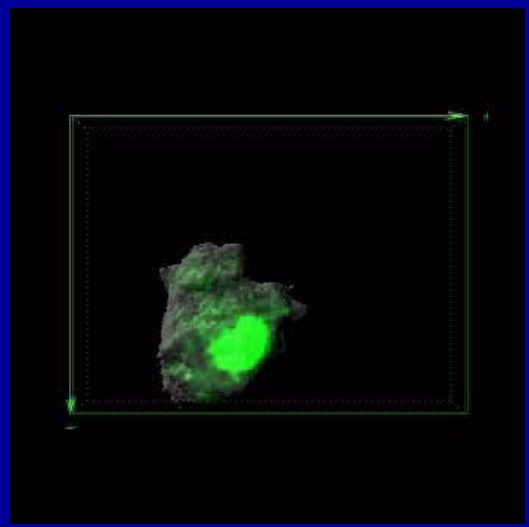
Jens Rietdorf:

This presentation is meant to give some general hints for live specimen microscopy

Live Specimen Microscopy

Practical Course on
EMBO Live Specimen
Light Microscopy

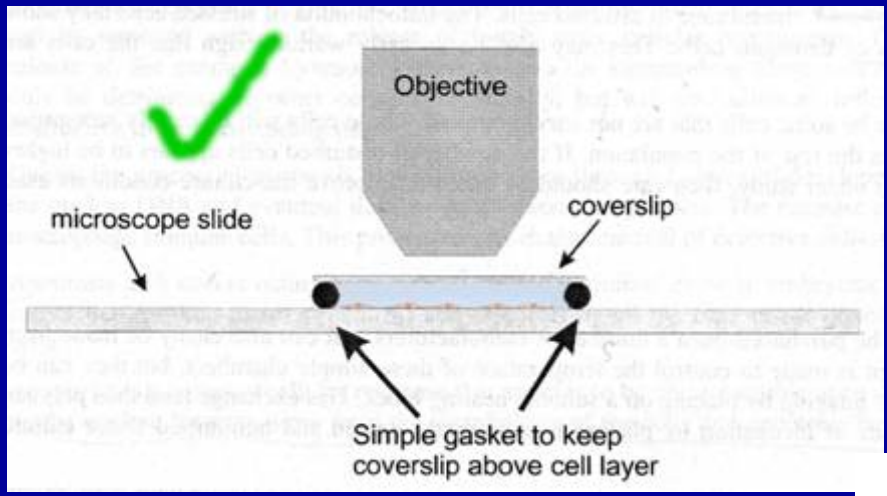
- **Environment**
  - Physical integrity
  - Attachment
  - Temperature
  - Gases (CO_2, O_2 , H_2O), pH.
  - Osmolarity
- **Illumination**
  - Autofluorescence
  - Photodamage
- **Microscopy and image processing techniques**
  - ‘deconvolution’
  - Live specimen microscopes
- **(Time lapse sequence analysis)**

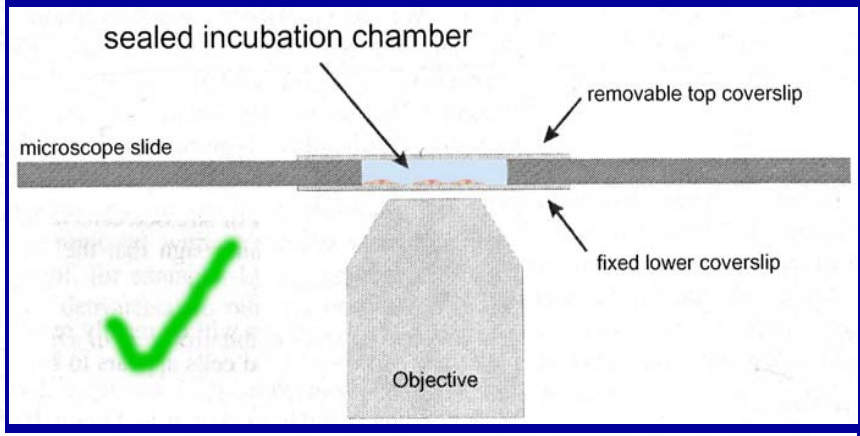


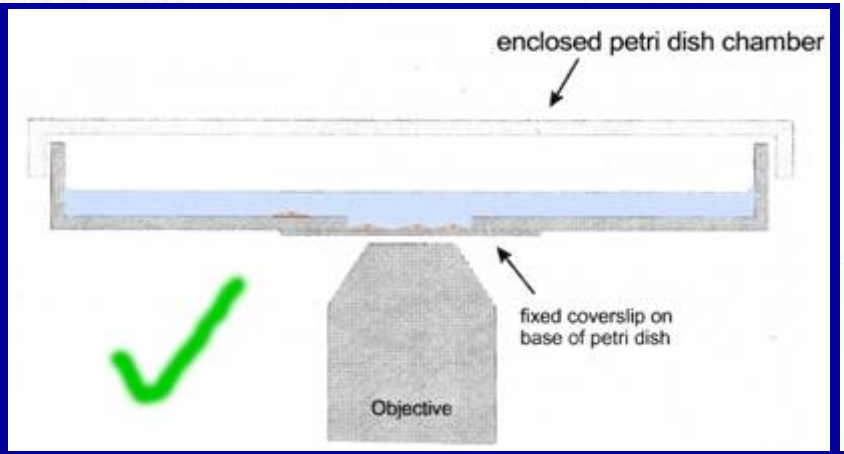
Jens Rietdorf:

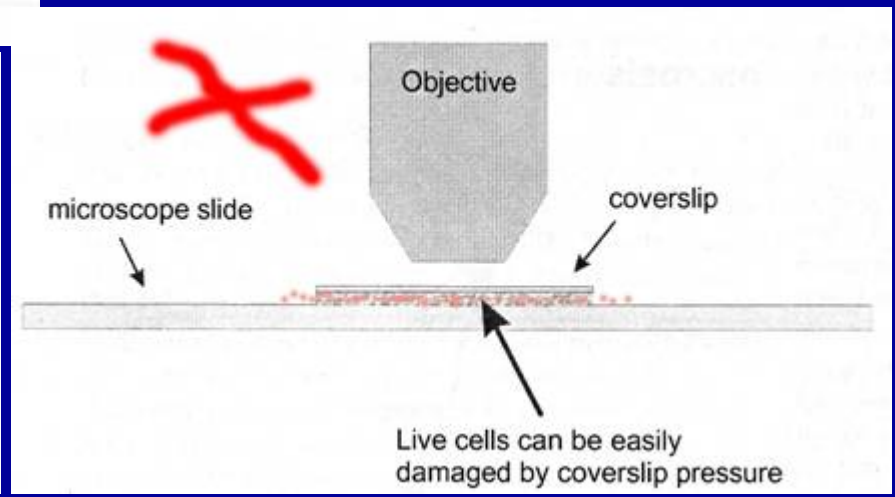
There are different possibilities to guarantee physical integrity of the specimen during imaging.

Physical integrity







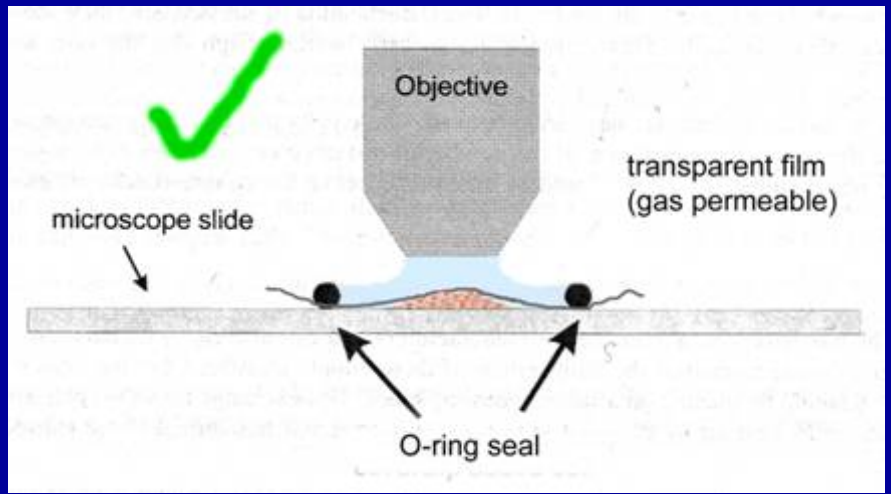


Jens Rietdorf:

Some tricks in case the sample does not adhere. Tissues or embryos can be trapped under transparent, gas permeable plastic films. Bacteria or nonadherent cells can be embedded in or overlayed with 0.1% low meltingpoint (LMP) agarose

Attachment

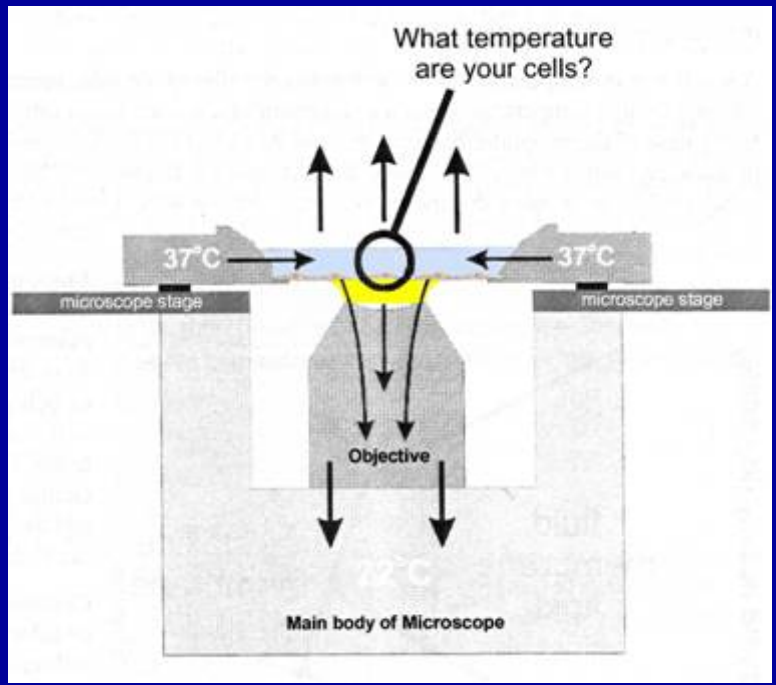
- Acid cleaning or flaming the coverglass
- Withdraw serum
- Coating the coverglass
  - Poly-L-Lysine
  - Concanavalin A
  - Other
- 0.1% LMP agarose
- Transparent films



Jens Rietdorf:

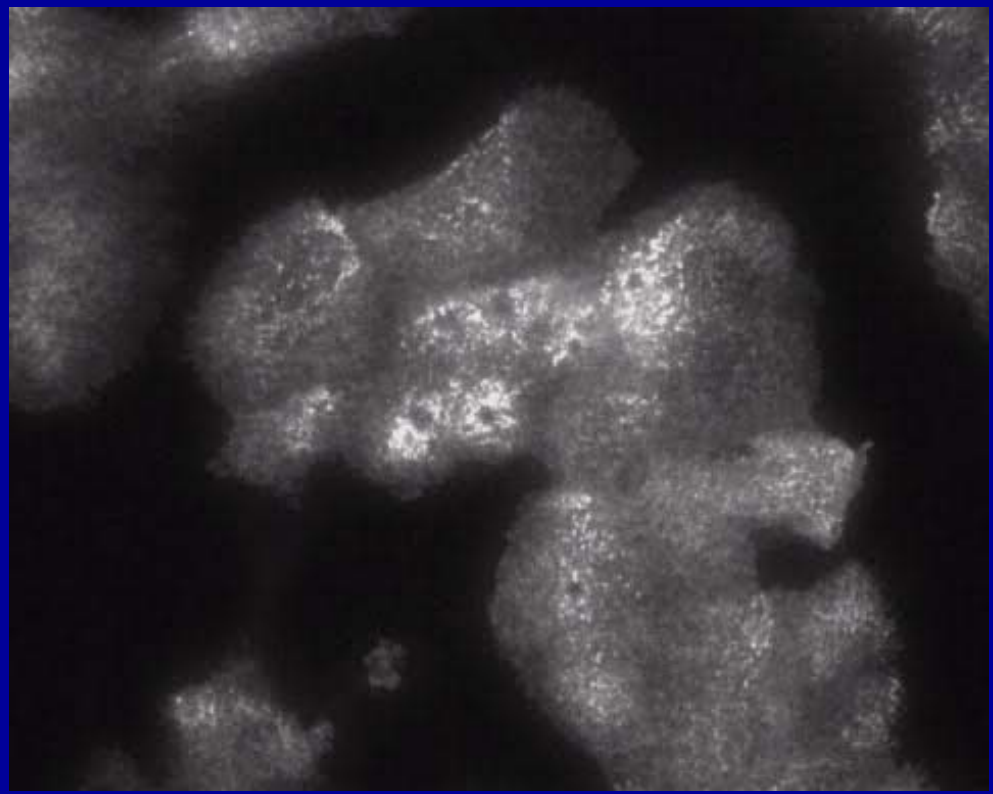
Temperature is critical both for integrity of the sample and stability of the microscope. Several solutions are discussed.

Temperature



Jens Rietdorf:

An example movie of a TIRF timelapse under bad temperature control. The feedback is too slow. Temperature shifts in the order of 0.1degC are visible with highNA lenses.



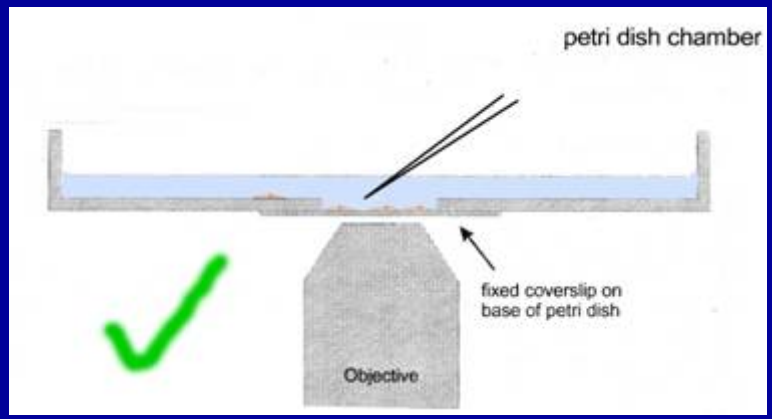
Gases (CO_2, O_2, H_2O), pH, osmolarity

- Replace carbonate buffer inside the medium by HEPES (e.g. 30mM HEPES, 0.5g/l Carbonate instead of 2.2g/l Carbonate).
- Seal the sample chamber (no gas exchange)
- Control CO_2, evaporation
  - Use perfusion chambers
  - Use incubators

Jens Rietdorf:

Regunly culture media contain carbonate buffers which are only stable under 5% CO2 atmosphere. Examples of open and closed incubation chambers are discussed.

HEPES buffered media



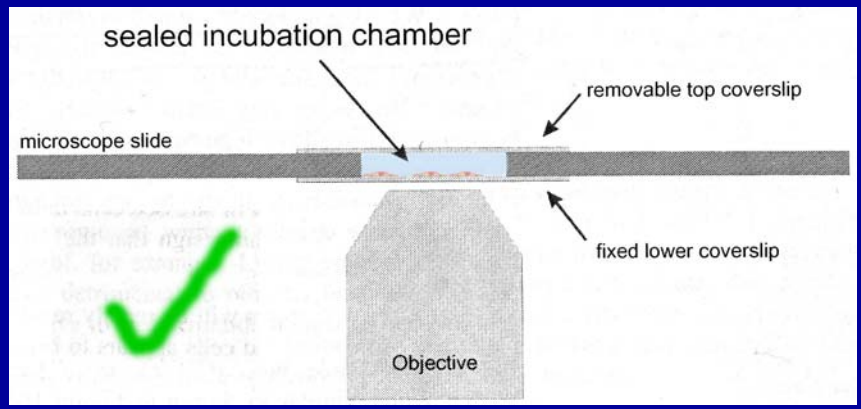
Advantages

- Open system, easy to manipulate.
- Easy to handle and control.

Disadvantages

- Usable for ca. 1 hour.
- Toxic conversion of HEPES by irradiation.
- Evaporation.

Sealed chambers



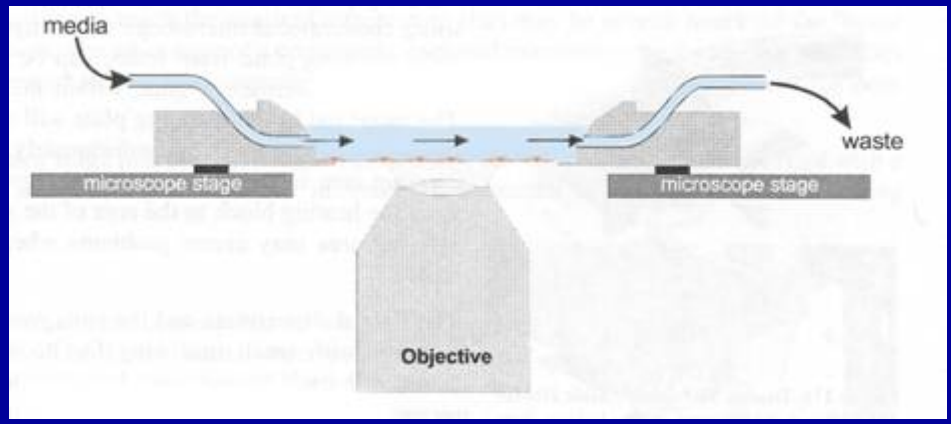
Advantages

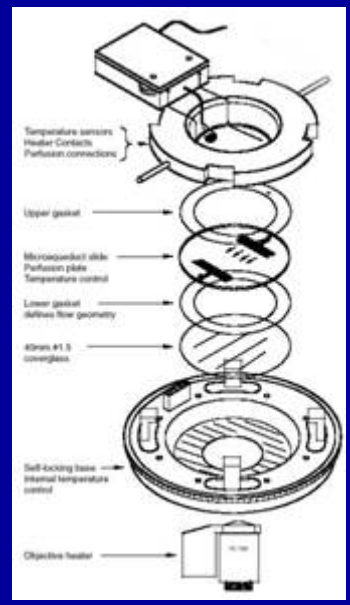
- Easy to handle and control.
- No evaporation.
- Cheap.

Disadvantages

- Usable for max 3 hours depending on volume.
- No manipulation.

Perfusion chambers





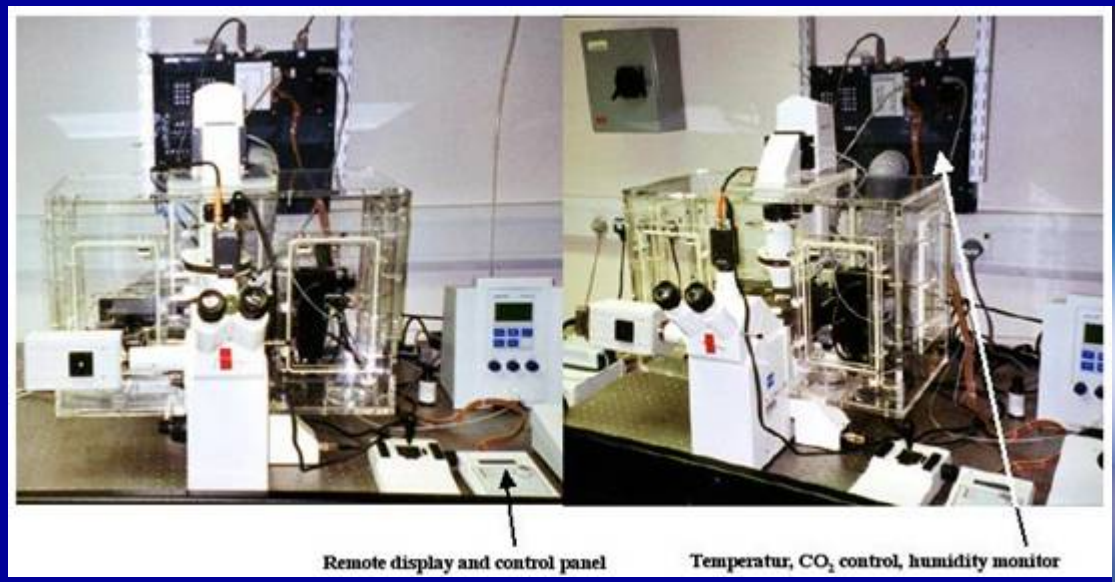
Advantages

- Constant conditions.
- Manipulation of media.
- Usable for days.

Disadvantages

- Hard to assemble and control.
- Expensive.

Microscope Incubators



Advantages

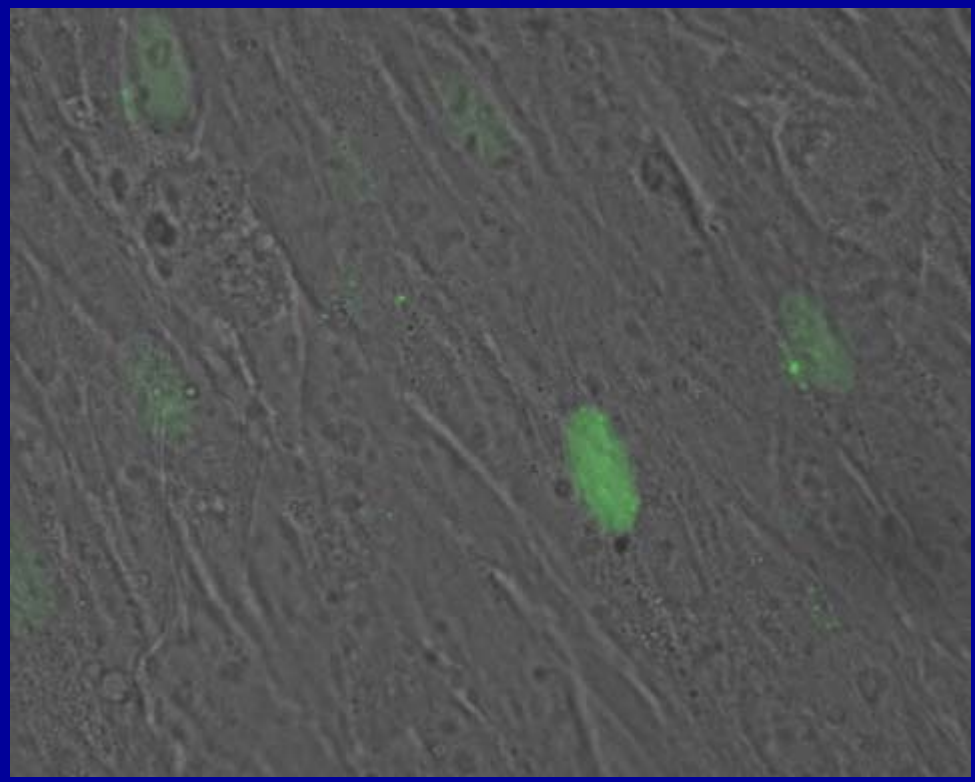
- Constant conditions.
- Manipulation.
- Usable for days.

Disadvantages

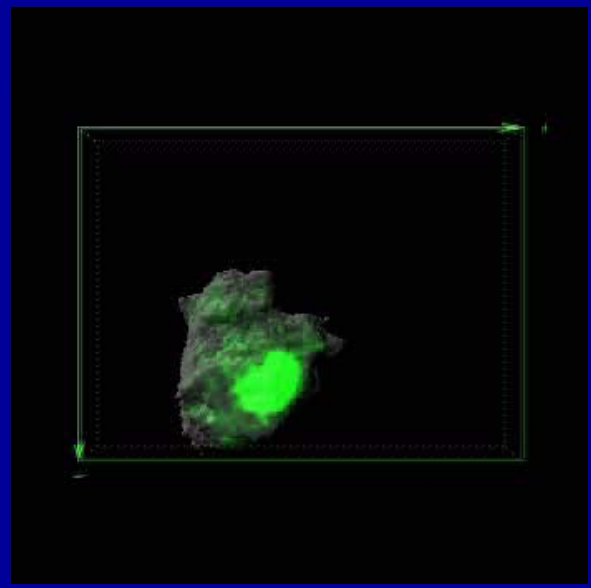
- Expensive.
- Microscope access impaired.

Jens Rietdorf:

Example movie of good environment control. 72hour spanning timelapse without focus shift, cells divide and express gfp which is good indication they are in good shape.



- **Environment**
  - Physical integrity
  - Attachment
  - Temperature
  - Gases (CO_2, O_2 , H_2O), pH.
  - Osmolarity
- **Illumination**
  - Autofluorescence
  - Photodamage
- **Microscopy and image processing techniques**
  - deconvolution
  - Live cell microscopes
- **(Time lapse sequence analysis)**



Jens Rietdorf:

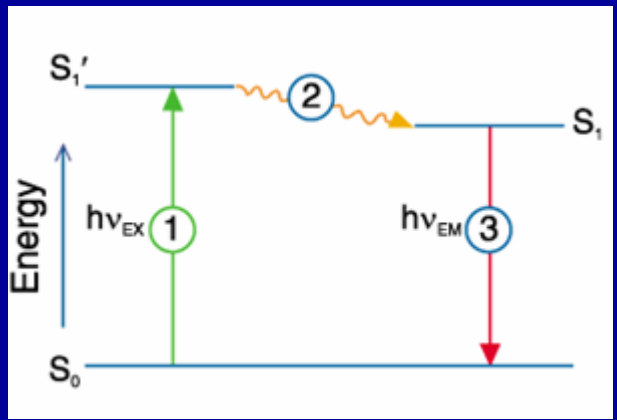
Autofluorescence may have different reasons, but is generally stronger, the shorter the wavelength and the higher the intensity of the excitation light is. Stressed or damaged specimen show AF.

Autofluorescence

- Specific sources of autofluorescence (excitation):
  - Aromatic amino acid residues (UV).
  - Reduced pyridine nucleotides (UV).
  - Flavins (UV, blue).
  - Chitin (broad).
  - Chlorophyll (blue, green).
- General sources of autofluorescence:
  - Dead cells (broad).
  - Lipofuscin (UV, blue).
- Cures:
  - Long wavelength (also lower energy) light.[except 2-Photon]
  - Avoid stress.

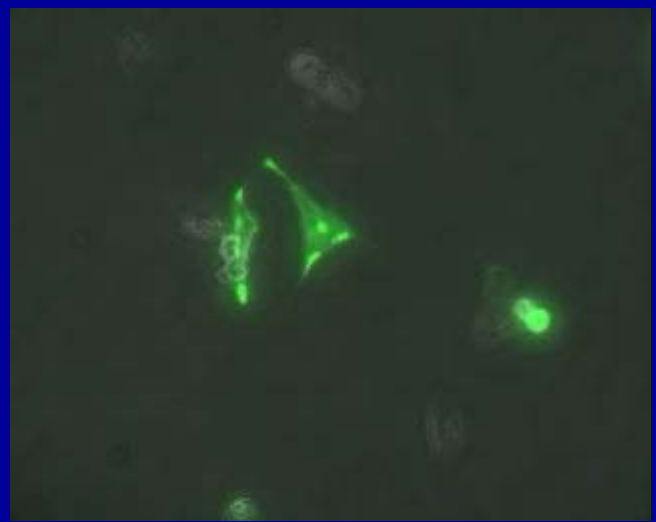
Photodamage

- Illumination energy not converted into emitted light (typ. <1%) or heat can enforce chemical reactions.



Recognise damaged cells

- Cells detach.
- Blebs form.
- Mitochondria swell.
- Cells do not make it through mitosis.
- Necrosis, Apoptosis



Jens Rietdorf:

Different microscopy techniques will be discussed which are very light efficient and avoid photodamage are discussed in the following

Avoid photodamage

- Use decent dyes.
- Optimise illumination and detection:
  - Filtersets
  - Detectors
  - Resolution (xy,z,t,intensity value, channels)
  - Make use of image processing (‘deconvolution’).
- Add antioxidants (Trolox, ascorbic acid 2mg/ml)
- Use appropriate microscope techniques.

- **Environment**
  - Physical integrity
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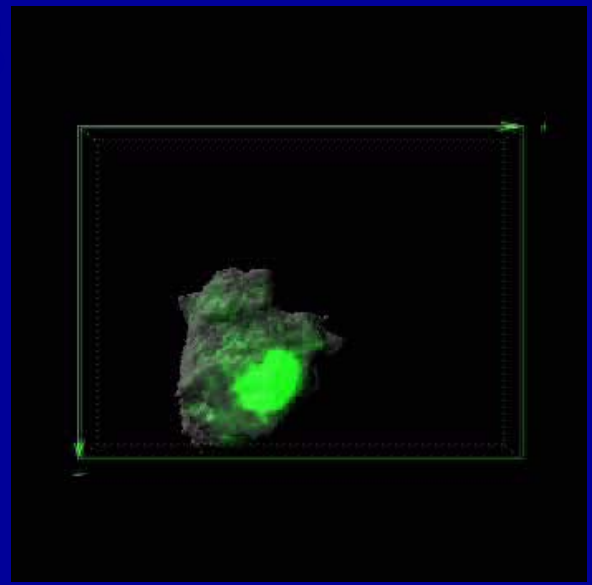
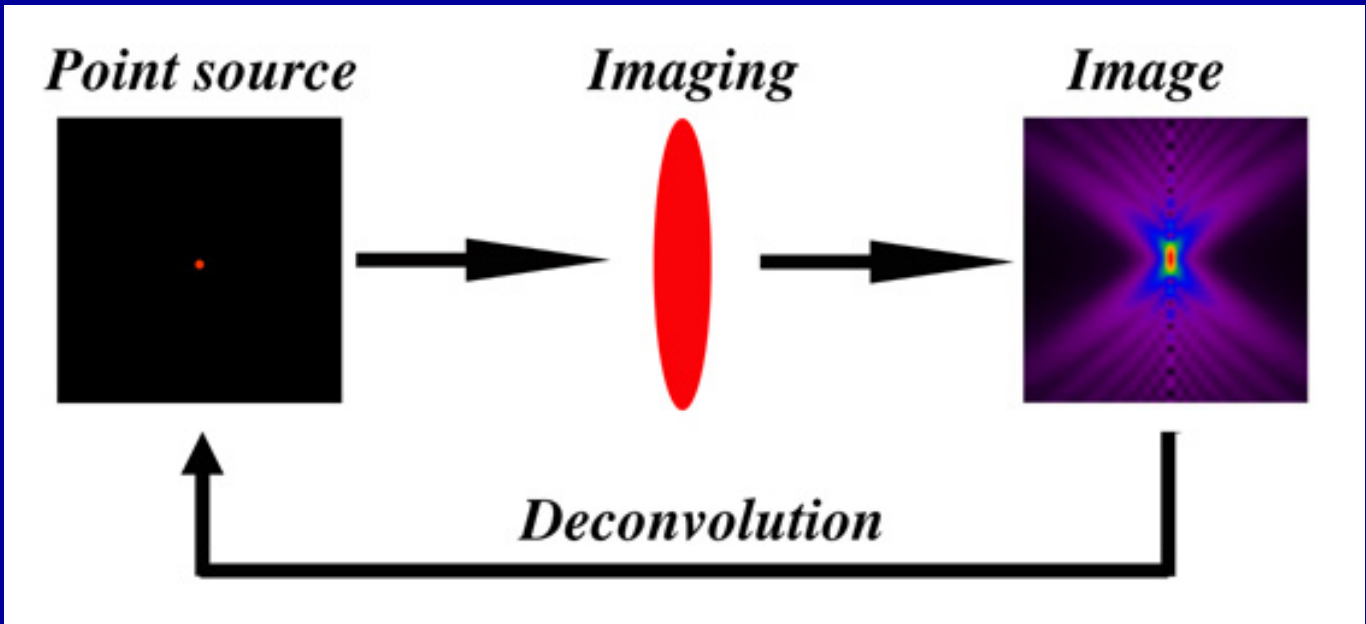


Wide-field microscopy + deconvolution

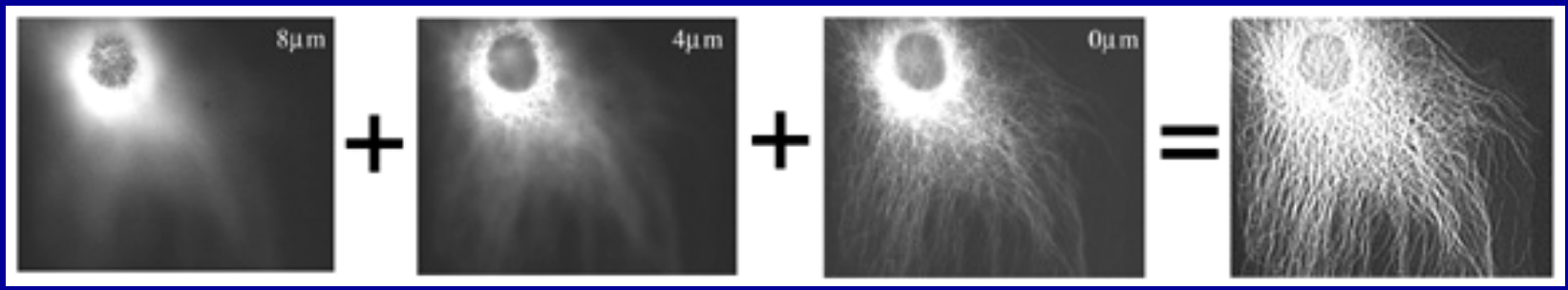


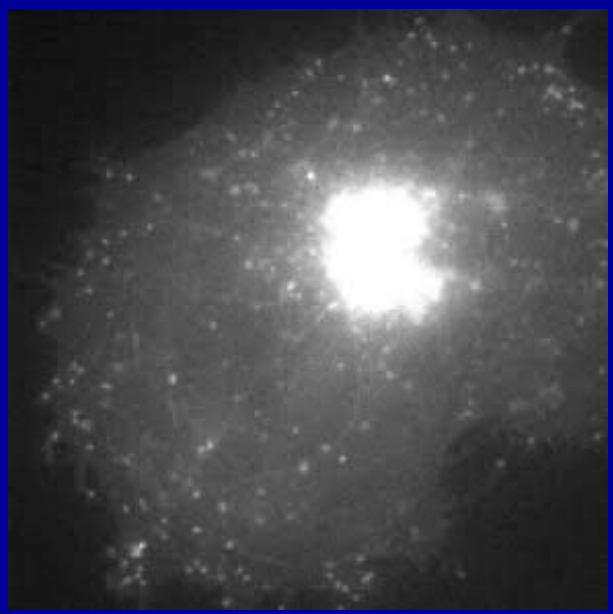
-Use *a priori* knowledge to improve image quality
-deconvolution is possible for all image 'dimensions':
Along optical axis, time-lapse, color

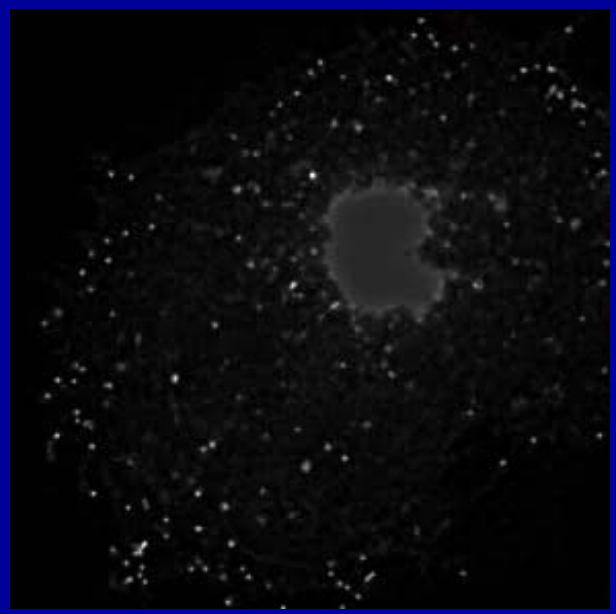
Jens Rietdorf:

Deconvolution can increase the signal-to-noise ratio and thereby allows reduction of excitation light. Always use deconvolution before estimating how much light has to be put into the sample to reveal the relevant information.

deconvolution



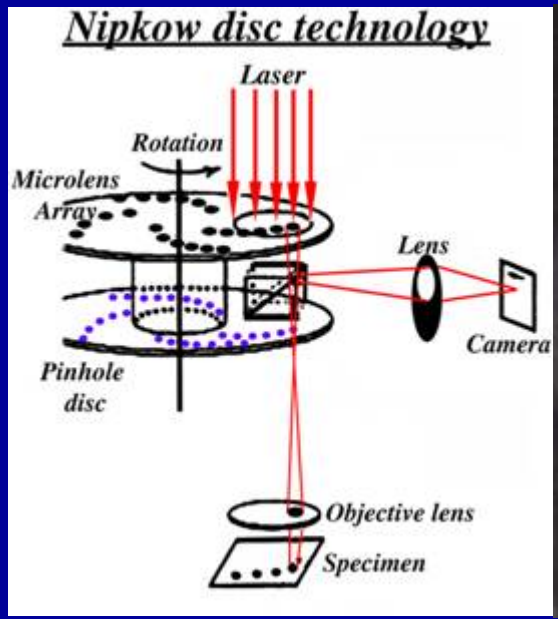


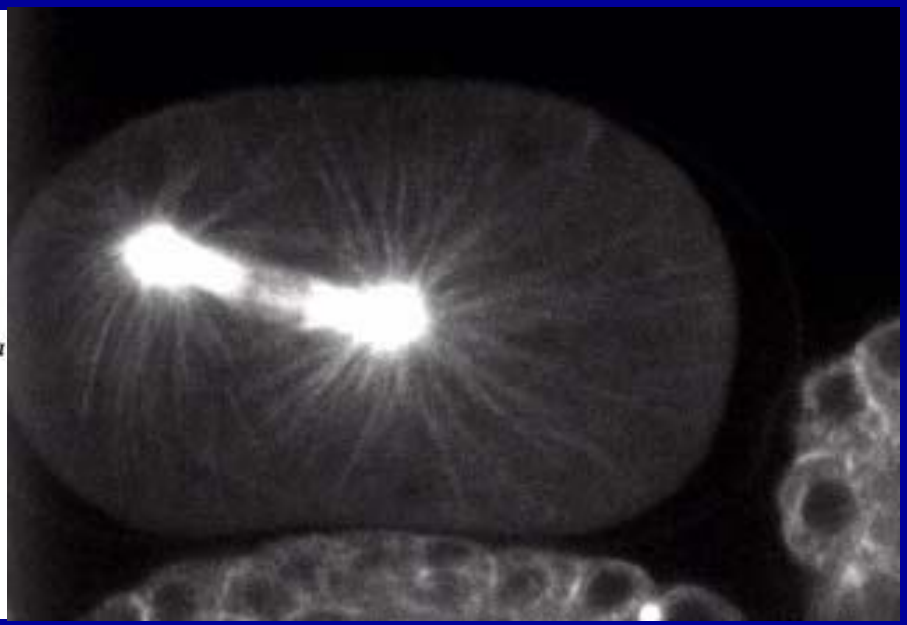


Jens Rietdorf:

Spinning disc confocals are a good alternative to single beam scanning confocals as they use very low excitation light intensities.

Example Yokogawa unit

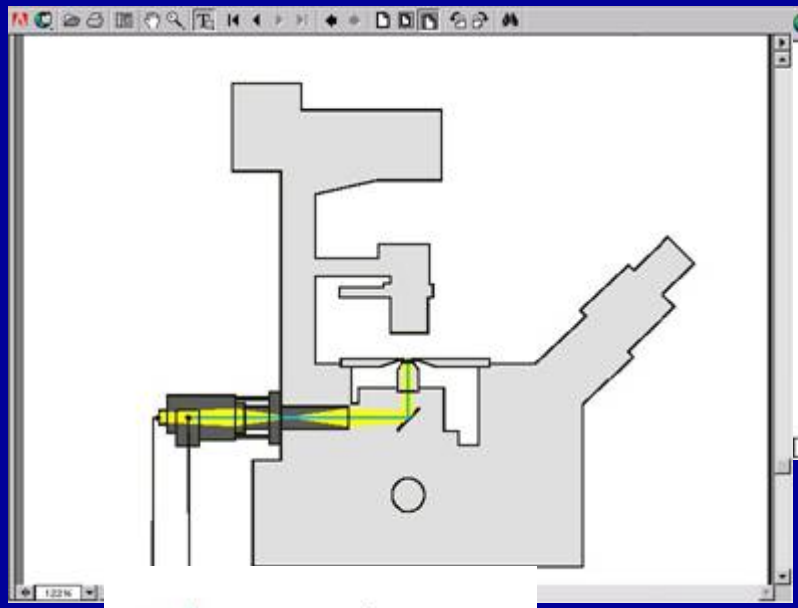


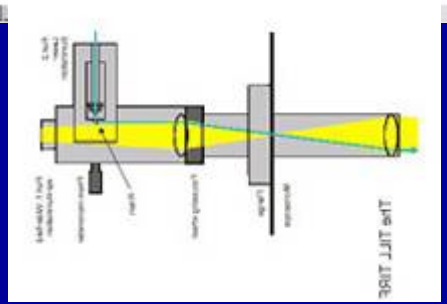


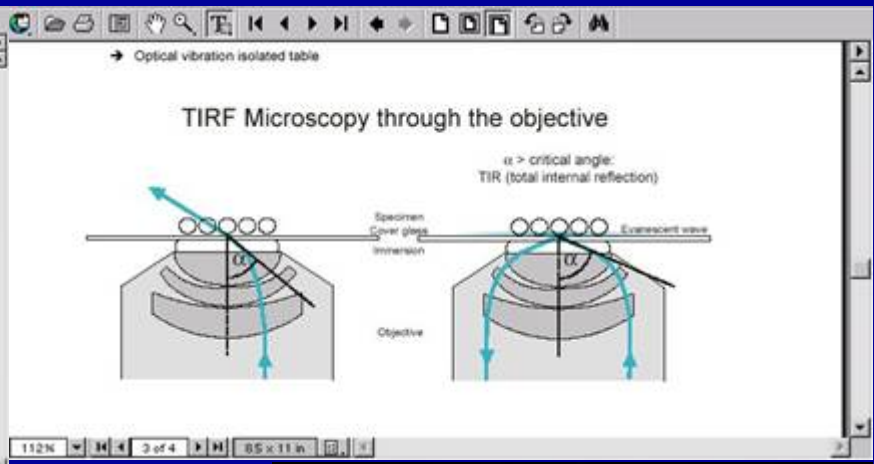
Jens Rietdorf:

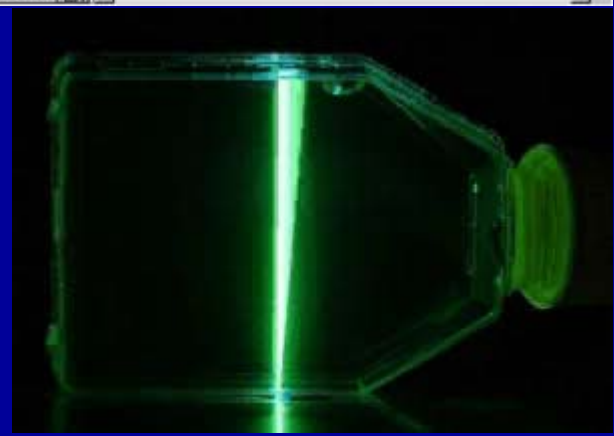
TIRF microscopy limits the excitation to a small area close to the coverglas and provides excellent contrast.

Total internal reflection fluorescence microscopy
TIRFM 'prismless' system

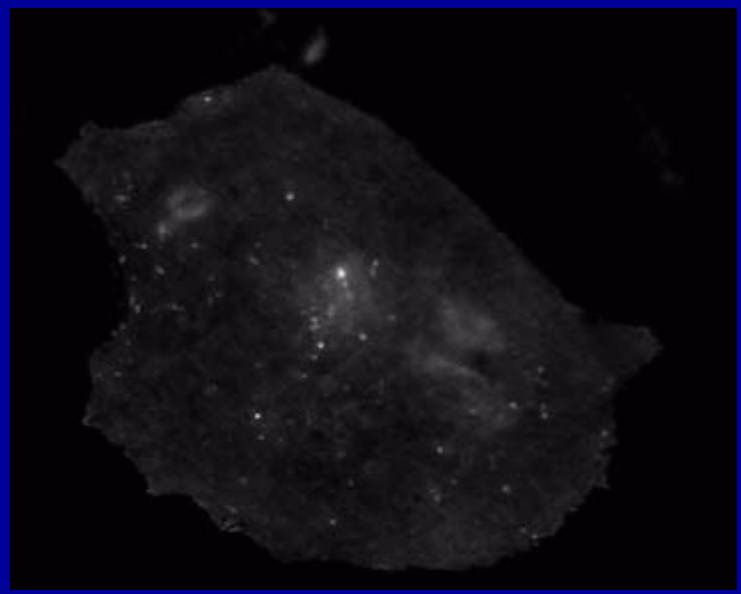


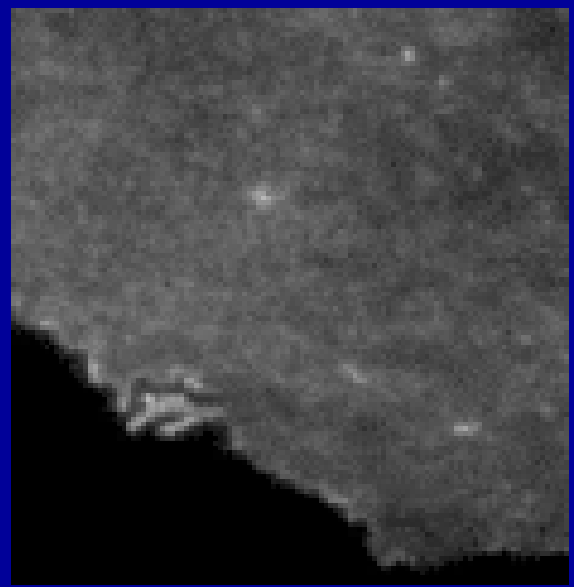


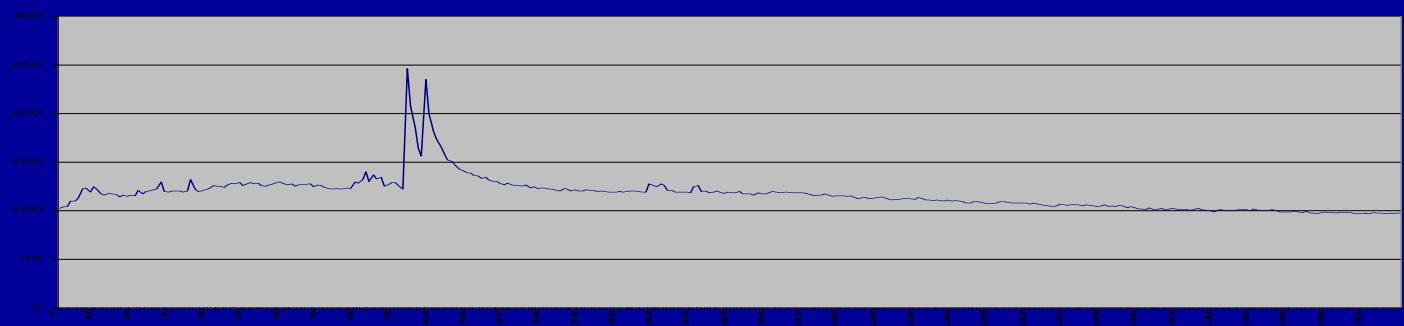




Example membrane fusion in TIRF

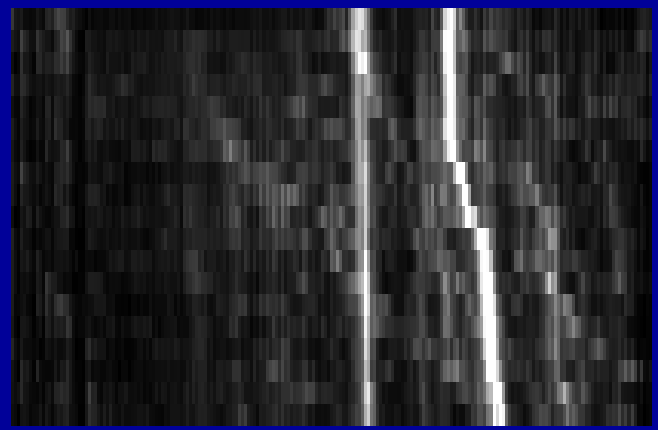






Jens Rietdorf:

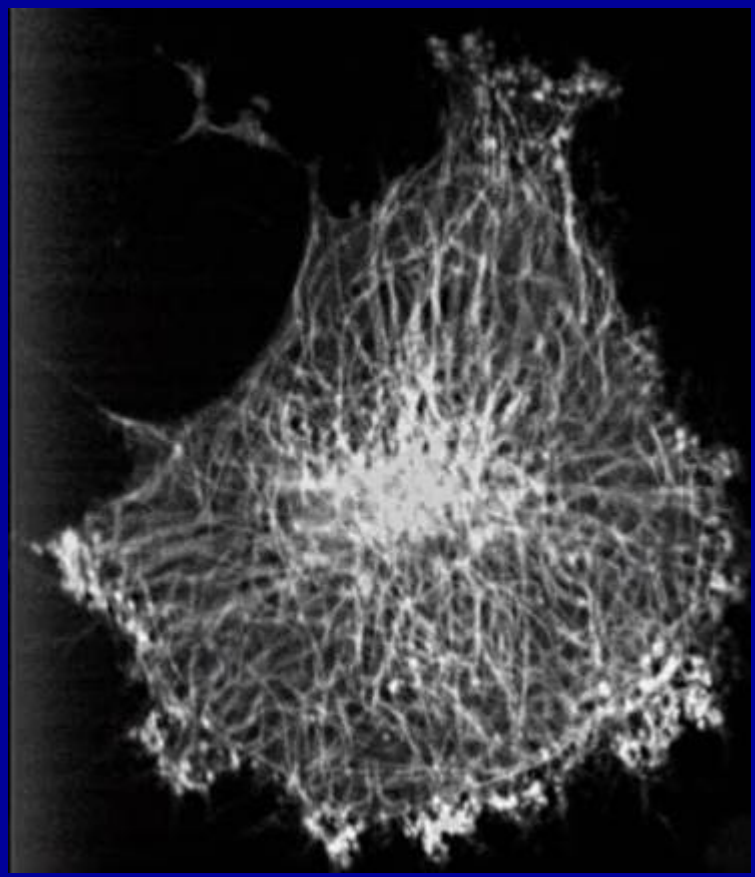
Example single molecule TIRF.

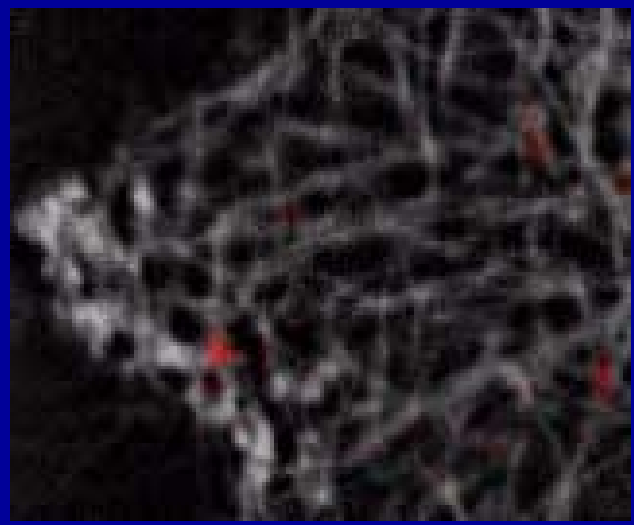


Jens Rietdorf:

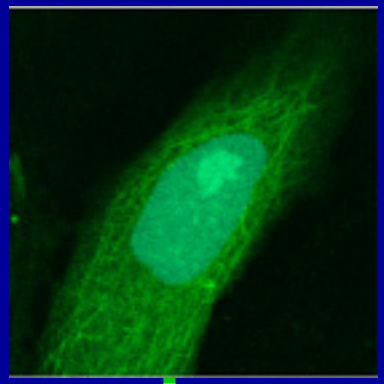
Differently shaped structures may be labeled with the same dye and are still saparable into different channels by object detection approaches. Double exposure for different fluorophores can be avoided.

Simultaneous multichannels I



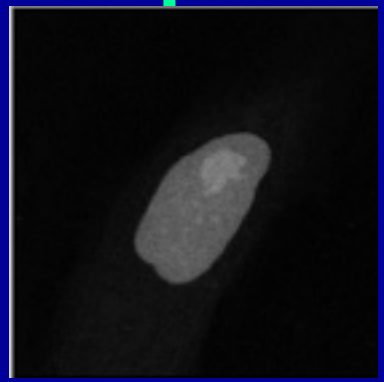


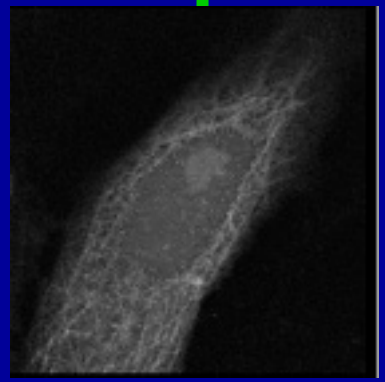
scope



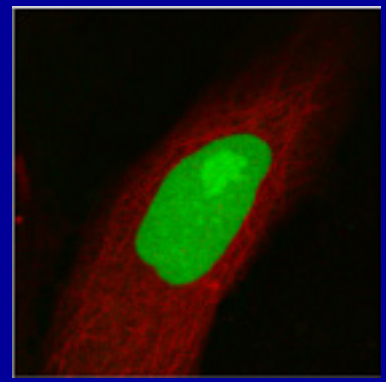
microimager

camera





unmix



Jens Rietdorf:

Different microscopy methods are more or less suited for different applications. Mark 1=good. A very rough estimate made to emphasize pros and cons of different methods with respect to live cell imaging.

Comparision of tools

	'Light efficiency'	Depth discrimination	Acquisition speed	Volume imaging	Timelapse imaging	Flexibility
Widefield (+deco)	2	3	2	3	2	2
Confocal	5	2	4	1	4	1
Multibeam confocal	4	3	3	2	3	3
2-Photon	2	3	4	3	4	4
TIRF	1	1	1	4(n.p)	1	5

Conclusions

- Keep environment constant and convenient
- Use powerful dyes
- Think about resolution required (xy,z,t,intensity value, channels) to minimize photostress
- Use appropriate microscopy method
- Use ‘deconvolution’

People involved

articles

Kinesin-dependent movement on microtubules precedes actin-based motility of vaccinia virus

Jens Rietdorf*, Aspasia Ploubidou*, Inge Reckmann, Anna Holmström, Friedrich Frischknecht, Markus Zettl, Timo Zimmermann and Michael Way†

ALMF:

Rainer Pepperkok
Timo Zimmermann
Andreas Girod
Kota Miura