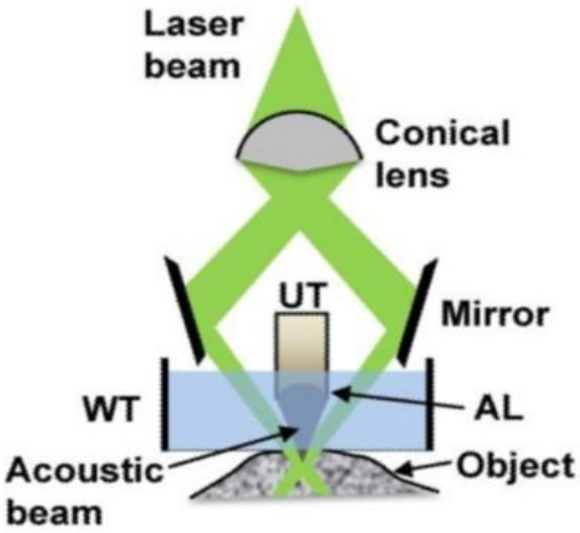


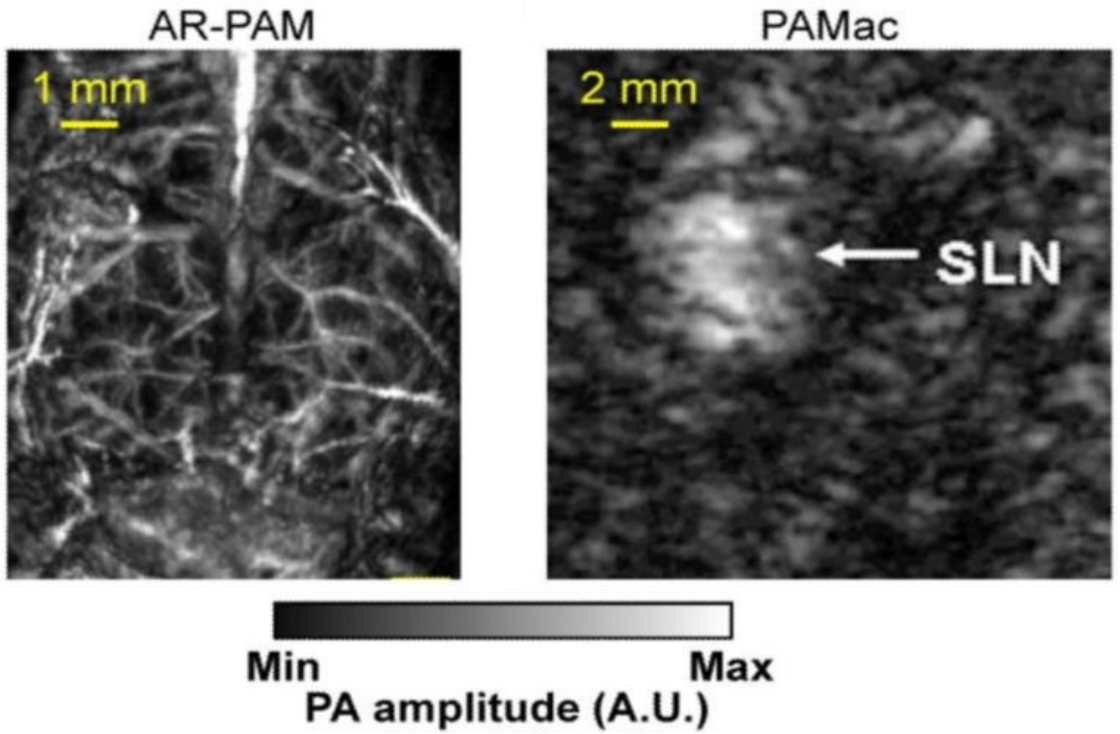
PAM of a melanoma cell, where single melanosomes can be resolved

Acoustic Resolution AR – PAM implementation

(lower lateral resolution higher depth)

$$R_{L,AR} = 0.71 \frac{\lambda_A}{NA_A} = \sim 45\ \mu m$$





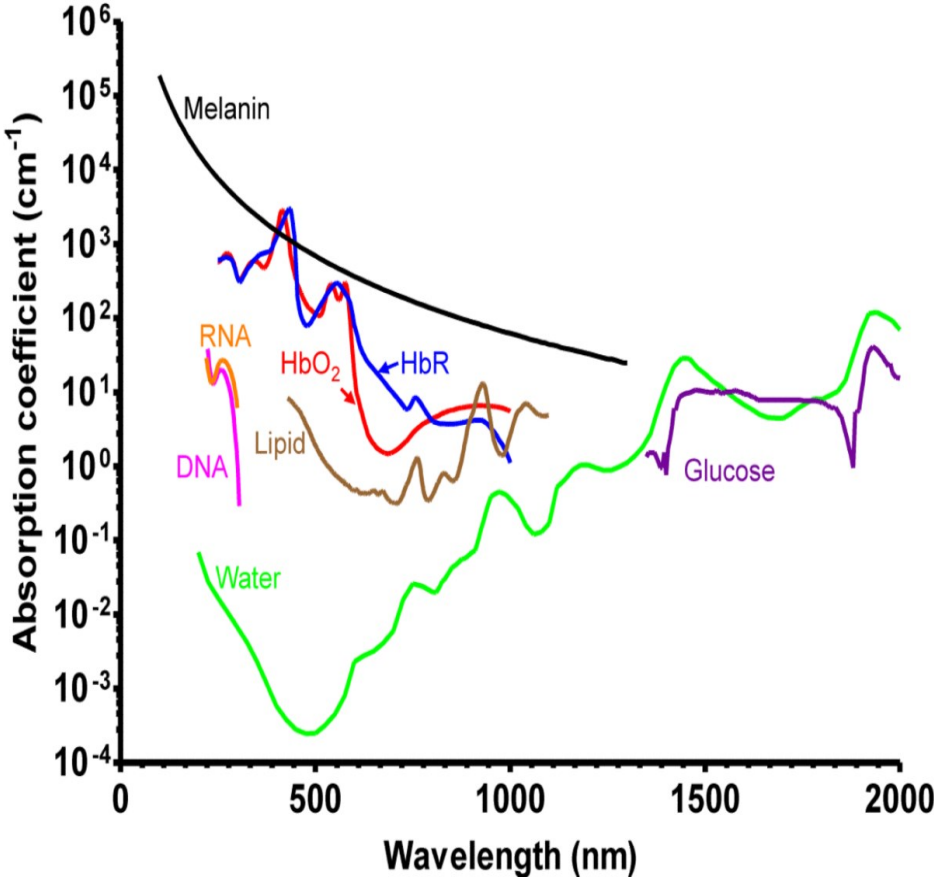
Dark-field of the cortical vasculature in a living mouse with both the scalp and skull intact.

Deep photoacoustic macroscopy (PAMac) of the sentinel lymph node (SLN) in a living rat. The SLN was about 18 mm deep

Multi - contrast PAM

PAM vs fluorescence microscopy: both start with photon excitation of molecules, but **PAM can potentially image all molecules**. Fluorescence microscopy can image only a small fraction of molecules, those that exhibit fluorescent relaxation.

Absorption spectra of major **endogenous contrast** agents in biological tissue:



Oxyhemoglobin, red line (150 g/L in blood);

Deoxy-hemoglobin, blue (150 g/L in blood);

Lipid, brown (20% by volume in tissue);

Water, green (80% by volume in tissue);

DNA, magenta (1 g/L in cell nuclei);

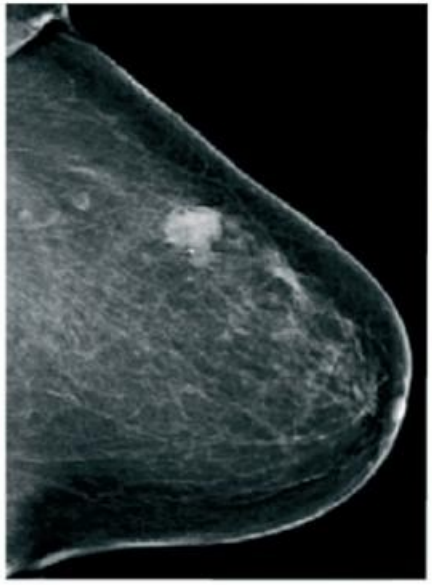
RNA, orange (1 g/L in cell nuclei);

Melanin, black (14.3 g/L in human skin);

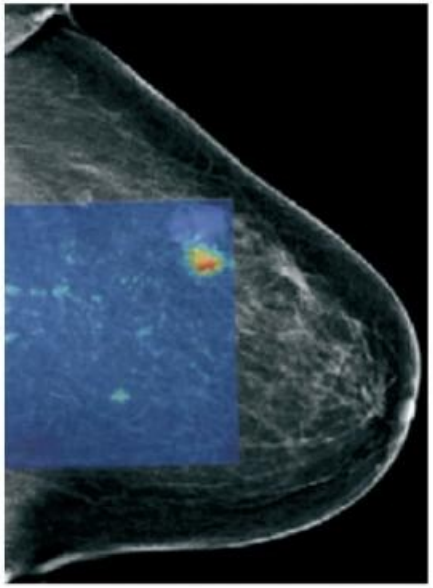
Glucose, purple (720 mg/L in blood).

Clinical applications

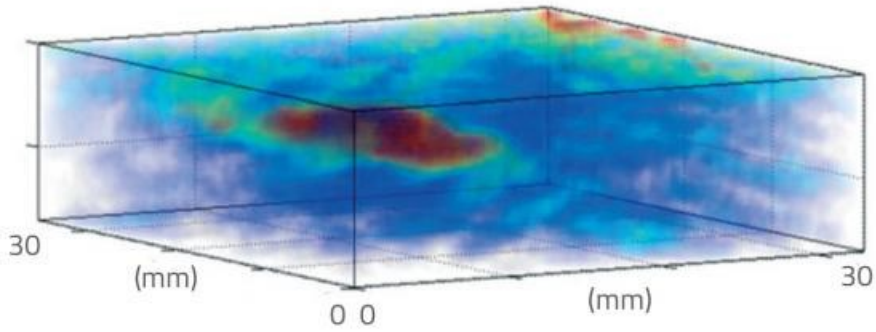
One application lies in tumour detection When tumours are growing, they trigger the reorganisation of surrounding blood vessels, often causing many new blood vessels to form in neovascularisation.



X-ray image

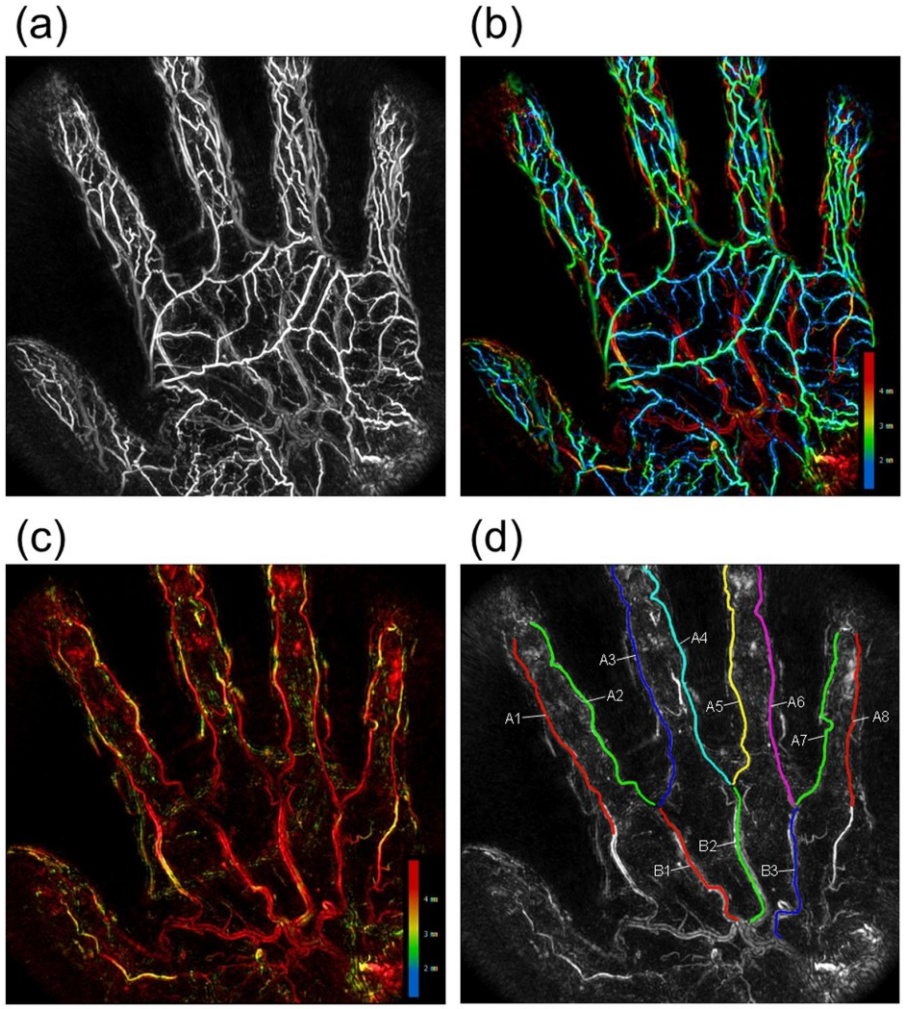


Combined X-ray and photoacoustic image



3D photoacoustic image

Comparison between X-ray and photoacoustic tomography for breast tumour imaging. As well as avoiding harmful radiation, photoacoustic tomography can reveal an increase in blood vessels associated with tumour growth. Heijblom et al. (2016) *European Radiology*



Example of a blood vessel image of the palm taken at a wavelength of 795 nm using.

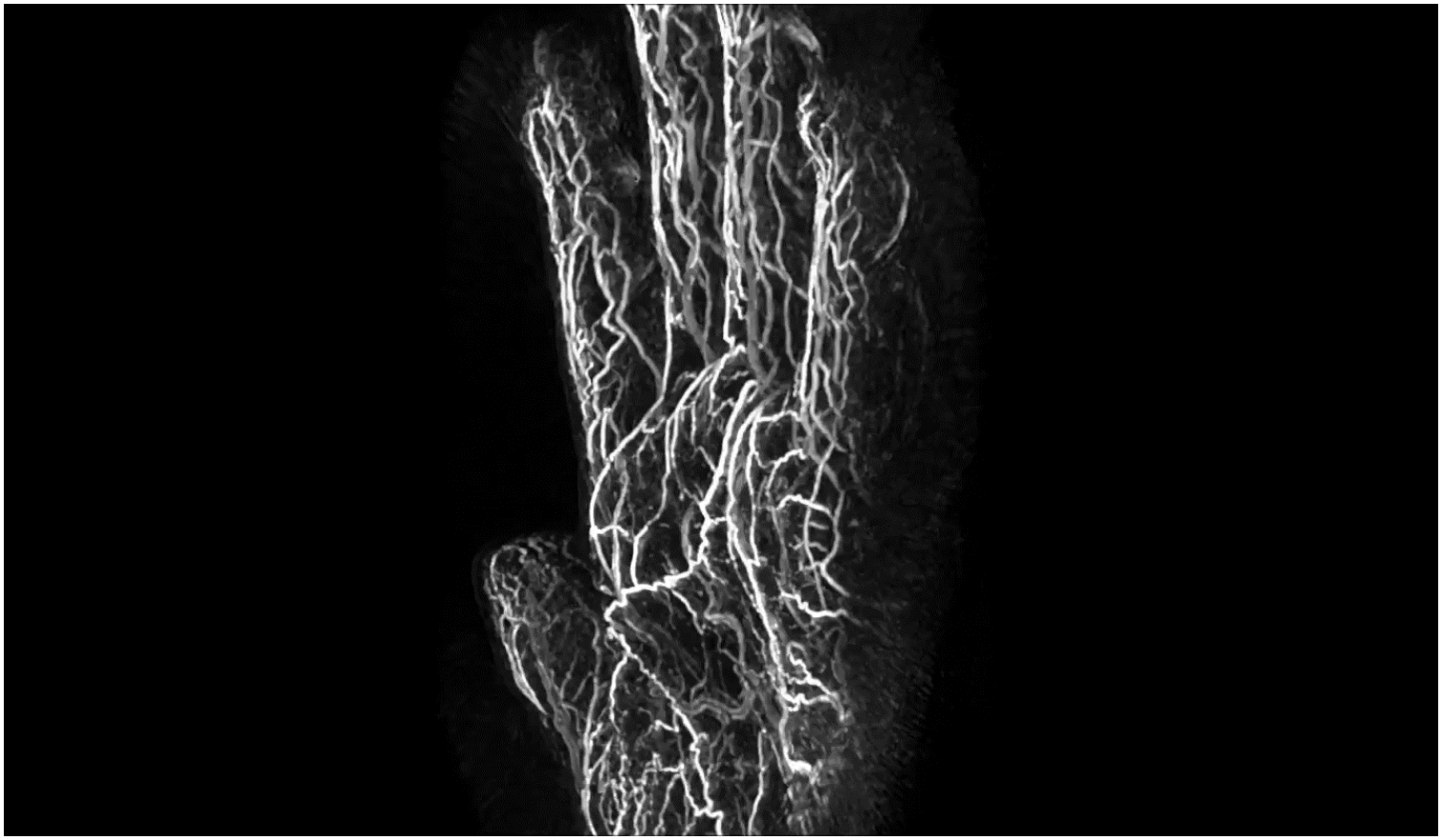
(a) Complete maximum intensity projection (MIP) image of the hand;

(b) image representing the depth as a colour parameter;

(c) MIP image after deletion of the vein image near the skin surface;

(d) superimposed image of the binarized coloured lines extracted from the common and proper palmar digital arteries over the original PA image.

Matsumoto et al.
(2018) *Scientific Reports*



Matsumoto et al. (2018) *Scientific Reports*

PAM has the following notable features:

(1) PAM breaks through the optical diffusion limit, with highly scalable spatial resolution and maximum imaging depth in both the optical and acoustic domains.

(2) PAM images optical absorption contrast with 100% sensitivity, and provides images without speckle artifacts.

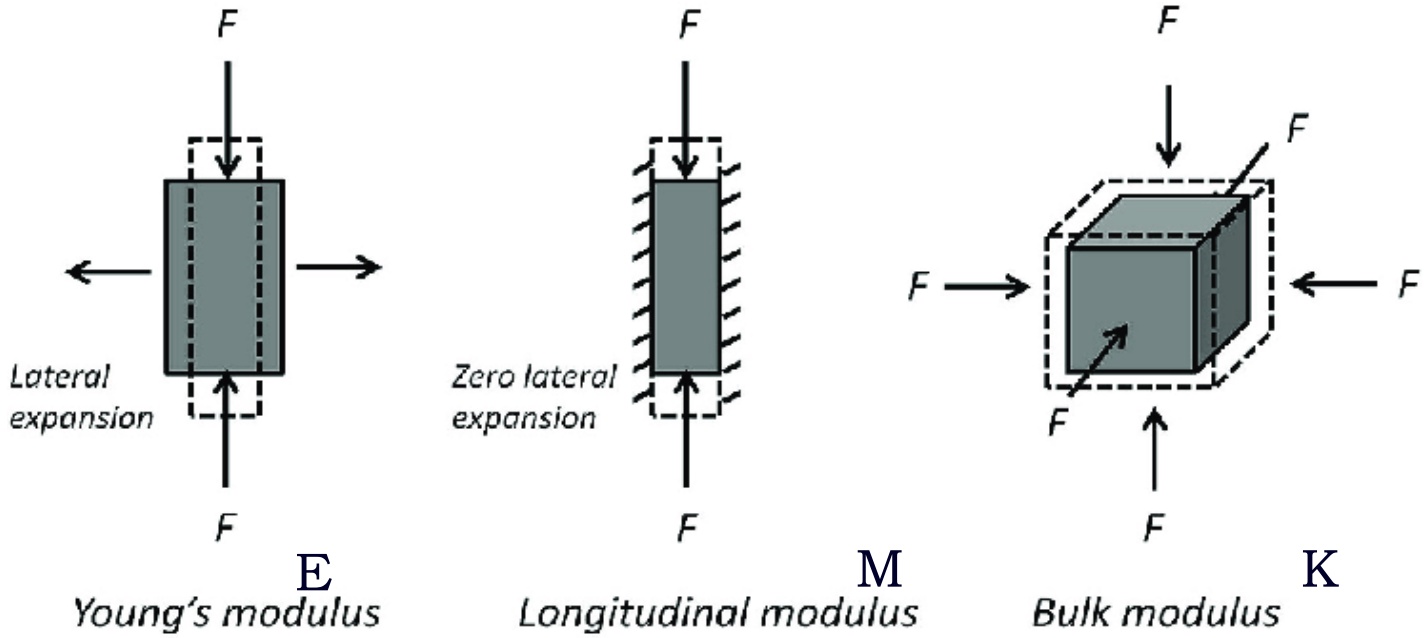
(3) PAM can essentially image all molecules at their absorbing wavelengths.

(4) PAM is capable of functional and metabolic imaging in absolute units using endogenous contrast agents.

PAM is expected to find new applications in both fundamental life science and clinical practice, which include but are not limited to tumor angiogenesis, lymphatic dynamics, neural activity, brain metabolism, cancer detection, drug delivery and intraoperative monitoring.

Brillouin Microscopy

Brillouin microscopy provides an alternative and complimentary assessment of material elasticity and viscosity through measurement of the longitudinal modulus in the GHz frequency range.



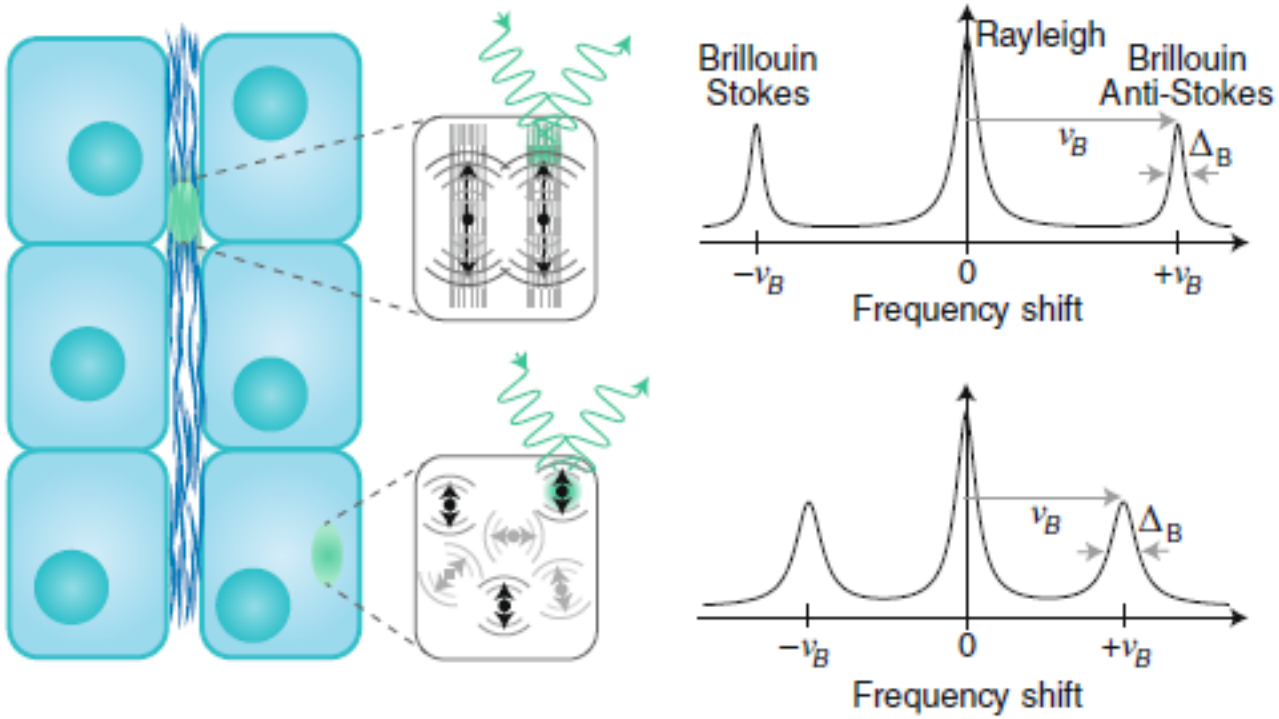
$$K = E/3(1 - 2\nu)$$

$$M = E(1 - \nu)/(1 + \nu)(1 - 2\nu)$$

ν Poisson ratio:
ratio between strain in the transverse direction to strain in the axial direction

Brillouin scattering in heterogeneous biological samples

Brillouin light scattering is an inelastic process arising from the interaction of light with spontaneous, thermally induced density fluctuations. These can be described asna population of microscopic acoustic waves often called phonons.



Monochromatic laser light is inelastically scattered by phonons propagating in the longitudinal (axial) directions. Light scattered off solid components (for example, collagen fibers; top) experiences a high Brillouin frequency shift. In contrast, the spectrum from the liquid-like cytosol (bottom) results in lower shift (indicating a less rigid material) and larger linewidth (indicating a more viscous medium).

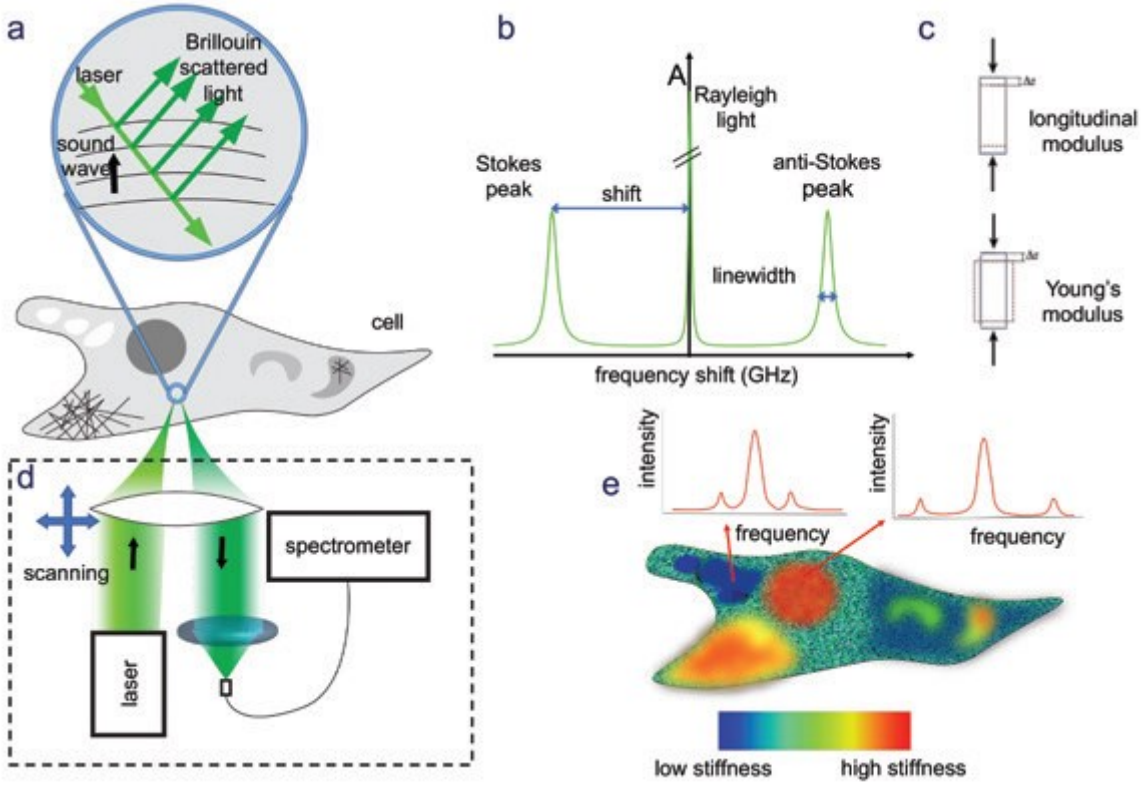
Prevedel R. et al Nature Methods 2019

Brillouin microscope

The speed of the soundwave induced by the laser beam and its lifetime determine the frequency shift and the linewidth of Brillouin scattered light, typically in the GHz regime.

Confocal implementation of Brillouin microscopy: the laser is focused on the sample by an objective lens and Brillouin scattered light is collected by the same lens. Subsequently, a single mode fiber acts as a confocal pinhole and delivers the light to the spectrometer.

A stiffness map can be constructed by scanning the focal point across the sample and plotting the frequency shift at each position.



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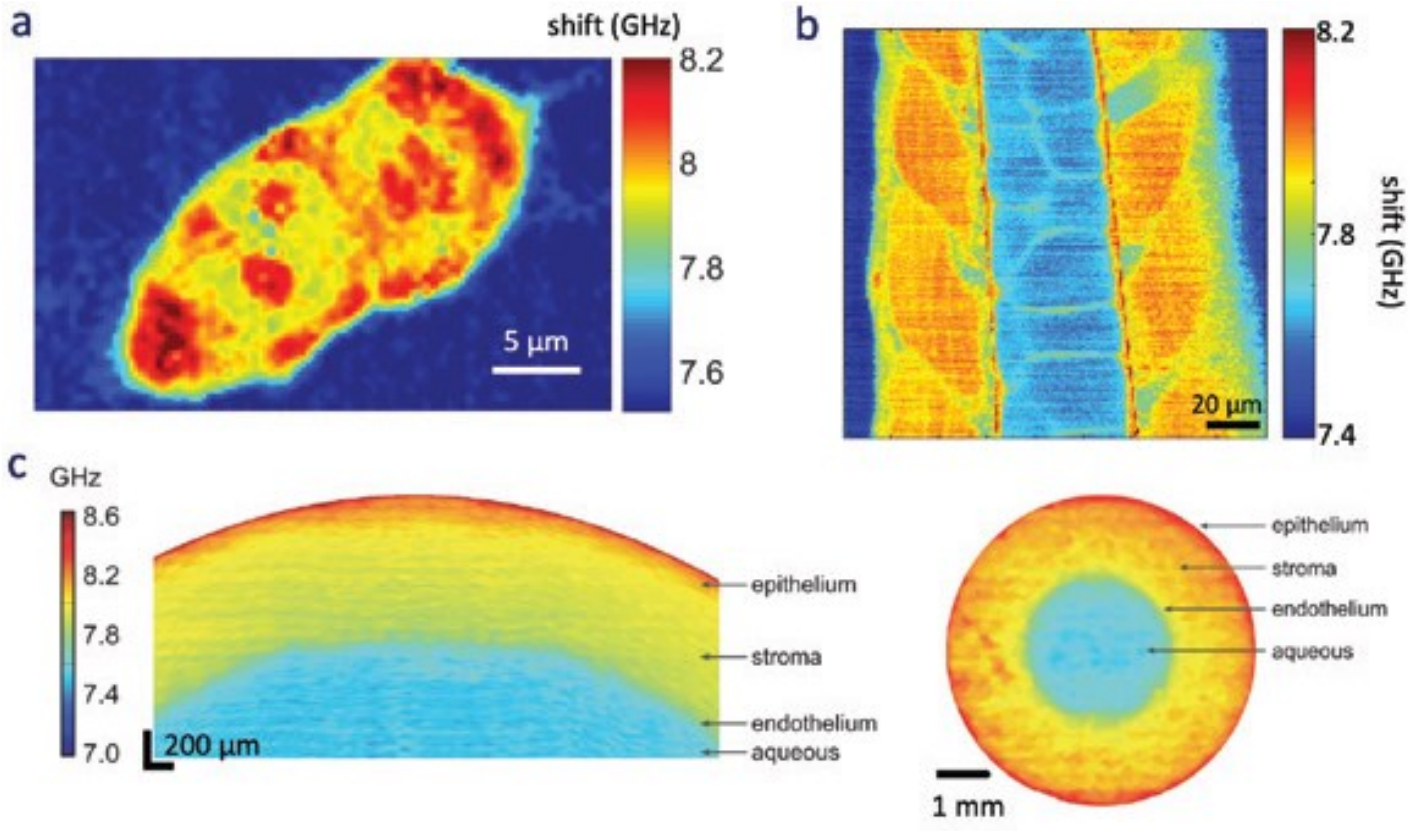
Brillouin images - biological samples examples

a) A mouse embryonic stem cell, showing sub-cellular scale stiffness differences.

b) Tail of a zebrafish larva where anatomical structures (muscles, sheath cells, notochord) can be clearly distinguished based on their stiffness properties.

c) Left: Sections of a bovine cornea showing the heterogeneity of mechanical properties. Left: Cross section. Right: Optical section from a deep layer.



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Optical Microscopy - CONCLUSIONS

- The optical microscope offers the unique advantage to study living cells
- The image contrast is formed by the changes introduced by the sample to the amplitude, phase, polarization of the intensity of the electrical field (non-stained samples)

WOPS !

Optical Microscopy to be continued with

NON LINEAR OPTICS Microscopy

molecule ! There are also other techniques as BALM based on the nucleic acid stains binding on DNA strand. Spatial resolution can thus be pushed to 1 nm !and less using visible light microscopy !!!

- Photobleaching-based Techniques for Assessing Cellular Dynamics (FRAP, FLIP, FLAP, PA)
- Photoacoustic Microscopy – no staining, deeper penetration, essentially image all molecules at their absorbing wavelengths
- Brillouin Microscopy – no staining, probes elasticity and viscosity