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

Live Specimen Microscopy
• **Environment**
  – Physical integrity
  – Attachment
  – Temperature
  – Gases (CO₂, O₂, H₂O), 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

- Simple gasket to keep coverslip above cell layer
- Enclosed petri dish chamber
- Fixed coverslip on base of petri dish
- Sealed incubation chamber with removable top coverslip
- Fixed lower coverslip

Live cells can be easily damaged by coverslip pressure
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.
Jens Rietdorf:

An example movie of a TIRF timelapse under bad temperature control. The feedback is too slow. Temperature shifts in the order of 0.1 degC are visible with highNA lenses.
Gases (CO$_2$, O$_2$, H$_2$O), 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.
Example movie of good environment control. 72 hour spanning timelapse without focus shift, cells divide and express gfp which is good indication they are in good shape.
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• **(Time lapse sequence analysis)**
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.

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.
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
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.
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• **(Time lapse sequence analysis)**
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.
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 coverglass and provides excellent contrast.

Total internal reflection fluorescence microscopy
TIRFM ‘prismless’ system
Example membrane fusion in TIRF
Example single molecule TIRF.
Jens Rietdorf:
Differently shaped structures may be labeled with the same dye and are still separable into different channels by object detection approaches. Double exposure for different fluorophores can be avoided.

Simultaneous multichannels I
Comparision of tools

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.

<table>
<thead>
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<th>Method</th>
<th>‘Light efficiency’</th>
<th>Depth discrimination</th>
<th>Acquisition speed</th>
<th>Volume imaging</th>
<th>Timelapse imaging</th>
<th>Flexibility</th>
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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

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

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