Royal College of Surgeons in Ireland

RCSI

Imaging (subcellular) events during cell death

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1. Added value and benefits from (timelapse) imaging

2. Key information and concepts for image acquisition and processing

3. Examples for imaging applications in cell death research

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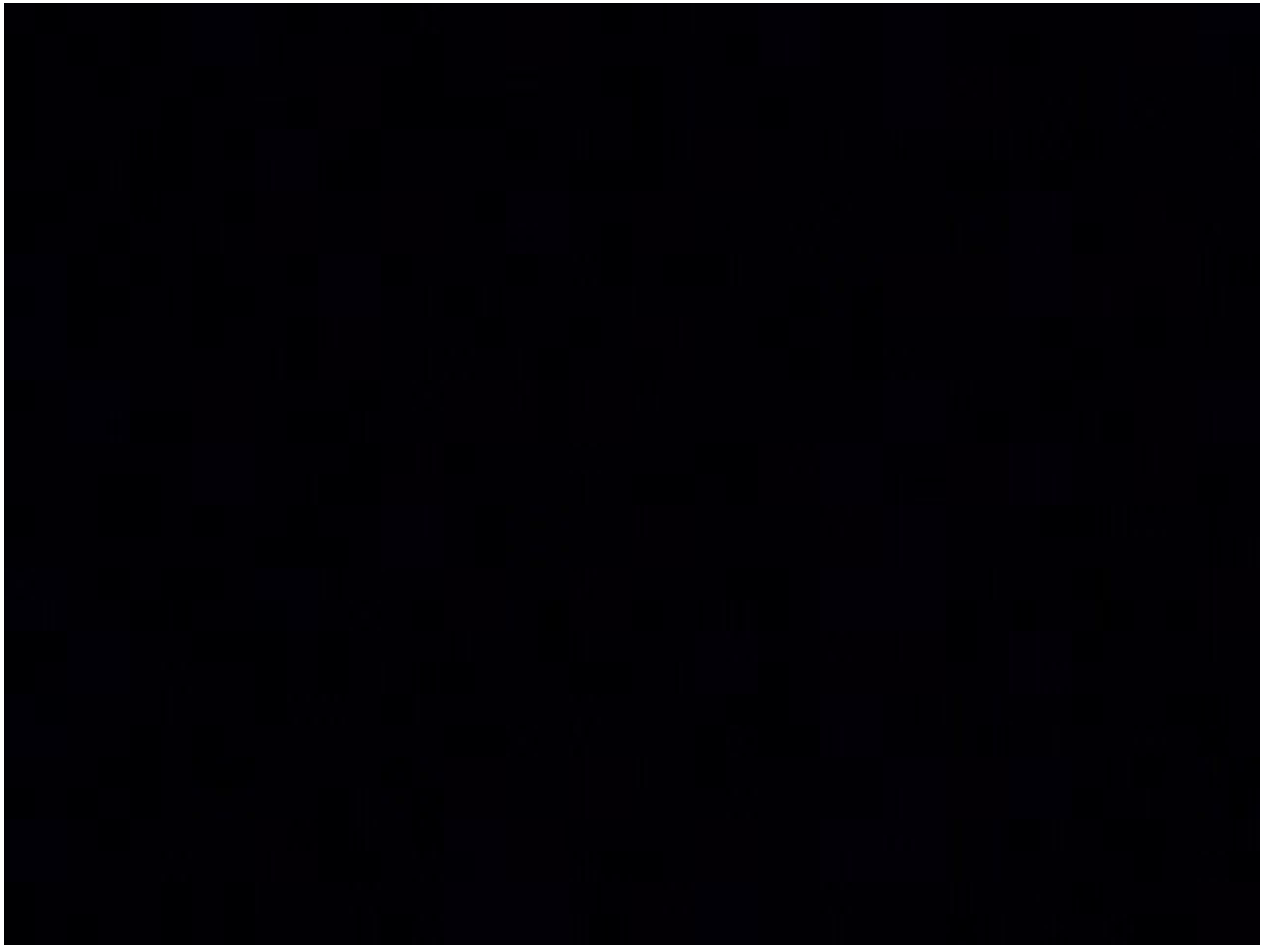
1. Added value and benefits from (timelapse) imaging

We often measure input/output behaviour



How can imaging help?

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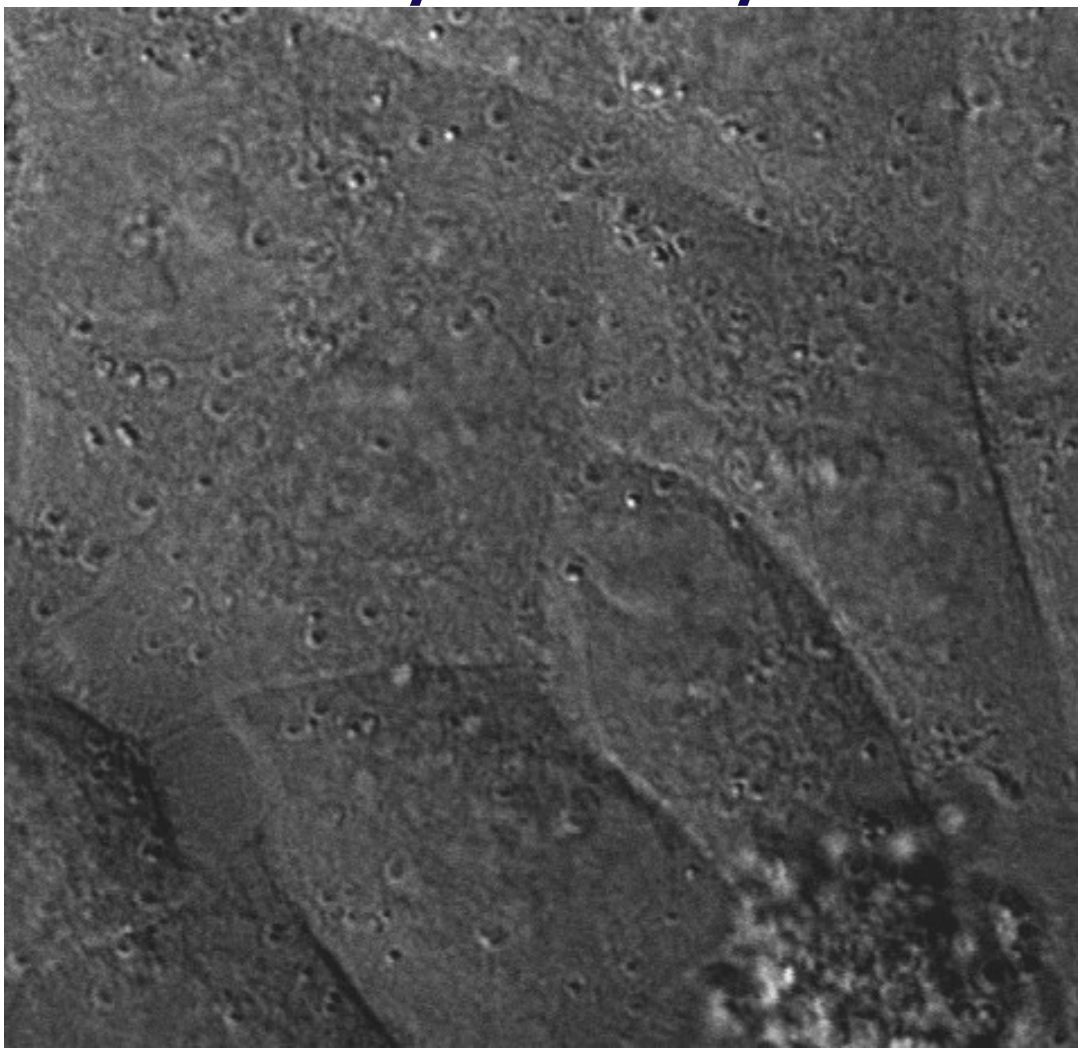
Cells undergo apoptosis asynchronously



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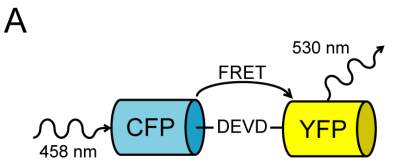
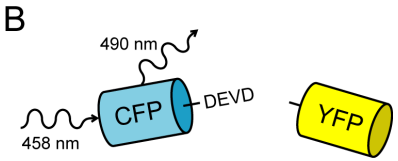
Intracellular Signalling Kinetics during Apoptotic Cell Death

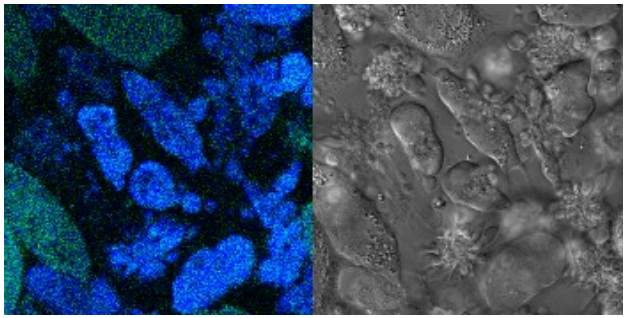
Example: Activation of effector caspases

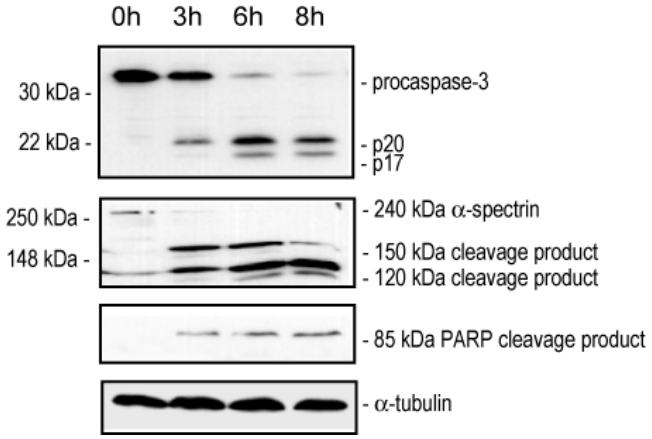
- High sensitivity
- Ratiometric (Low noise)
- Measure Enzymatic Activity

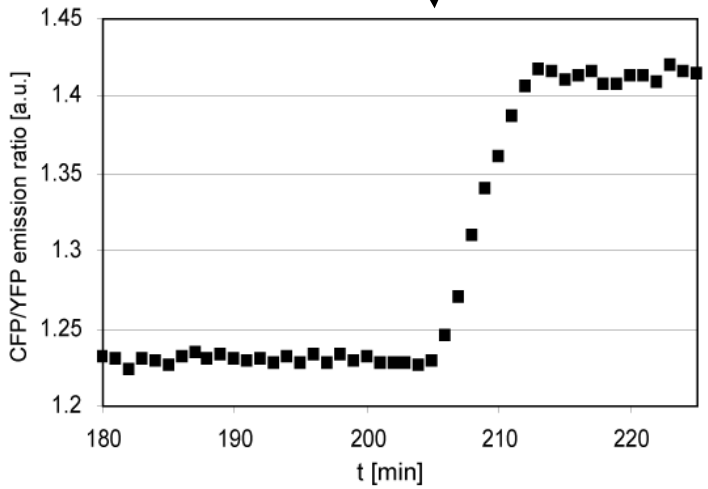




FRET Probe







Bulk: Hours
Kinetics look dose dependent

Single Cell: Minutes
Kinetics are dose & drug independent → switch-like "all or none" behaviour

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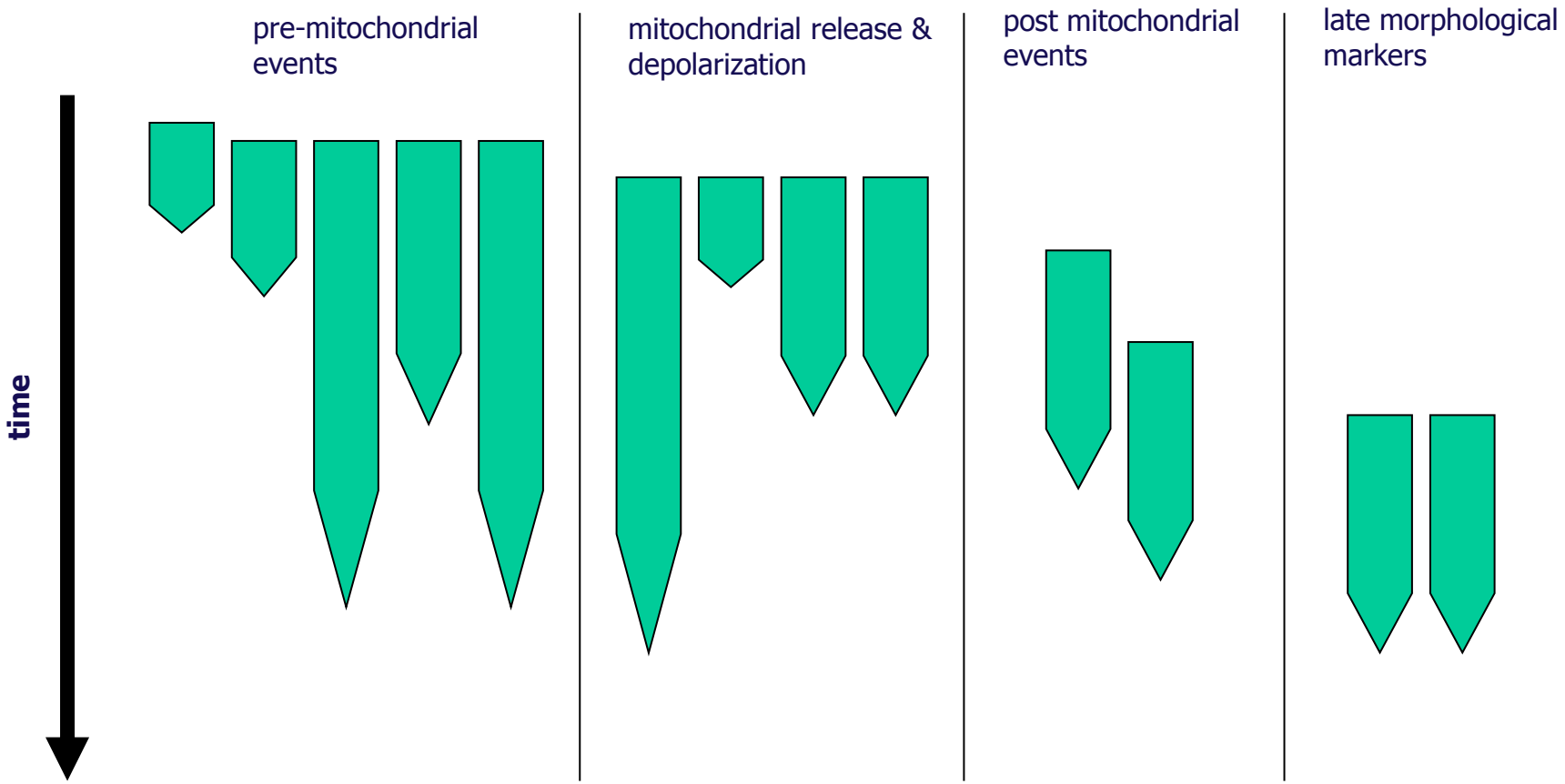
Single Cell Imaging

Signalling sequences and networks

Death receptor-mediated apoptosis



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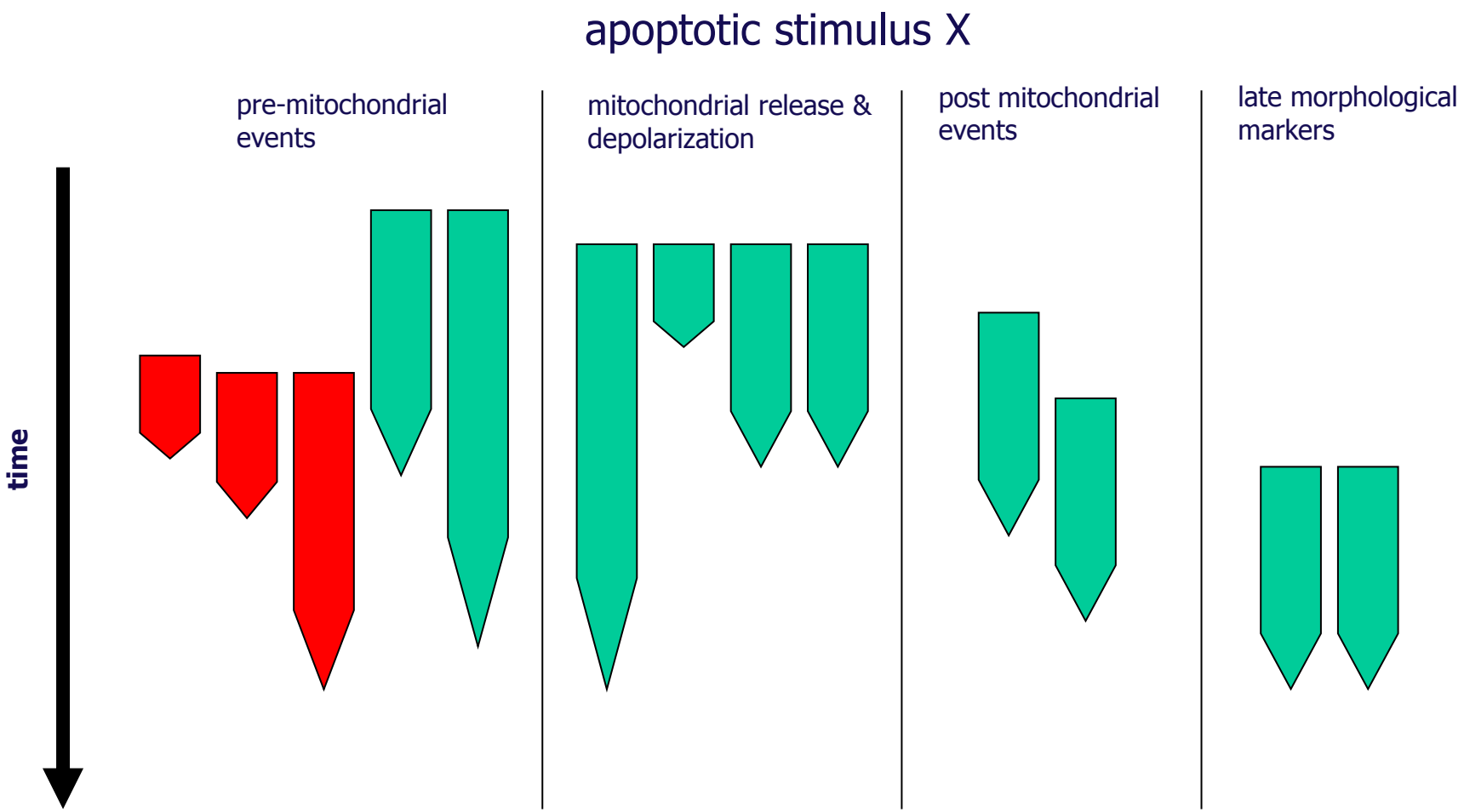
Single Cell Imaging

Signalling sequences and networks



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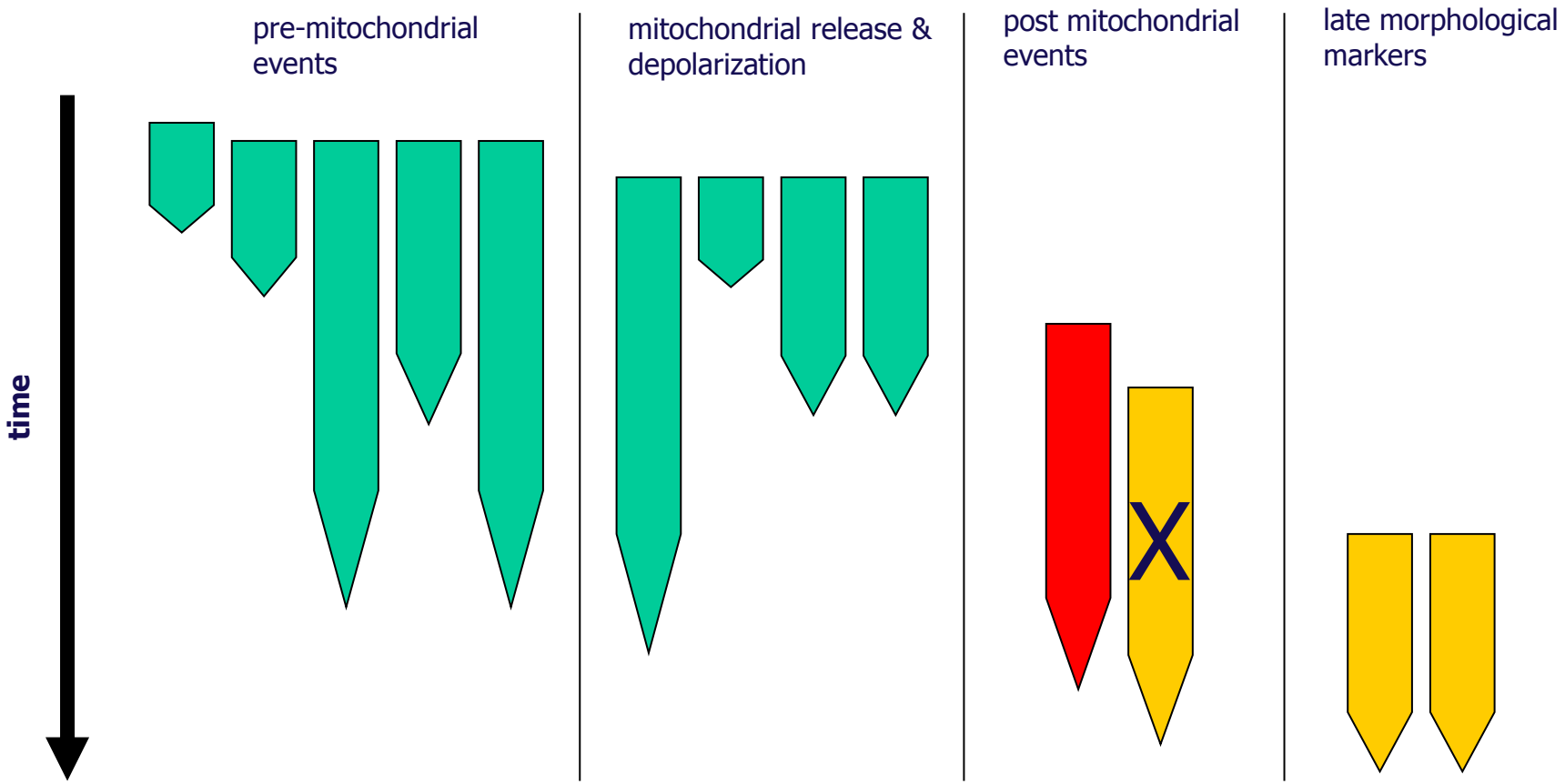
Single Cell Imaging

Signalling sequences and networks

Overexpression/knock out of protein Y



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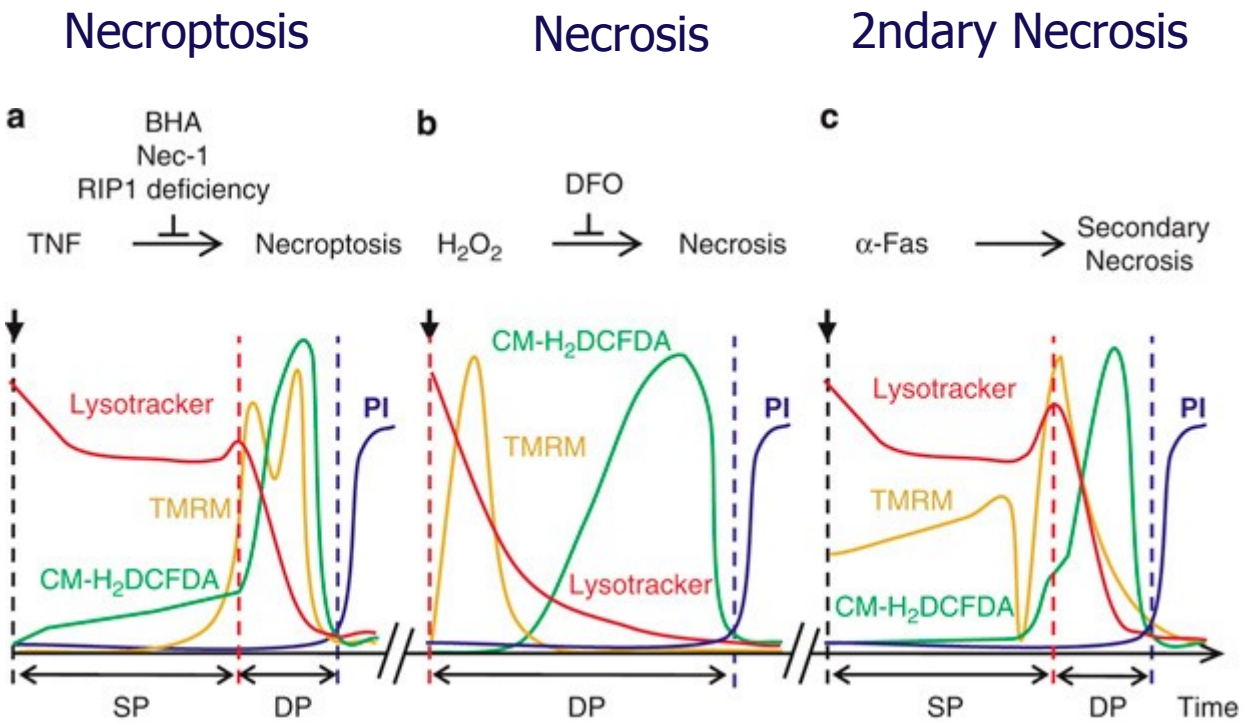
Does not only apply to apoptosis…



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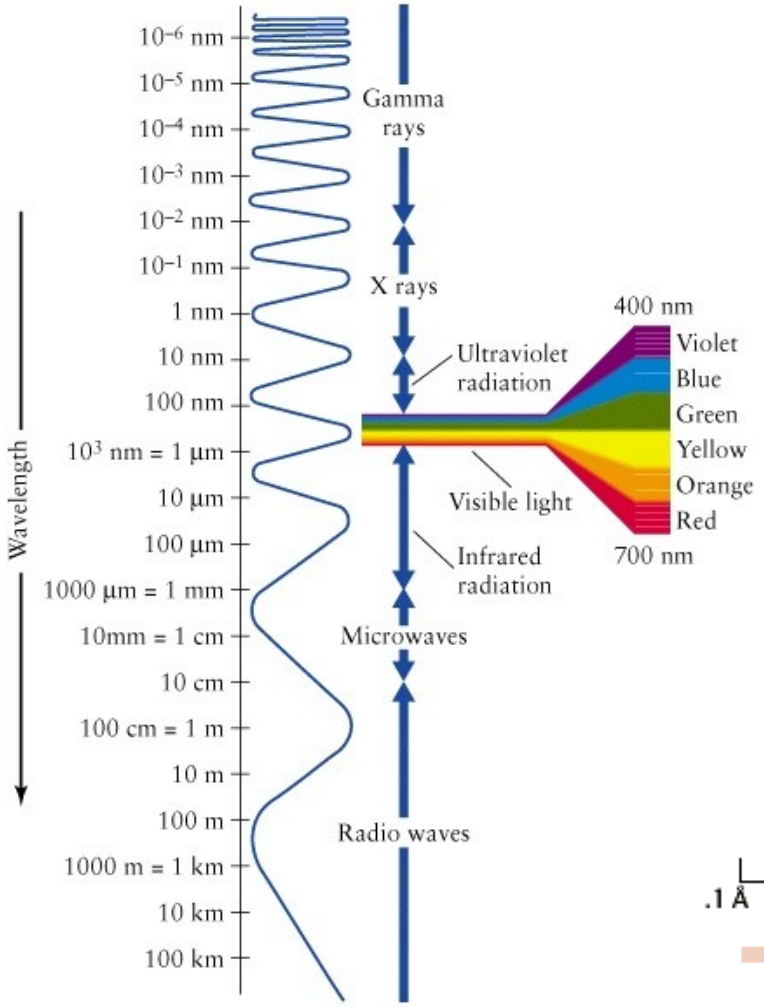
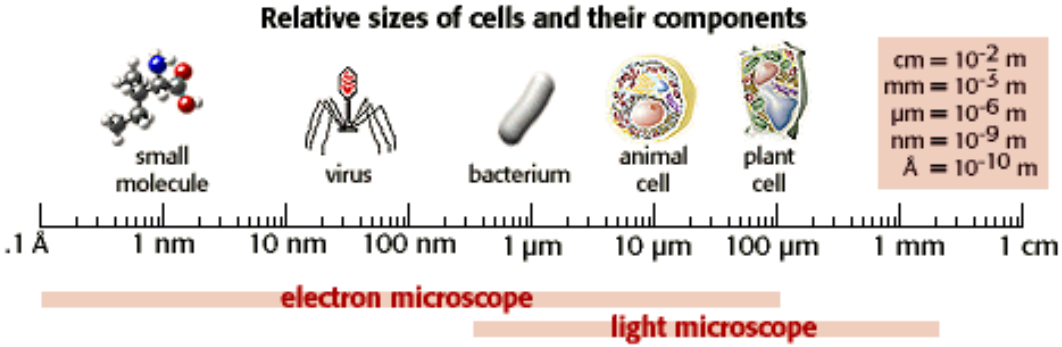
Vanden Berghe T et al., Cell Death Differ, 2010

2. Key information and concepts for image acquisition and processing



(Wavelength image from *Universe* by Freedman and Kaufmann.)

Visible Light
&
Resolution



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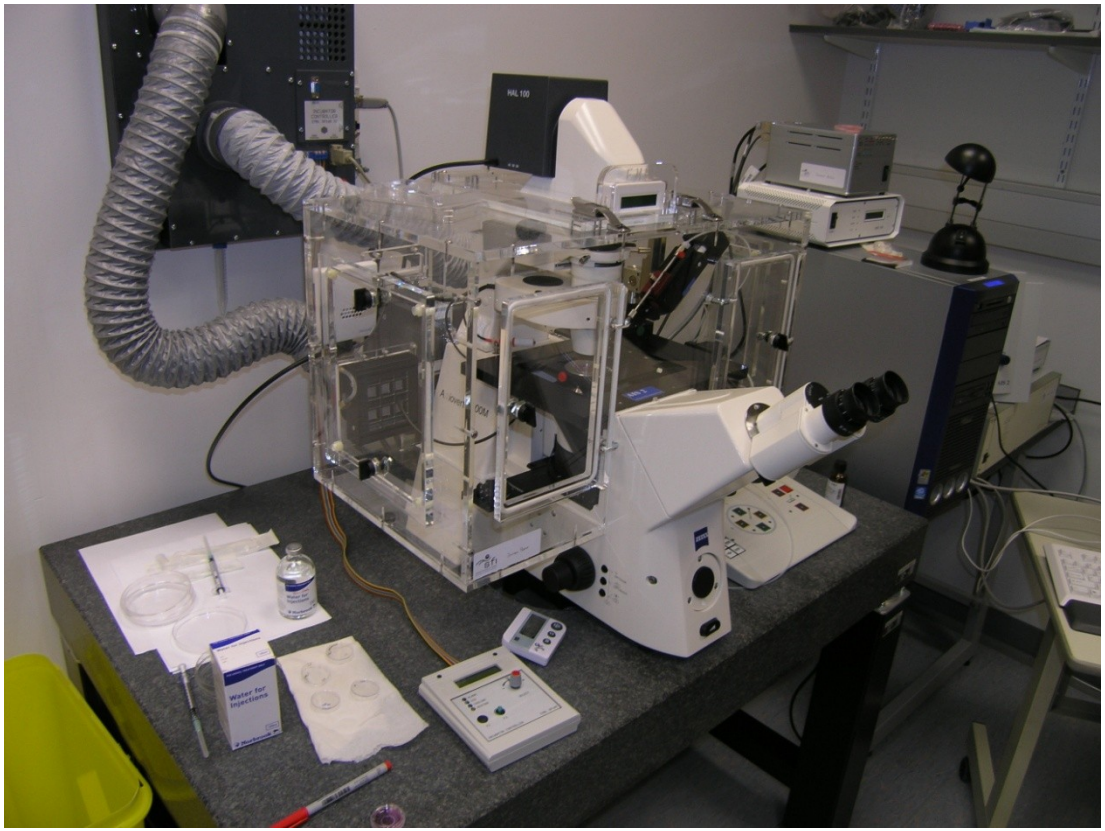
Imaging and image acquisition

Equipment changes...

17th century

21st century



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Relative performance of techniques & instruments

	Technique	Example ZEISS System	Performance Specification: Out-of-Focus Discrimination	Depth Penetration	Acquisition Speed	Thin Samples: Lateral Resolution	Thin Samples: Axial Resolution	Thick Samples: Lateral Resolution	Thick Samples: Axial Resolution	Spectral Flexibility	Simplicity
Flexible	Widefield Deconvolution	Cell Observer and Deconvolution	•	•	••••	•••••	•••••	•••	•	••	•••••
Flexible	Structured Illumination	ApoTome	•••	••	••	•••••	•••••	•••	•••	••	••••
Flexible	Single Point Laser Scanning	LSM 700	••••	••••	•••	••••	••••	•••••	•••••	••••	••••
Flexible	Single Point Laser Scanning	LSM 710 and LSM 780	••••	••••	•••	••••	••••	•••••	•••••	•••••	••
Deep	Multiphoton	LSM 710 NLO and LSM 780 NLO	•••••	•••••	•••	•••	••	••••	•••••	••••	•
Deep	Multiphoton	LSM 7 *MP*	•••••	•••••	•••	••	••	••••	•••••	•••	••
Fast	Aperture Correlation	VivaTome	•••	••	••••	•••••	•••••	•••	•••	••	••••
Fast	Spinning Disk	Cell Observer SD	••	••	•••••	•••	•••	•••	•••	••	•••
Fast	Line Scanning	LSM 7 *LIVE*	•••	•••	••••••	•••	•••	••••	••••	••	••
Fast	Total Internal Reflection	Laser TIRF 3	••••••		••••••	•••••				••	•••
Superres.	PAL-M	ELYRA P.1	••••••		•	••••••• •••••••				•	•
Superres.	SR-SIM	ELYRA S.1	•••	•	••	••••••	••••••	•••	•••	••	••

RCSI

1. Flexibility 2. Ease of Use 3. Spatial resolution 4. Temporal Resolution

Imaging equipment is expensive

Consumable costs are low

Don’t buy without consulting experienced users

Invest into training or trained personnel

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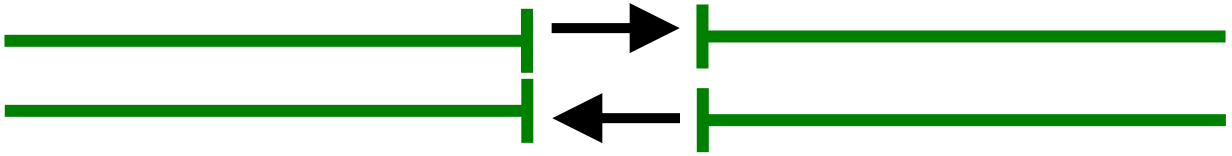
Research Interaction and Research Culture

Collaborative Interface

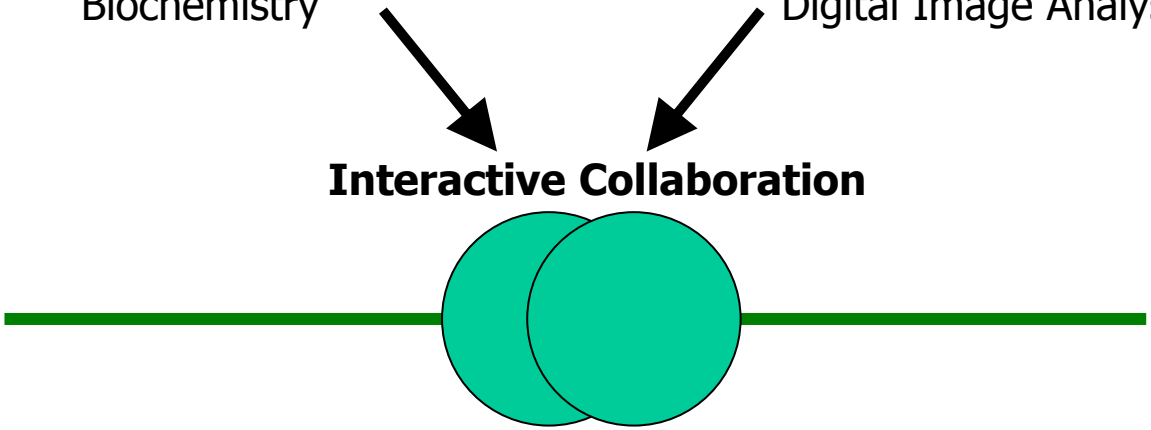


Command and Serve

Imaging is interdisciplinary

Biosciences
Cell Biology
Molecular Biology
Biochemistry

Physics & Engineering
Optics
Electronics
Digital Image Analysis



Interactive Collaboration

Developing towards an overlap in expertise

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Image acquisition changes...

Portrait of
Antonie van Leeuwenhoek
by Jan Verkolje

Born October 24, 1632
Died August 26, 1723
Delft, Netherlands

Discovery of protozoa
First red blood cell description

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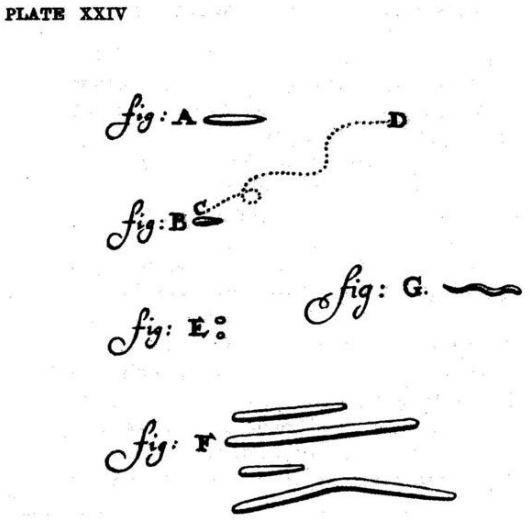
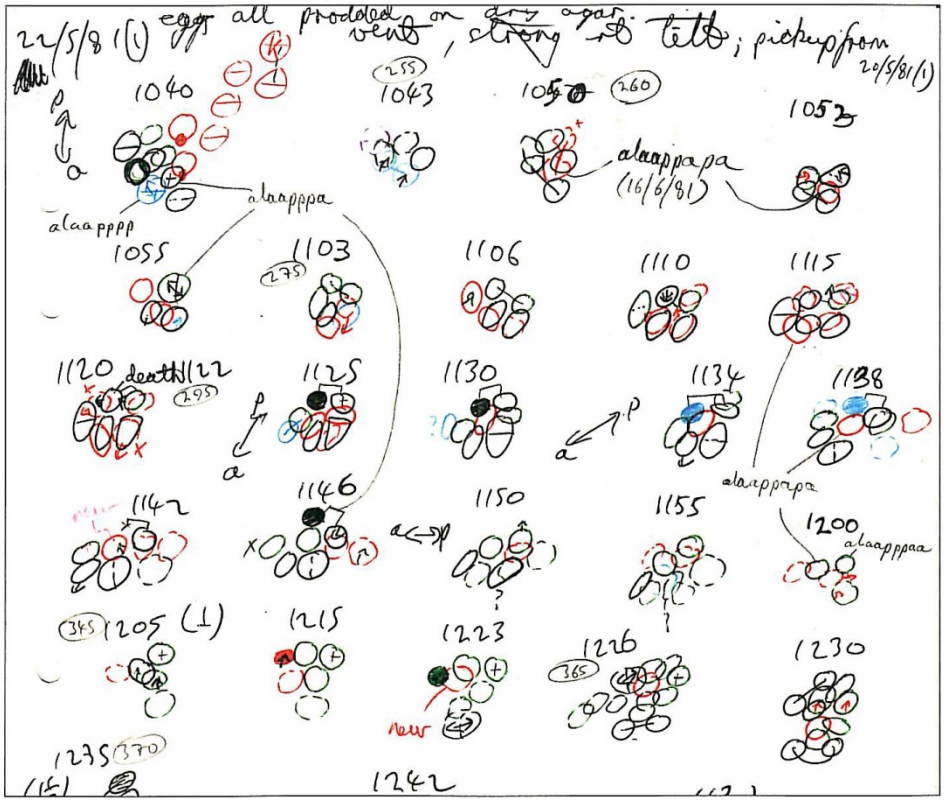
Antonie van Leeuwenhoek's drawings of bacteria in the human mouth (**1684**)





John Sulston's drawings of cell division and death in *C. elegans* (**1981**)

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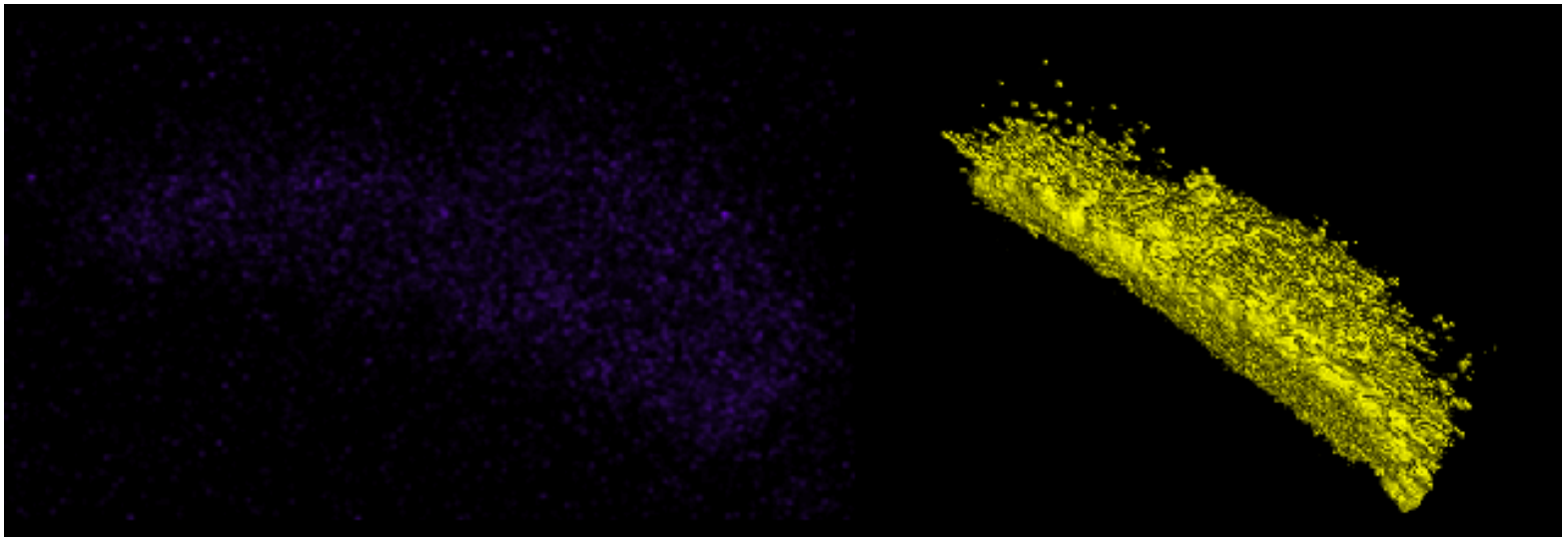
Heiko Dussmann's 3D image of a HeLa cell (**2007**)



Is this still an image?

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There is more than the eye can see…

There is more than the screen can show…

This screen/projector displays 256 levels of gray.

Enough to display a continuous gradient to the human eye.

0 $256 = 2^8 = 8$ bit 255

What about higher bit depth (e.g. 12 bit)?

0 4095

↓ Auto-scaling for display ↓

0 255

A **12 bit** image might contain
16 times more information than
you can see!

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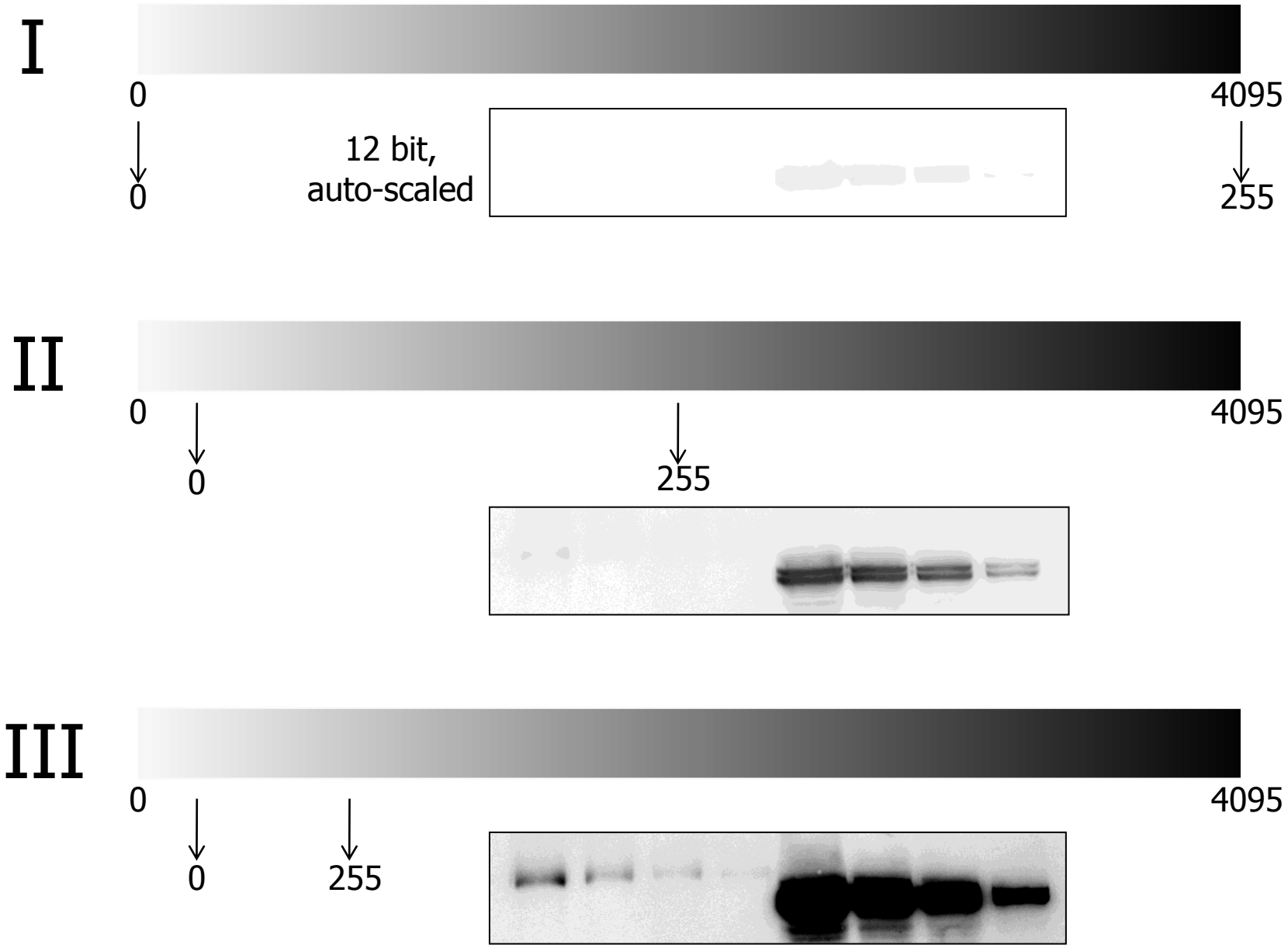

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How should I acquire an image?

1. Use high bit depth wherever possible
2. Avoid overexposure
3. Avoid underexposure

How should I display an image?

1. Adjust intensity range
2. Avoid signal saturation
3. Avoid white background
4. Convert to 8 bit
5. Repeat as required

Always keep a backup of your raw data and work on a copy

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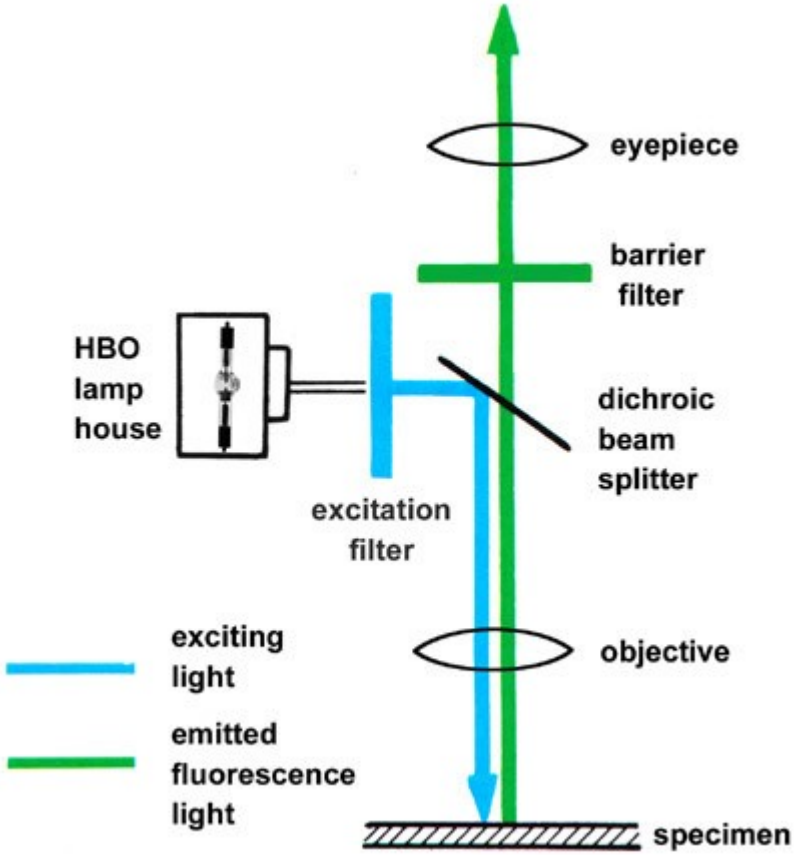
Typical light path



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Crosstalk/Bleed-Through

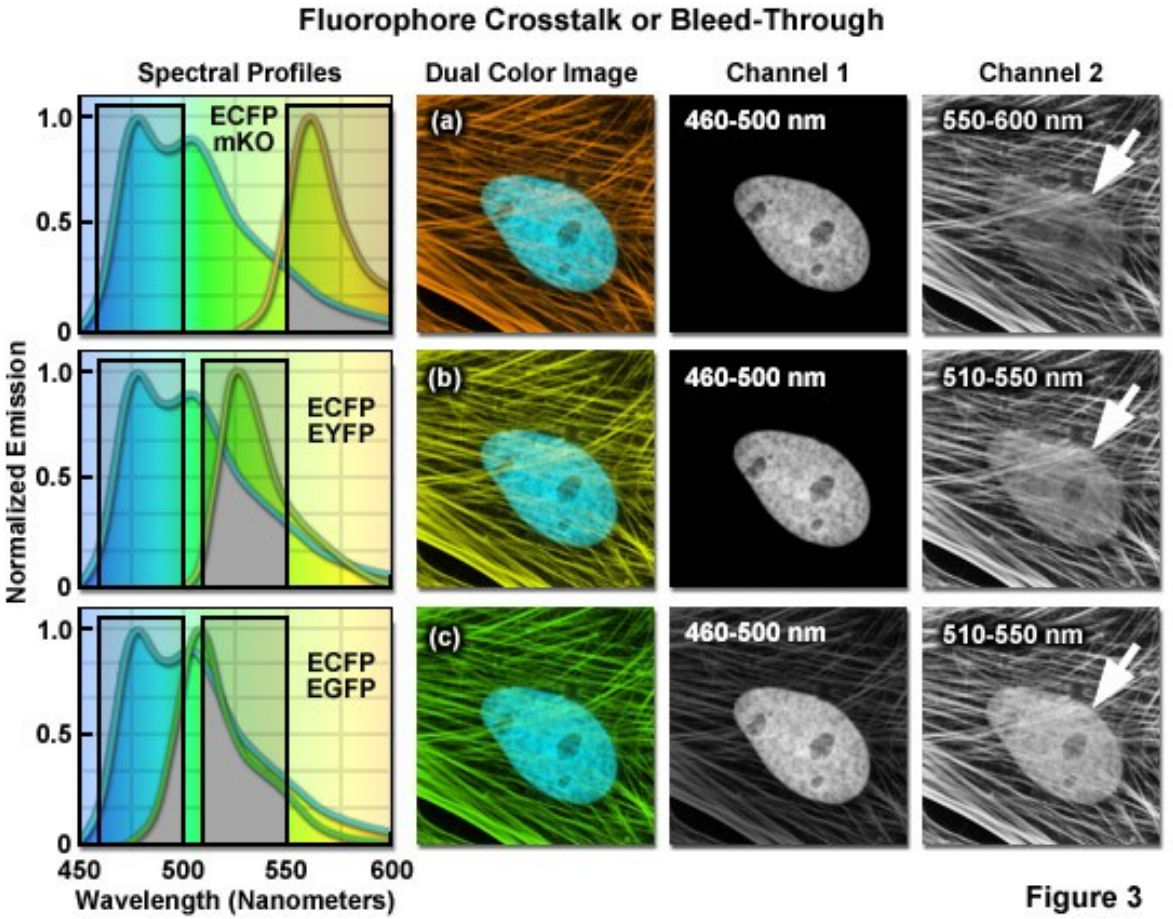

Figure 3

Even more problematic when staining identical regions

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Select fluorophores and filters wisely

Fluorophores:

- Quantum yield (brightness)
- Photostability
- Excitation/Emission spectra (check for overlap)

Filters:

- Spectral windows
- Transmission characteristics

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Do your Controls

Example: Dual-Color Immunofluorescence

- Measure sample stained with one dye only
    1. At optimized settings check for crosstalk into other channels
    2. Repeat for other dye
    3. Can cellular autofluorescence be detected at these settings?
- Measure sample stained with secondary antibodies only
    1. Determine whether unspecific staining can be observed

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Software-assisted Image Analysis

A digital image is an array of numbers

	1	2	3	4	5	6	7	8	9	10
1	92	99	1	8	15	67	74	51	58	40
2	98	80	7	14	16	73	55	57	64	41
3	4	81	88	20	22	54	56	63	70	47
4	85	87	19	21	3	60	62	69	71	28
5	86	93	25	2	9	61	68	75	52	34
6	17	24	76	83	90	42	49	26	33	65
7	23	5	82	89	91	48	30	32	39	66
8	79	6	13	95	97	29	31	38	45	72
9	10	12	94	96	78	35	37	44	46	53
10	11	18	100	77	84	36	43	50	27	59

Displaying an image is a visualization of these numbers

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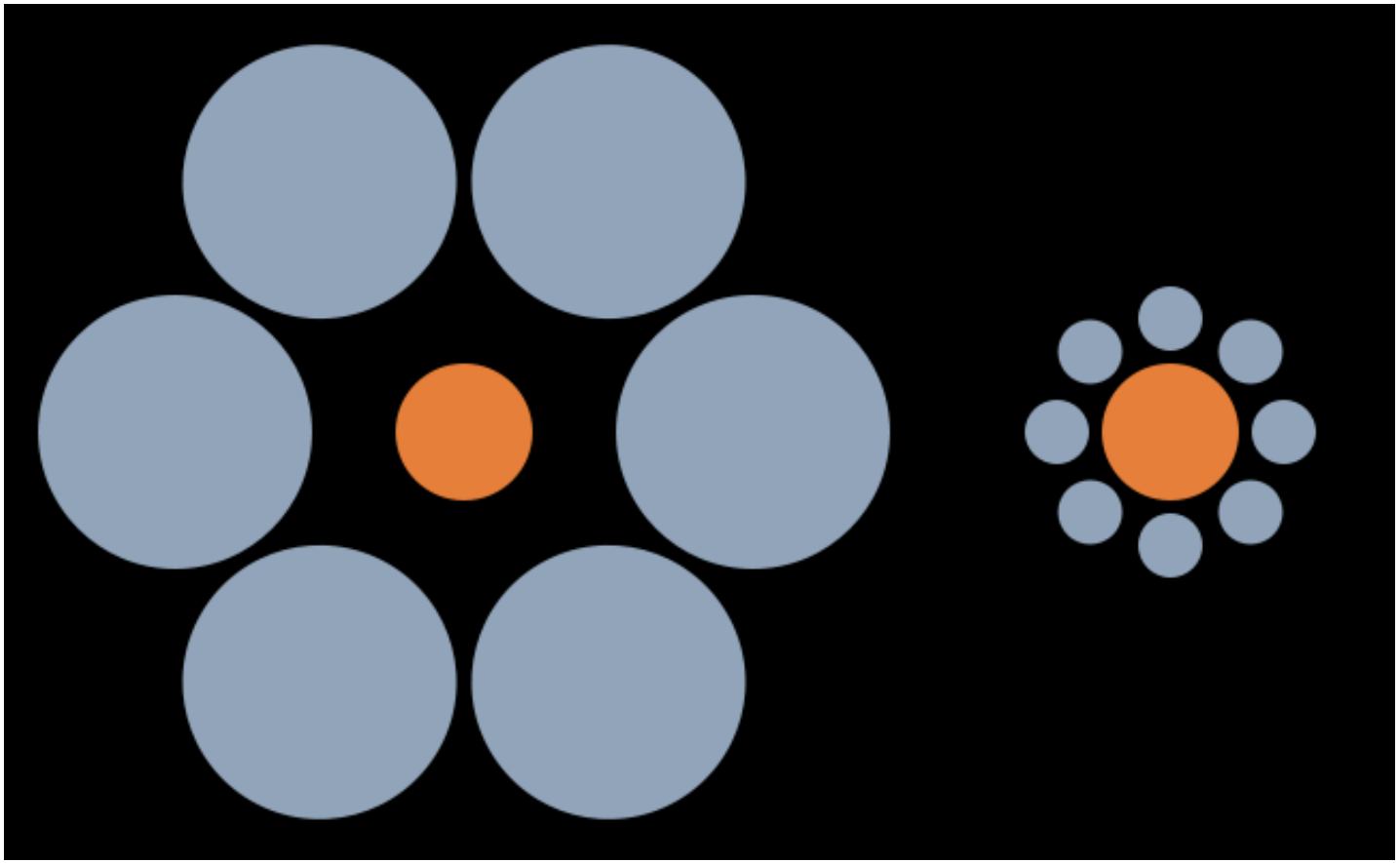
The eye can be tricked
→ **Size**



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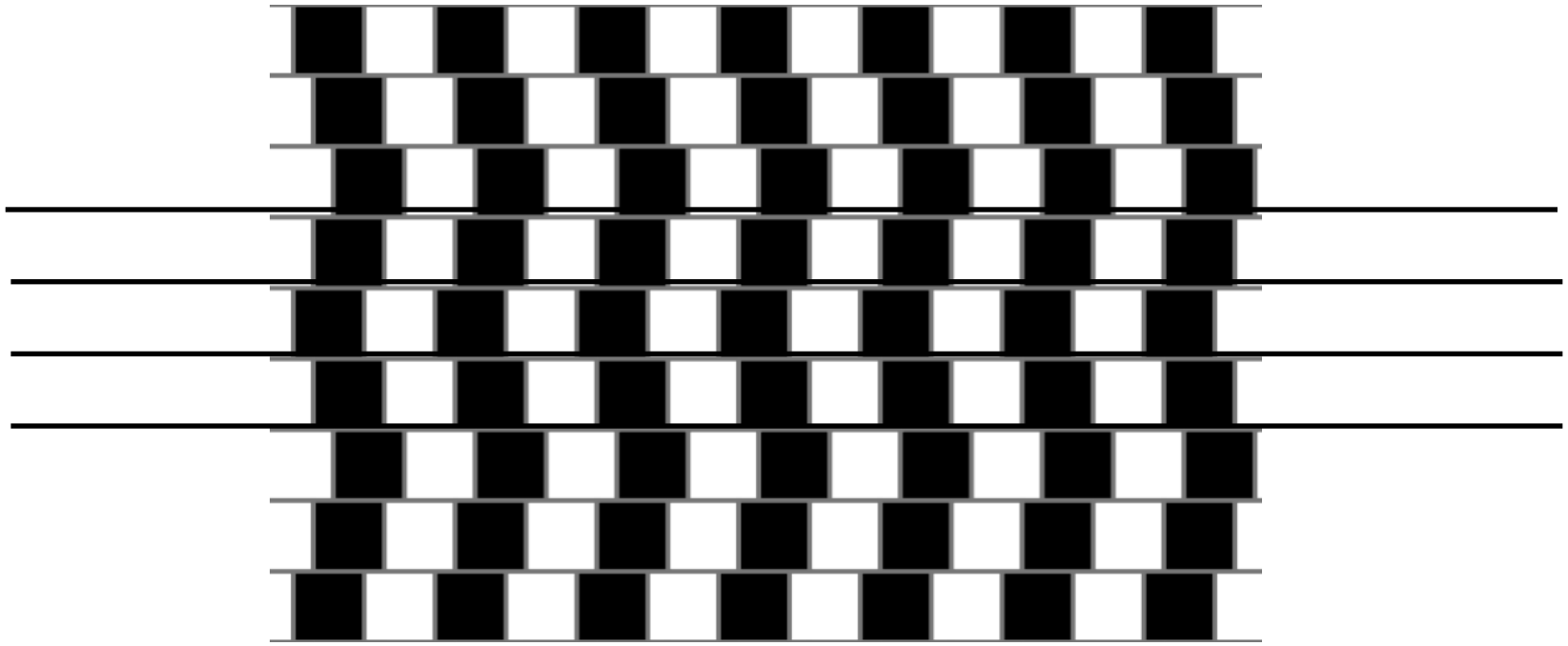
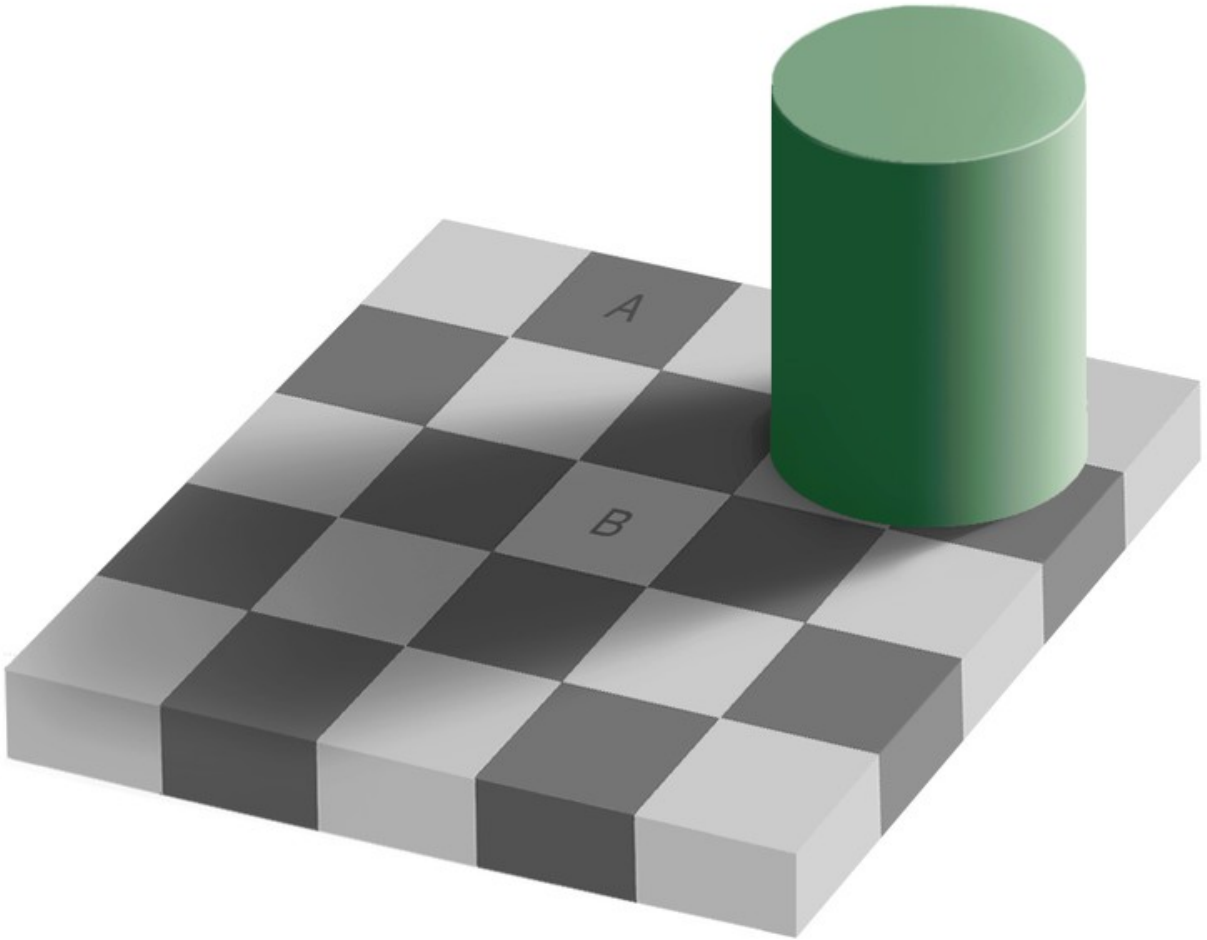
The eye can be tricked
→ **Geometry**



RCSI

The eye can be tricked
→ **Intensity**



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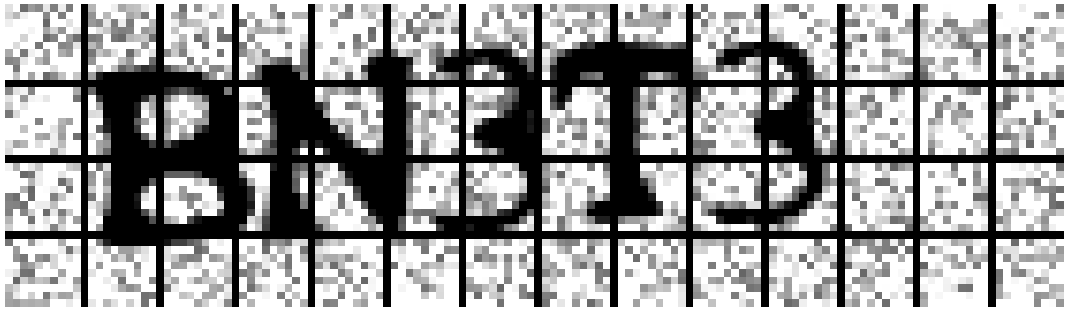
Correctly taken, a digital image or an image series contains a lot of **quantitative** information

- Advanced image analysis can provide quantitative, unbiased information
- Statistical analysis possible



- Pattern recognition and segmentation not trivial

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Imaging living cells

Apart from some instrument automation...

Optical components are critical!

- Numerical aperture of objective
- Sufficient neutral density filters (low light excitation)
- Excellent transmission of fluorescence emission

Detectors/Cameras are critical!

- High sensitivity, signal to noise

Everything else can be improvised

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Imaging living cells

Optimizing experimental conditions

1. **Make sure your fluorophores and probes work and are suitable**
    1. Spectral compatibility
    2. Signal to noise ratio
    3. FP maturation times
    4. Behaviour of fusion proteins

RCSI

Imaging living cells

Optimizing experimental conditions

2. Do cells grow properly on stage?

1. Measure proliferation rate on stage
2. Temperature and pH
3. How long are experiments expected to take?

3. Phototoxicity/bleaching controls

1. Does measured signal notably change due to imaging?
2. Do cells proliferate subsequent to imaging?
3. Is increased spontaneous cell death detectable?
4. Reduce excitation intensity, increase exposure time

Imaging living cells

Optimizing experimental conditions

4. Make sure your drugs etc are compatible with imaging

1. Are they coloured (do they absorb, are they fluorescent)

→ phototoxicity?!

2. Is amount of cell death notably different between imaged field of view and areas outside of the field of view (check after experiment)

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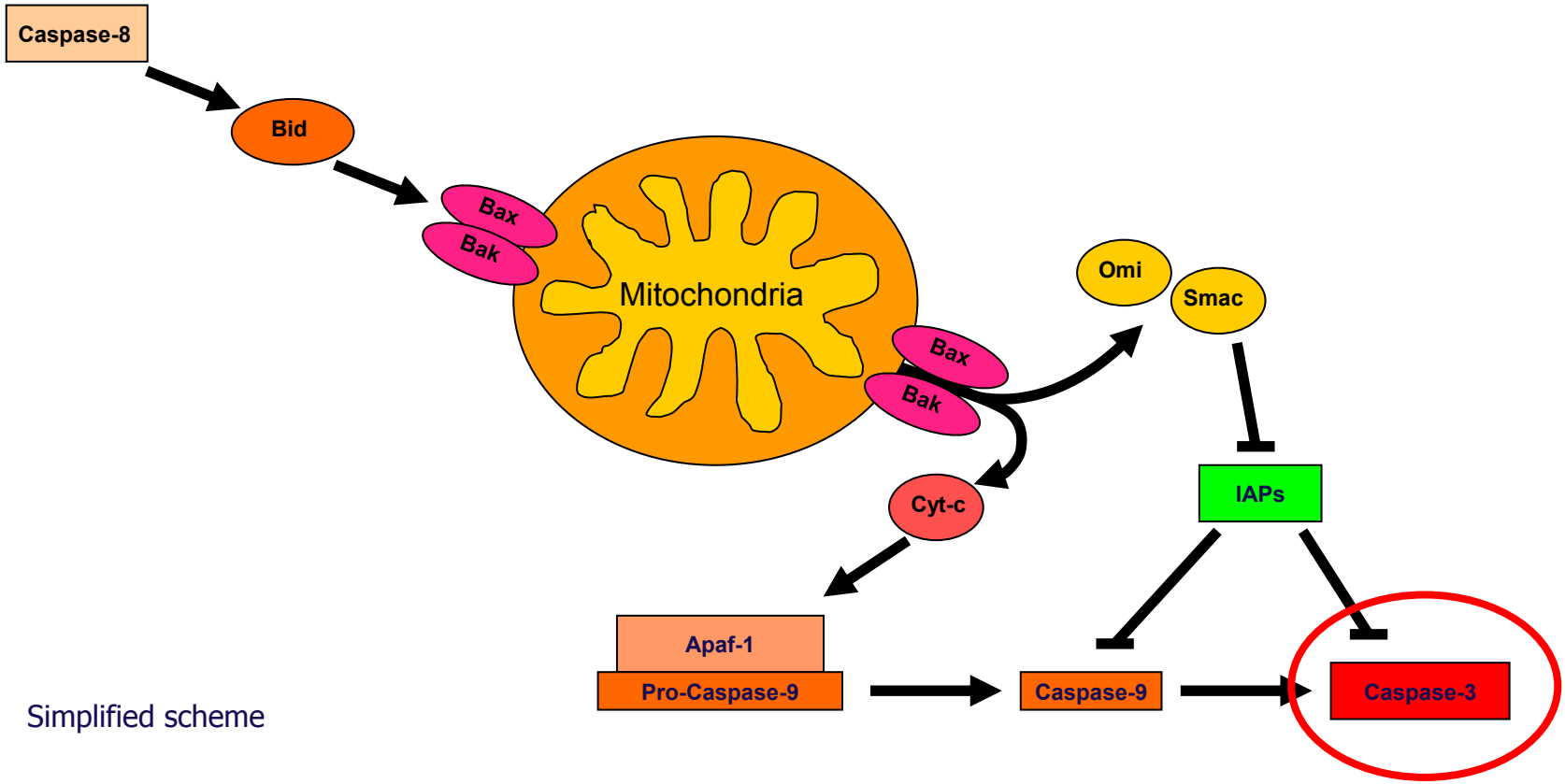
Imaging living cells

Optimizing experimental conditions

5. Think about non-obvious sources of problems

1. Eliminate vibrations
2. Eliminate stray light
3. Stabilise room temperature (+/- 2ºC changes a lot)
4. Think about (auto-)focussing (reflected light preferred)
5. Data storage during experiment (RAM vs. Hard drive)
6. Stabilise computer performance (avoid automatic updates, LAN connection required?)

Applications: Analyses of the apoptotic signalling cascades



Simplified scheme

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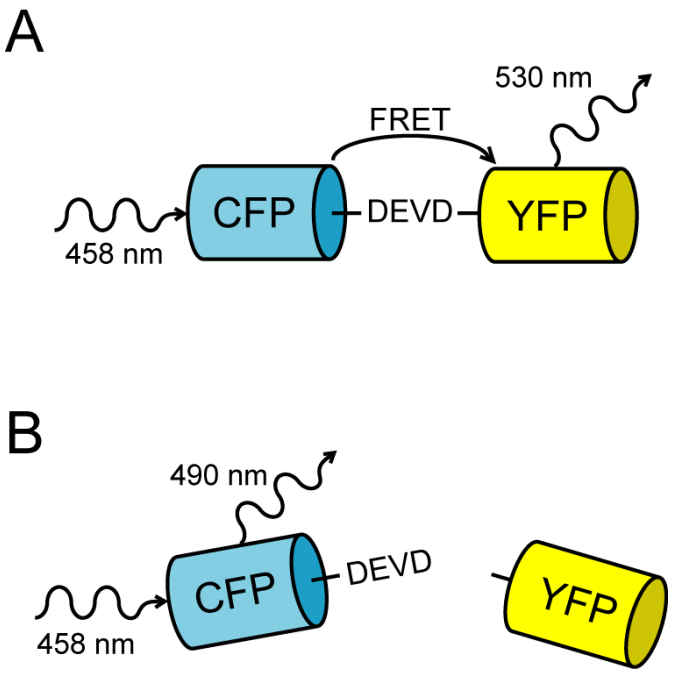
FRET Imaging – Use case I



Equimolar amounts of donor and acceptor

Imaging modalities:

CFP excitation – CFP emission (CFP)
CFP excitation – YFP emission (FRET)
YFP excitation – YFP emission (YFP)

Ratiometric analysis:

CFP/YFP
FRET/YFP

see e.g.
Tyas et al, Embo Rep, 2000
Rehm et al., J Biol Chem, 2002

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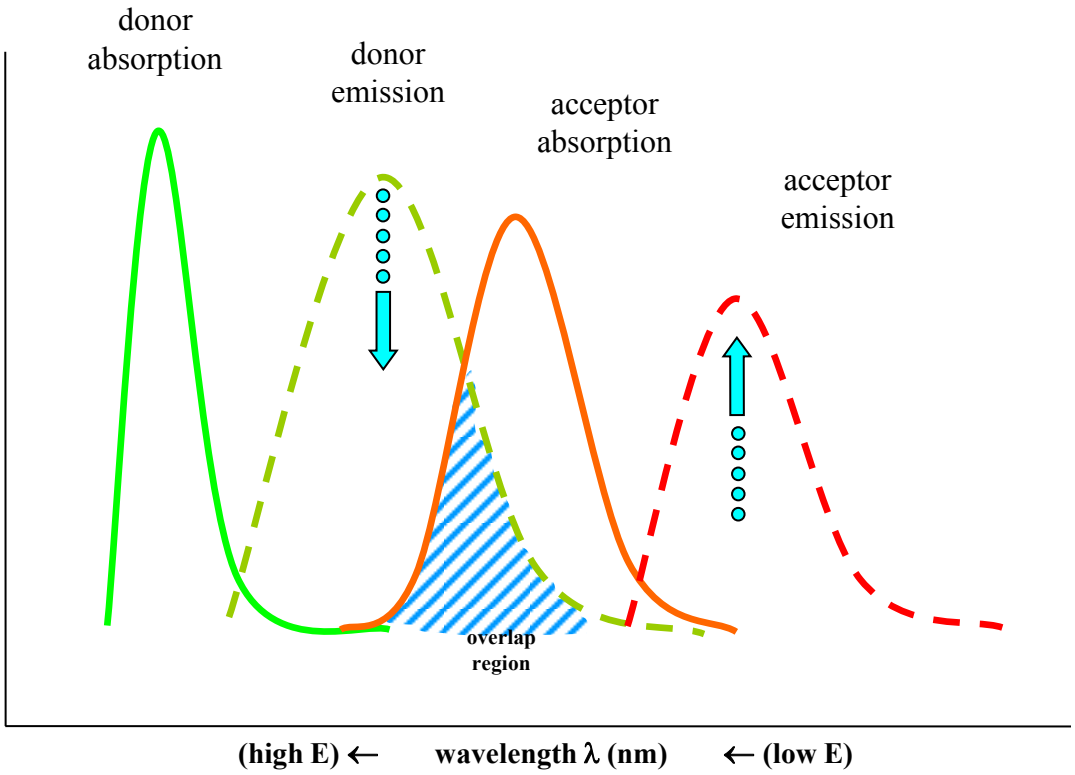
Förster Resonance Energy Transfer (FRET)

- radiationless energy transfer between donor and acceptor

- efficiency dependent on:
  - quantum yield
  - overlap region
  - transition dipol orientation
  - distance (~1/r ^{6})



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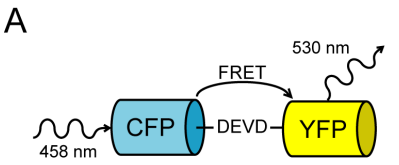
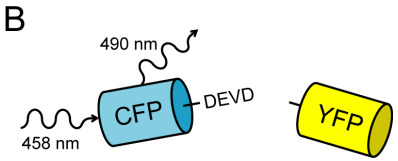
Intracellular Signalling Kinetics during Apoptotic Cell Death

Example: Activation of effector caspases

- High sensitivity
- Ratiometric (Low noise)
- Measure Enzymatic Activity





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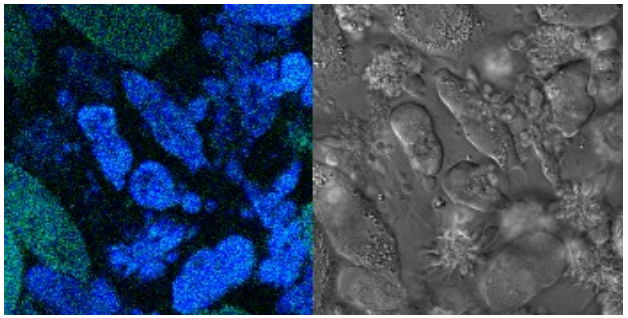

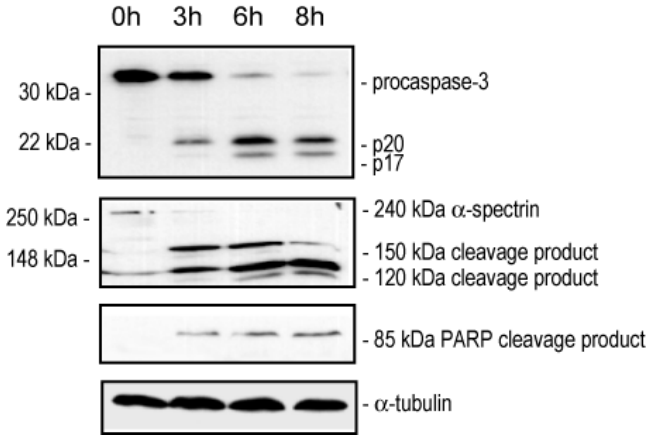

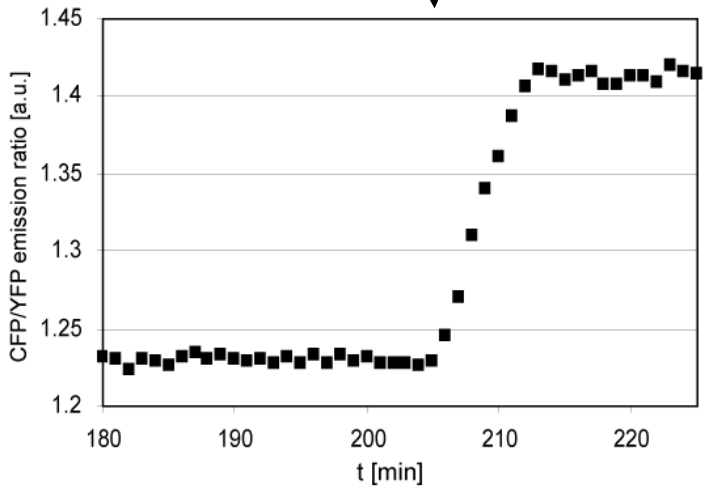

Bulk: Hours
Kinetics look dose dependent

Single Cell: Minutes
Kinetics are dose & drug independent → switch-like "all or none" behaviour

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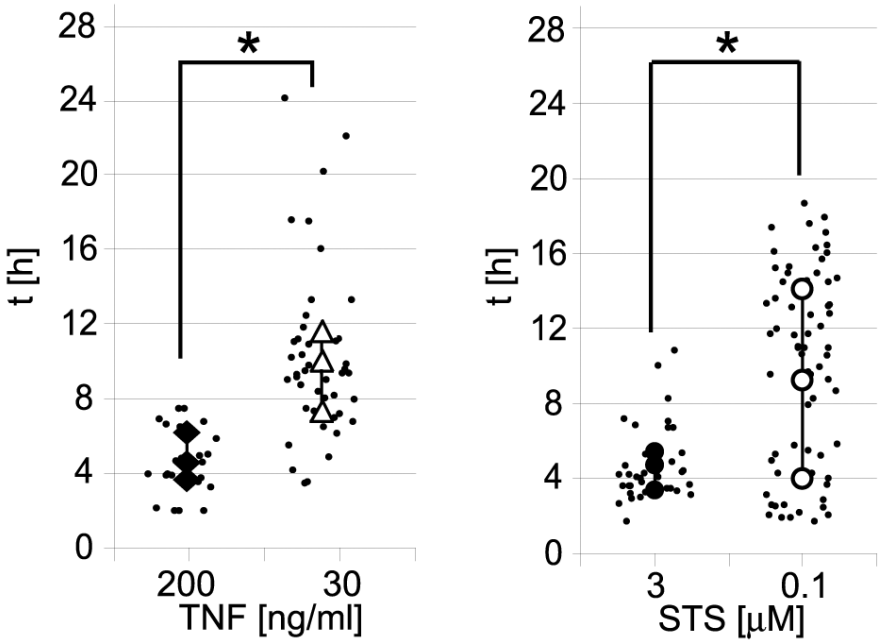
Single Cell Imaging

Activation of effector caspases

- Rapid activation of effector caspases

- activation kinetics independent of concentration of the stimulus



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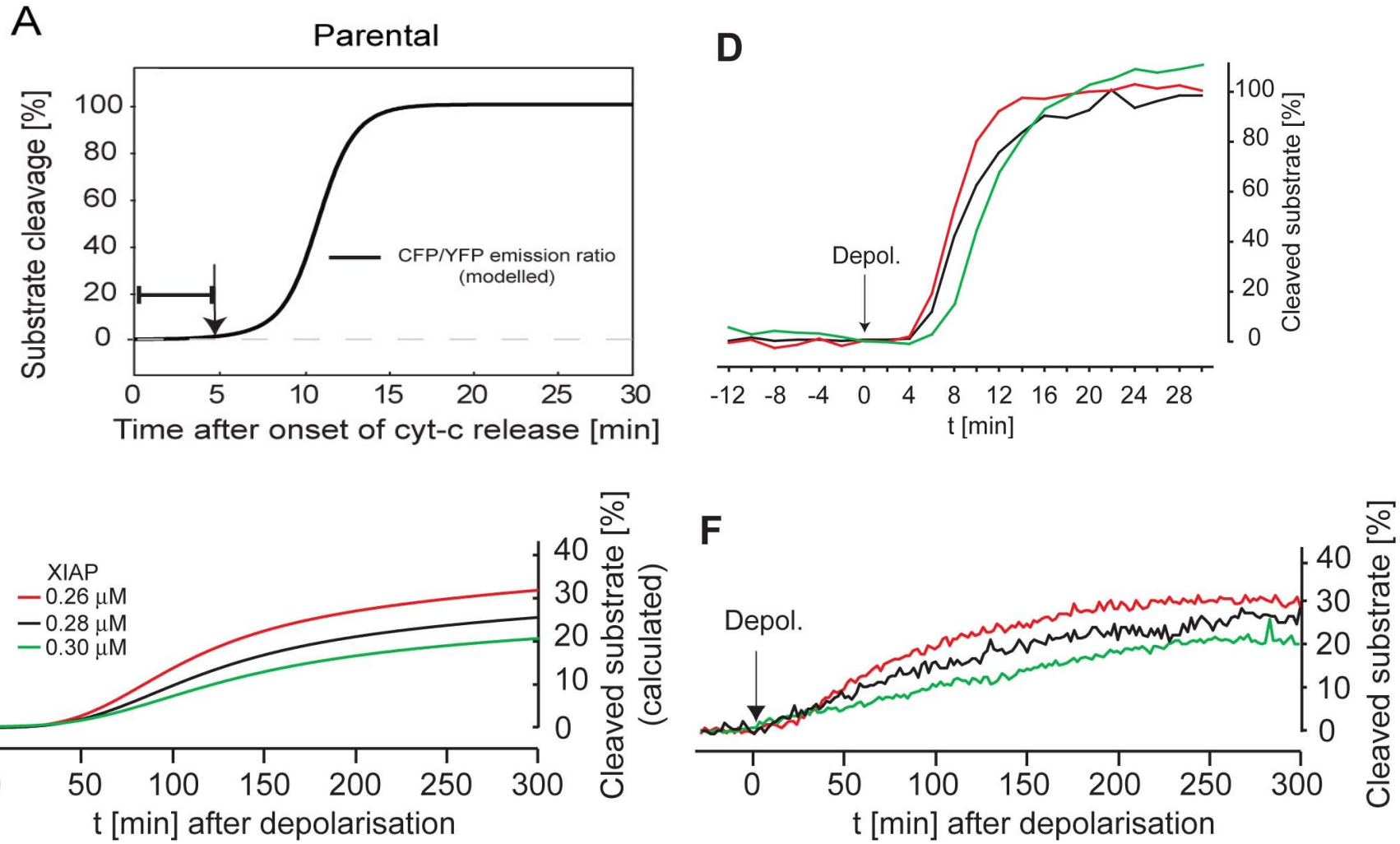
Single Cell Imaging & Systems Modelling

Mitochondrial Permeabilisation/Depolarisation and Activation of Effector Caspases



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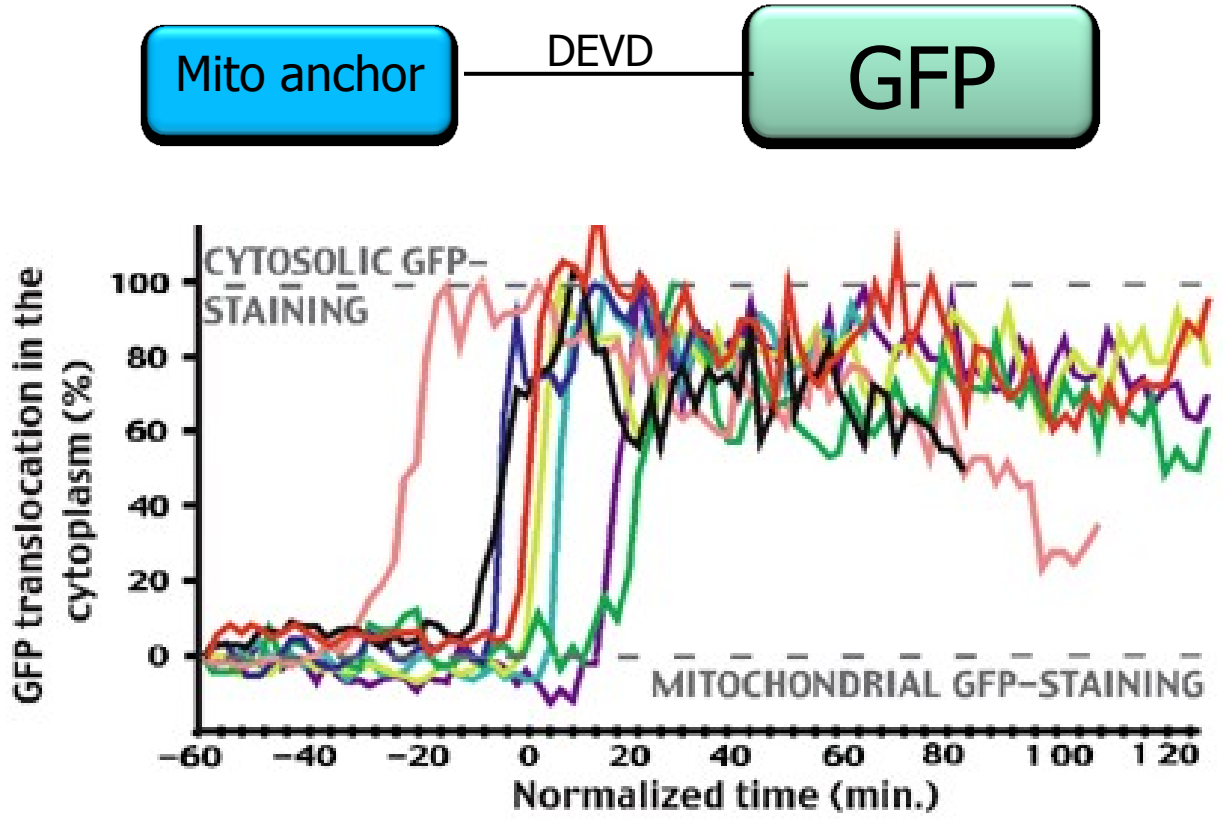
See e.g. Rehm et al, EMBO J 2006; Albeck et al., Mol Cell 2008



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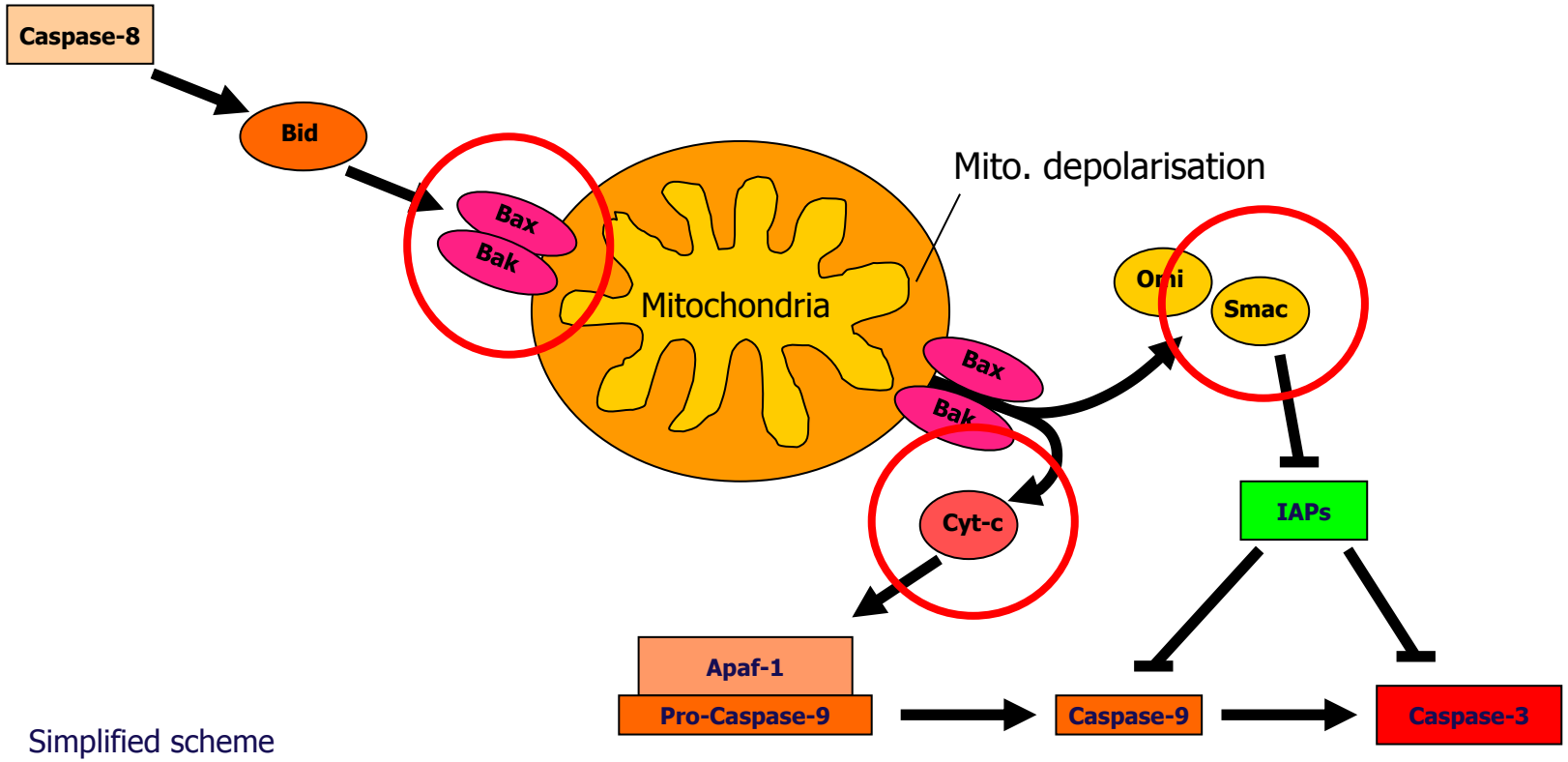
Henderson CJ, Cell Death Differ 2005

Investigation of pore formation



Simplified scheme

RCSI

Bax Interaction & Translocation

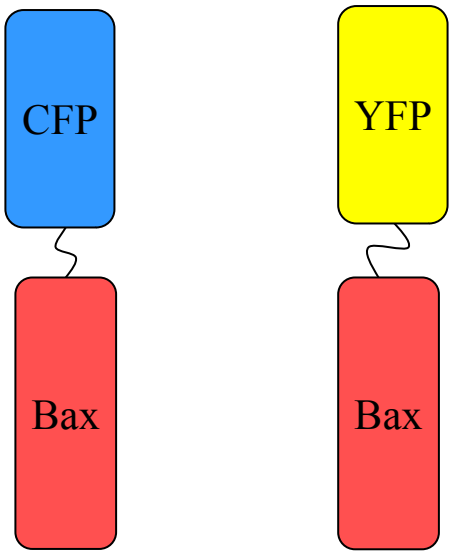
Donor/acceptor are NOT equimolar

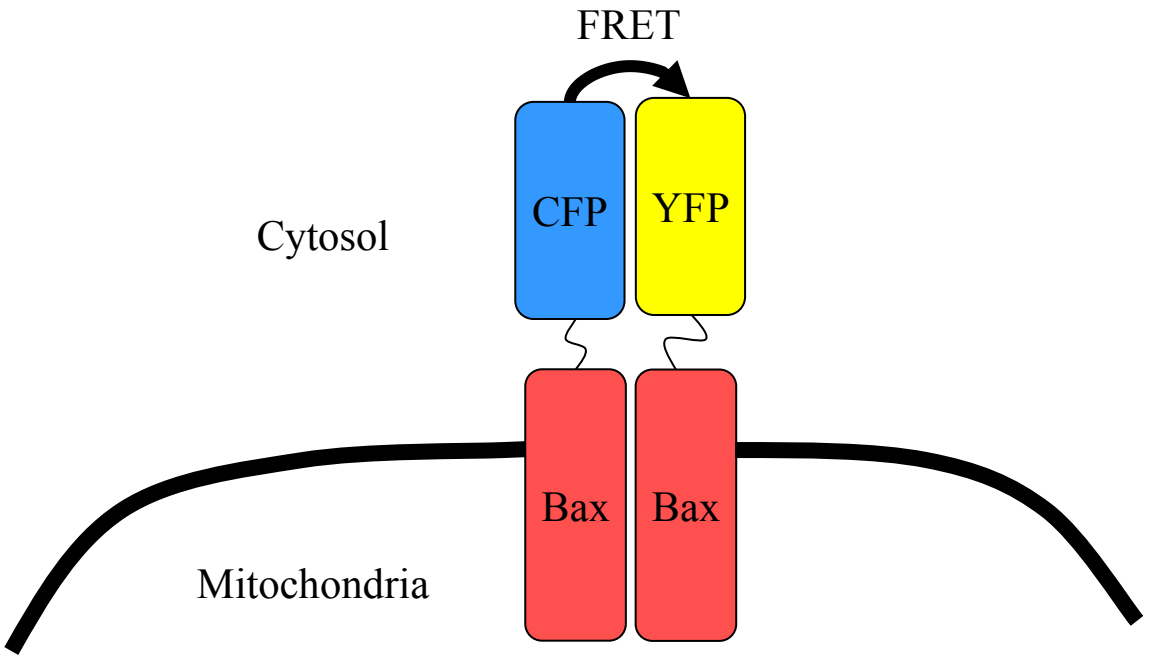
Simple ratiometry NOT good enough

Lower signal to noise ratio

Protein redistribution changes signal to noise ratio

→ Normalized FRET measurements more appropriate

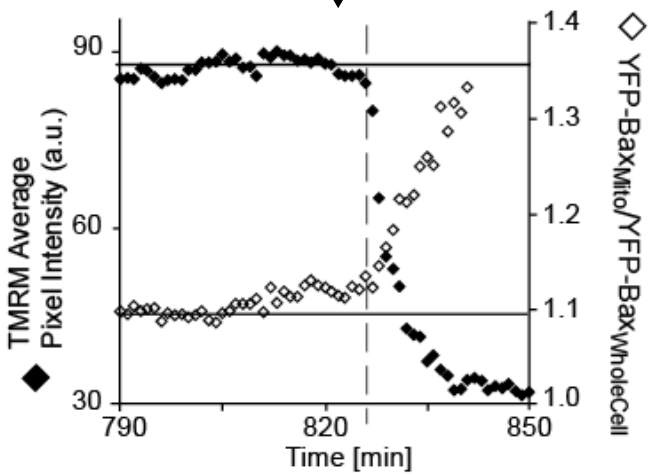




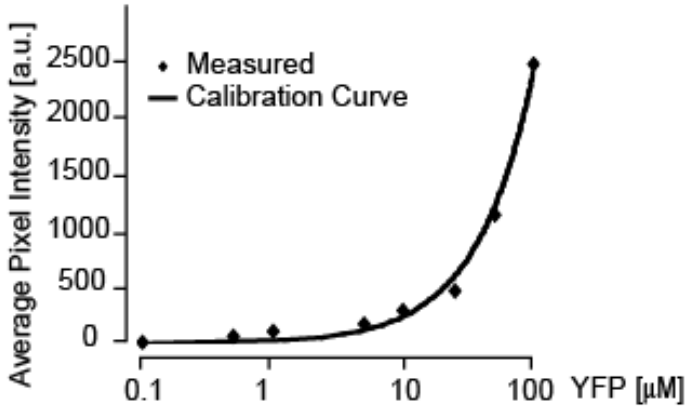
Dussmann et al. CDD 2010

RCSI

- Segmentation of mitochondrial regions from images
- Measurement of fluorescence intensities and intensity changes



- Coating of coverslips
- Application of liquid layer of fluorescent proteins in solution
- Determination of fluorescent intensities



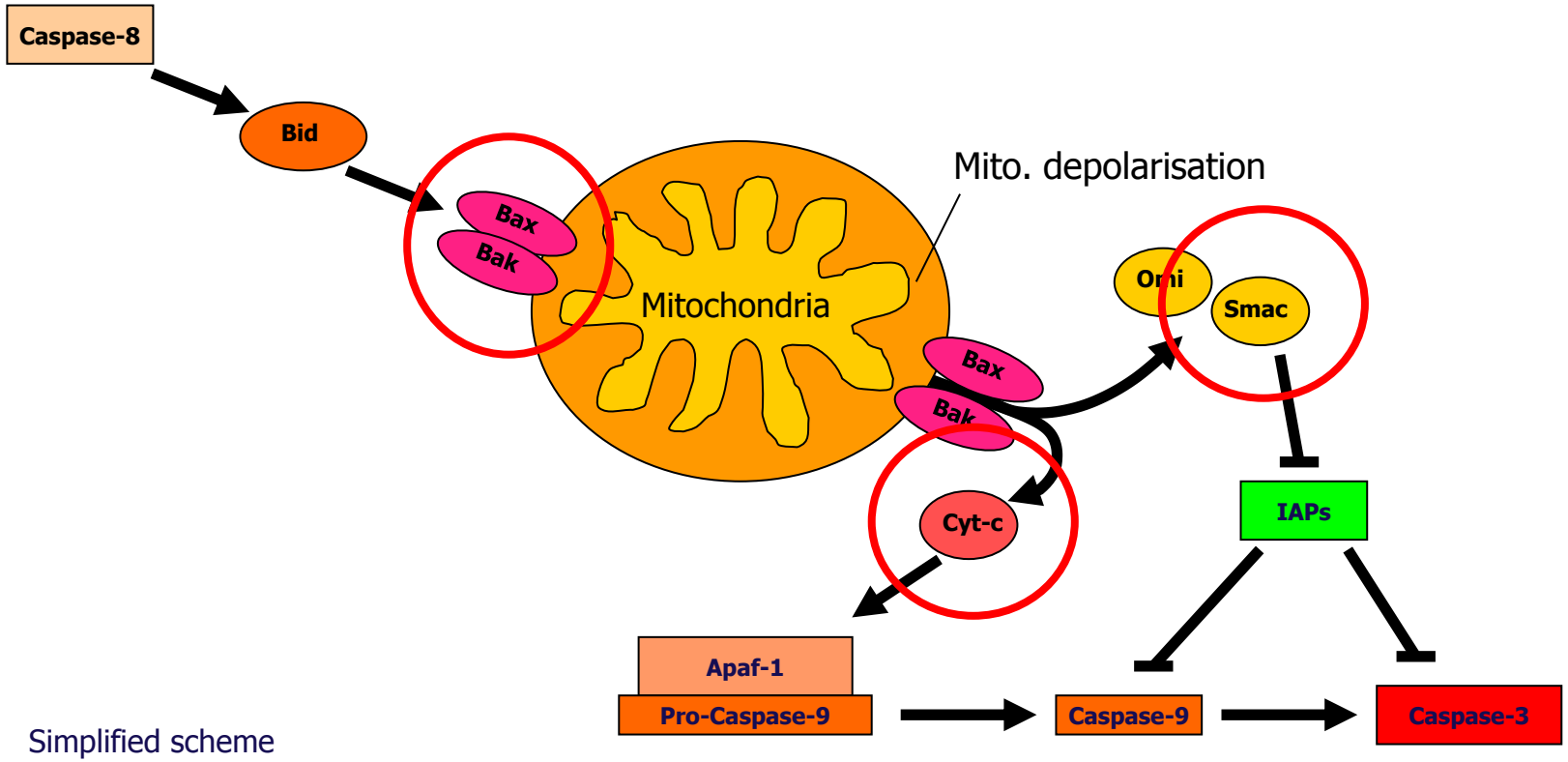
Calibration of intracellular signals
intensity → concentration

activation of approx. 5% of total Bax sufficient for pore formation

RCSI

Dussmann et al.
Cell Death Differ 2010

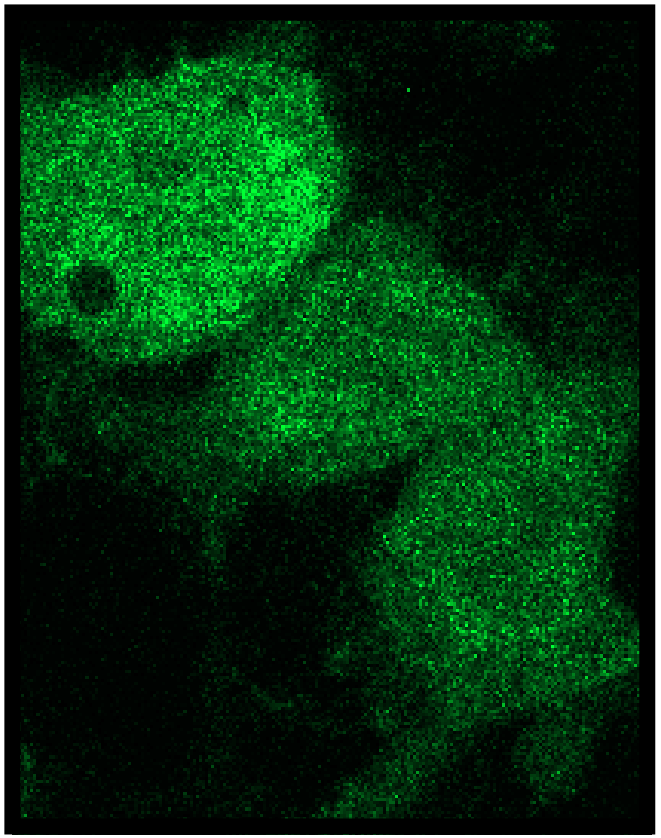
Investigation of pore formation



Simplified scheme

RCSI

Mitochondrial Permeabiliation and the Release of Intermembrane Space proteins



See e.g.

Goldstein et al., Nat Cell Biol 2000

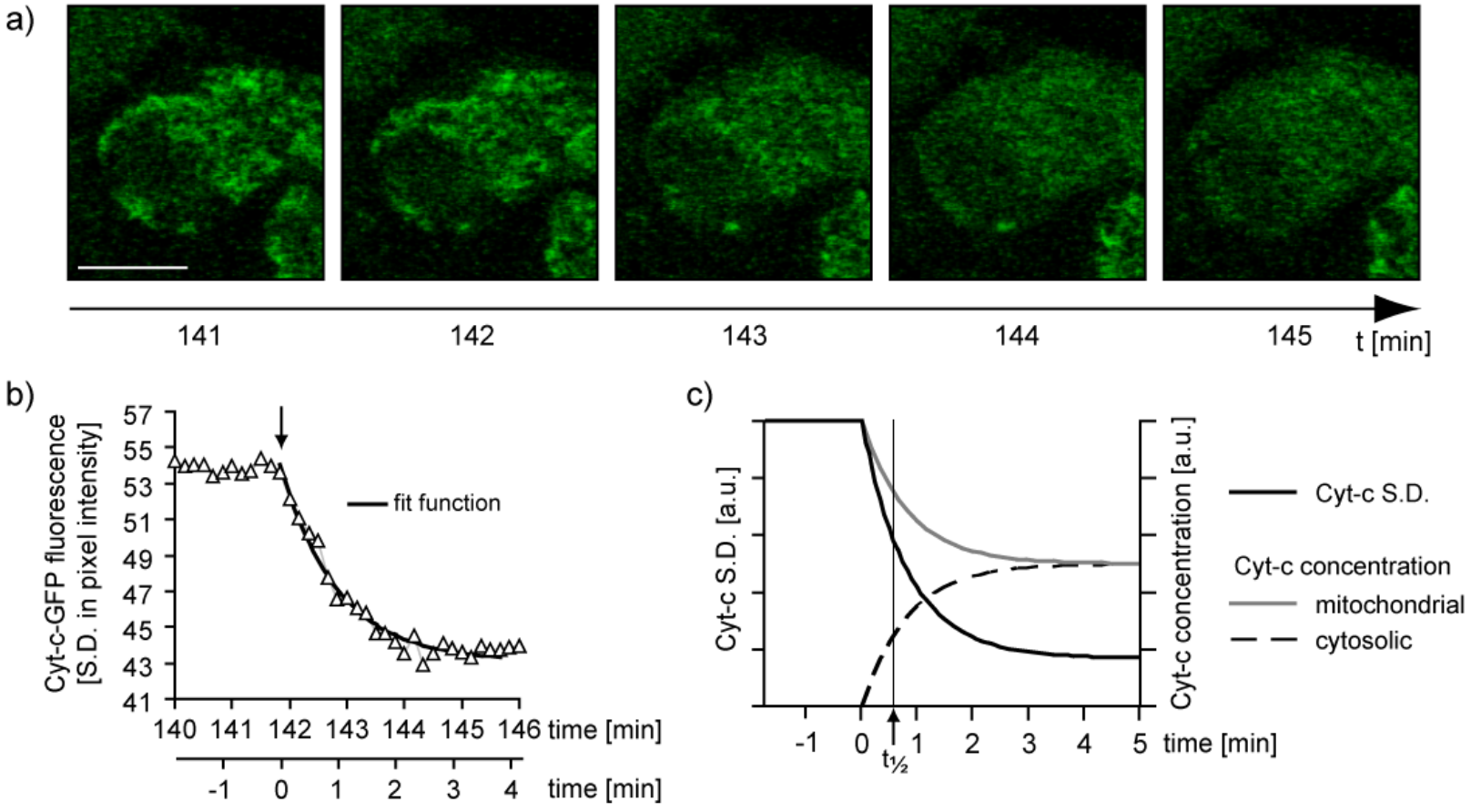
Dussmann et al., J Cell Sci 2002

Rehm et al., J Cell Biol 2003

Munoz Pinedo et al., PNAS 2005

RCSI

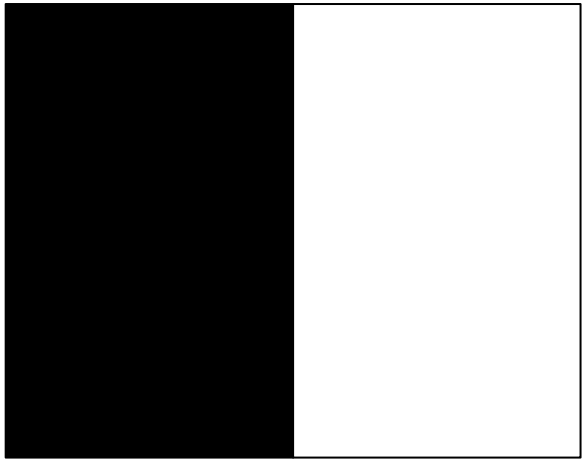
Cyt-c release visualised using GFP-fusion proteins

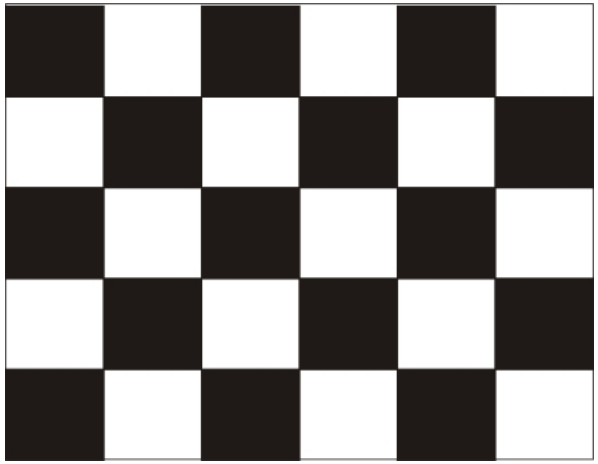


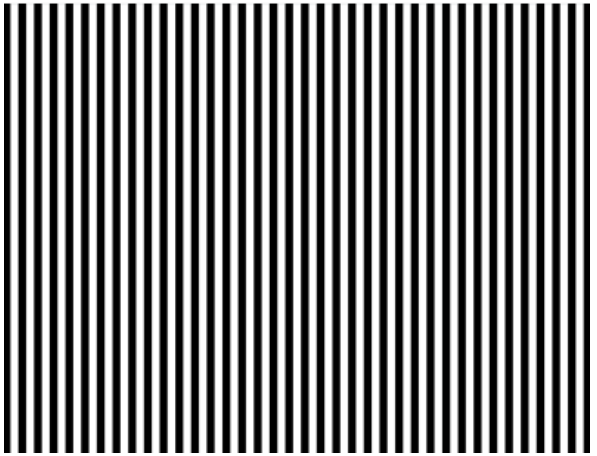
RCSI

Standard Deviation heavily depending on **Homogeneity** and **Signal to Noise** ratio
- fluorophore brightness
- expression level
- changes in cellular morphology
- imaging modalities

- **Standard Deviation is NOT a measure for clustering below the resolution of one pixel!!**

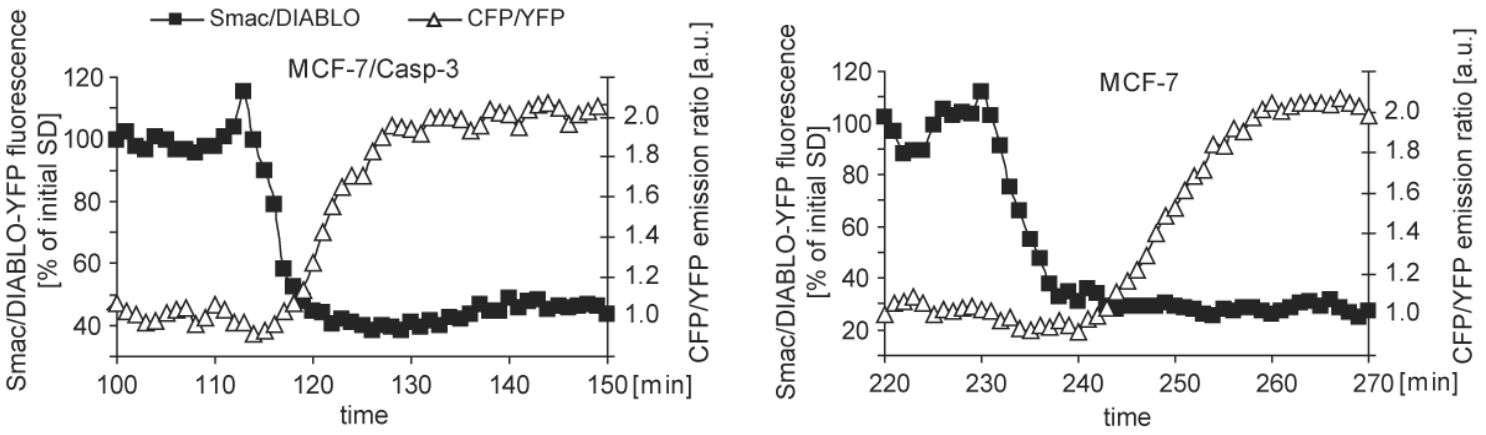






RCSI

Combination of SD and FRET analysis

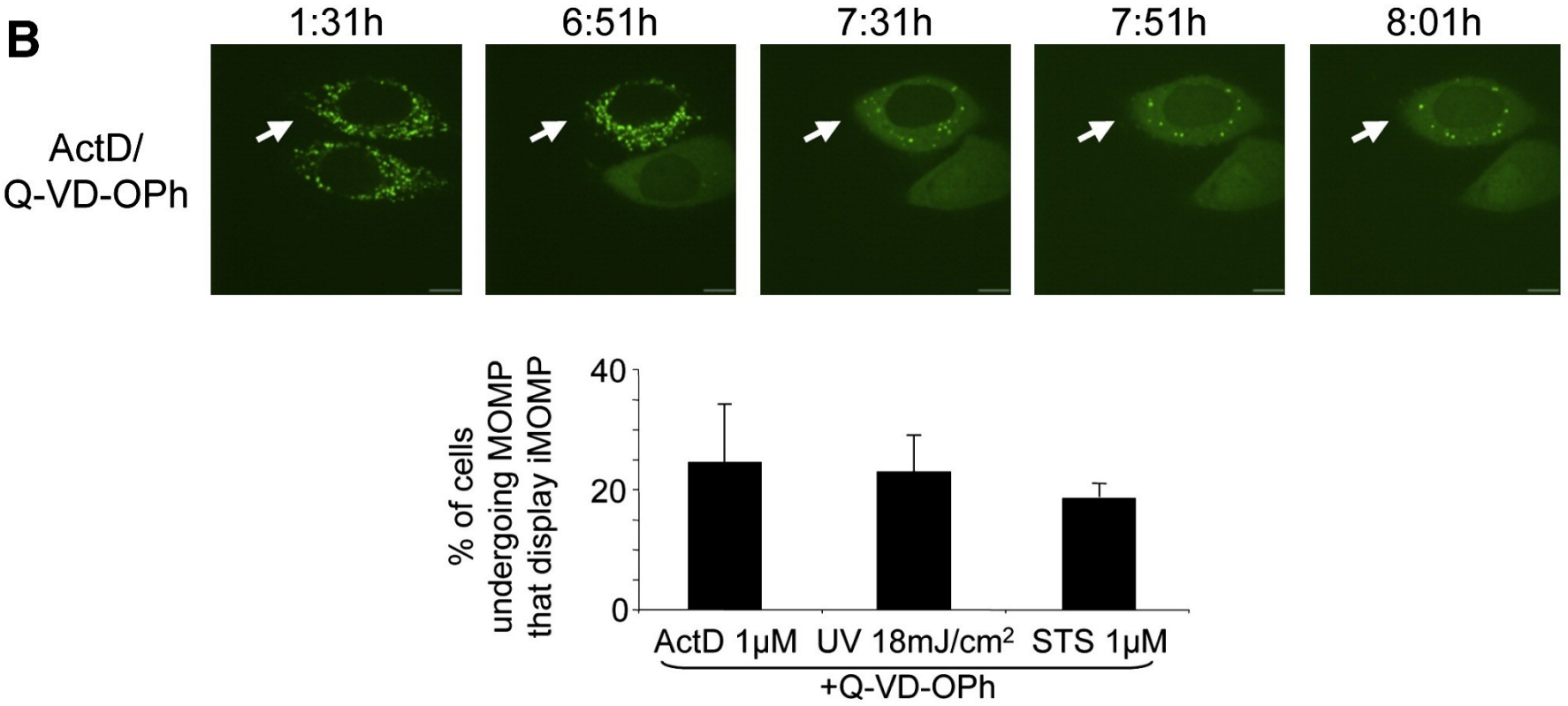


- Effector caspase activation within <10 min after mitochondrial membrane permeabilisation

RCSI

Incomplete Mitochondrial Outer Membrane Permeabilisation

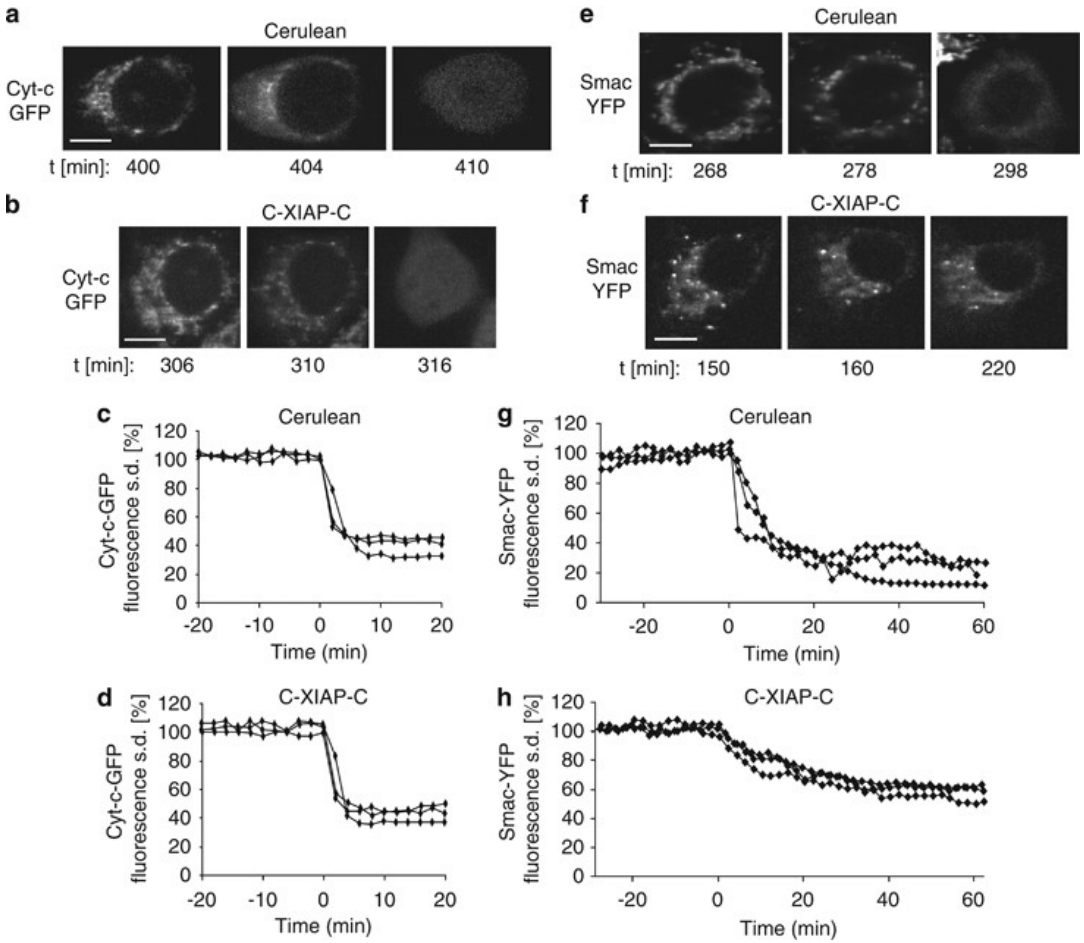
Smac-GFP in HeLa cells

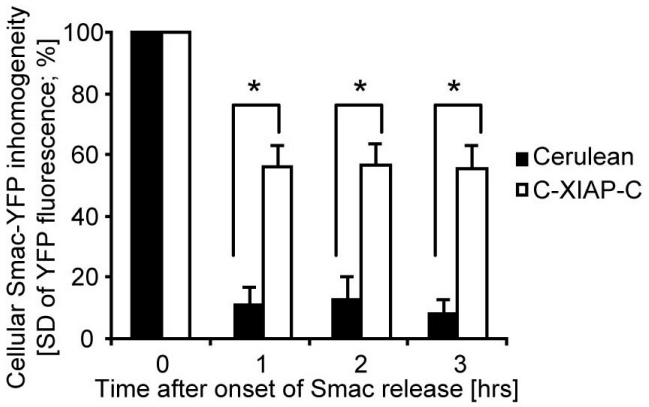


RCSI

Tait et al., Dev Cell 2010

XIAP impairs Smac release from mitochondria

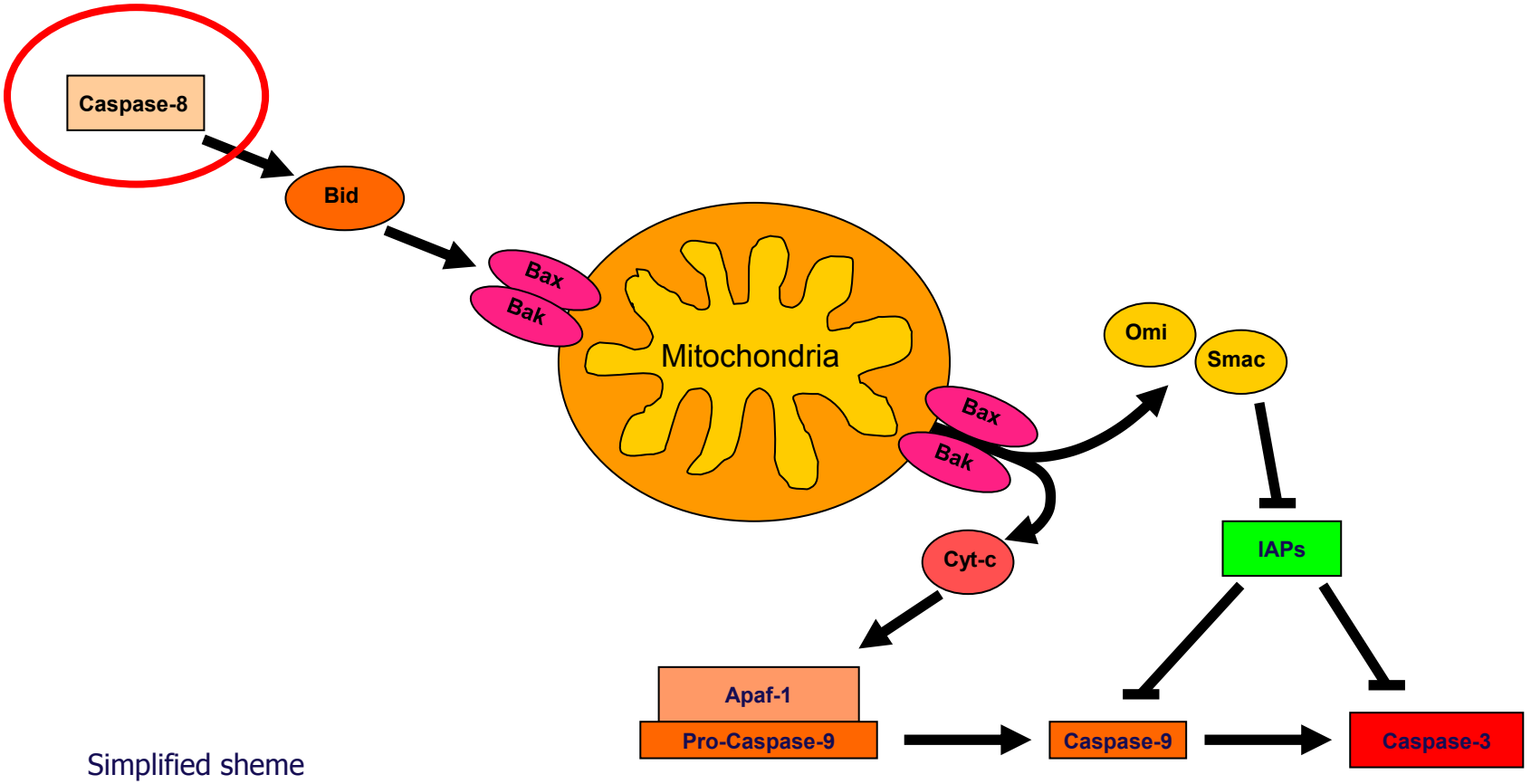




RCSI

Flanagan et al., Cell Death Dis 2010

Analyses of the apoptotic signalling cascades

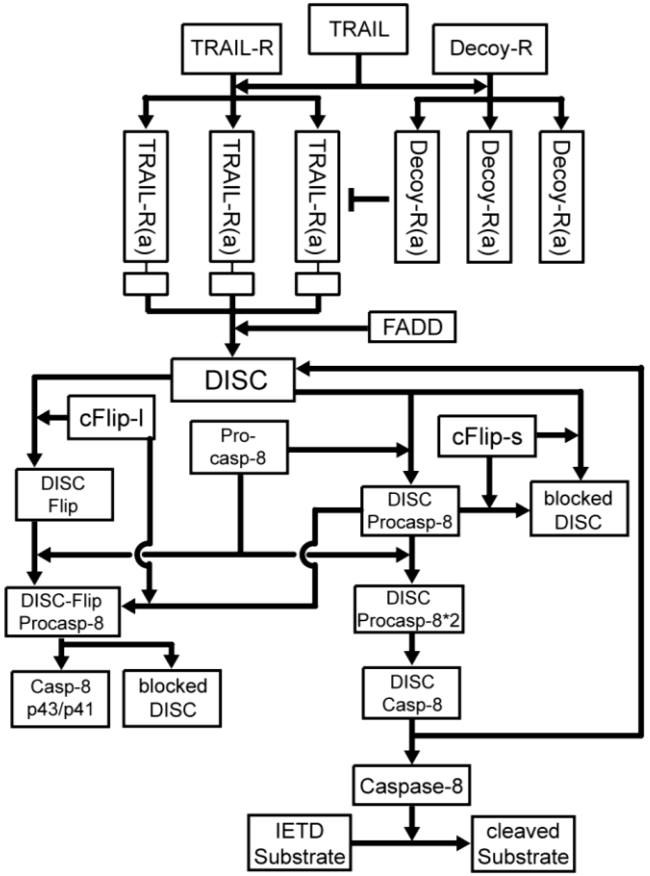


Simplified sheme

RCSI

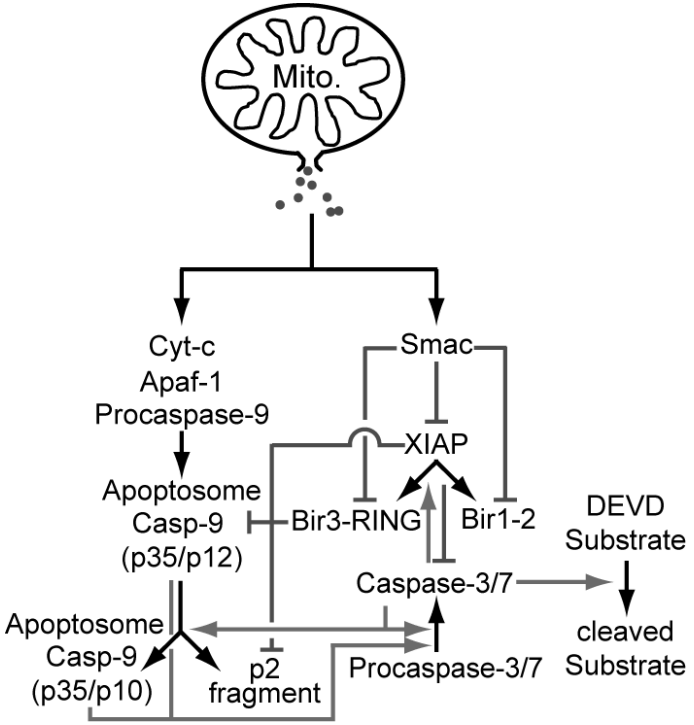
Distinct Topologies of Apoptosis Initiation and Execution

Apoptosis Initiation (extrinsic, TRAIL)



- Predominantly linear signalling sequences

Apoptosis Execution (mito. pathway)



- Prominent positive feed back loops

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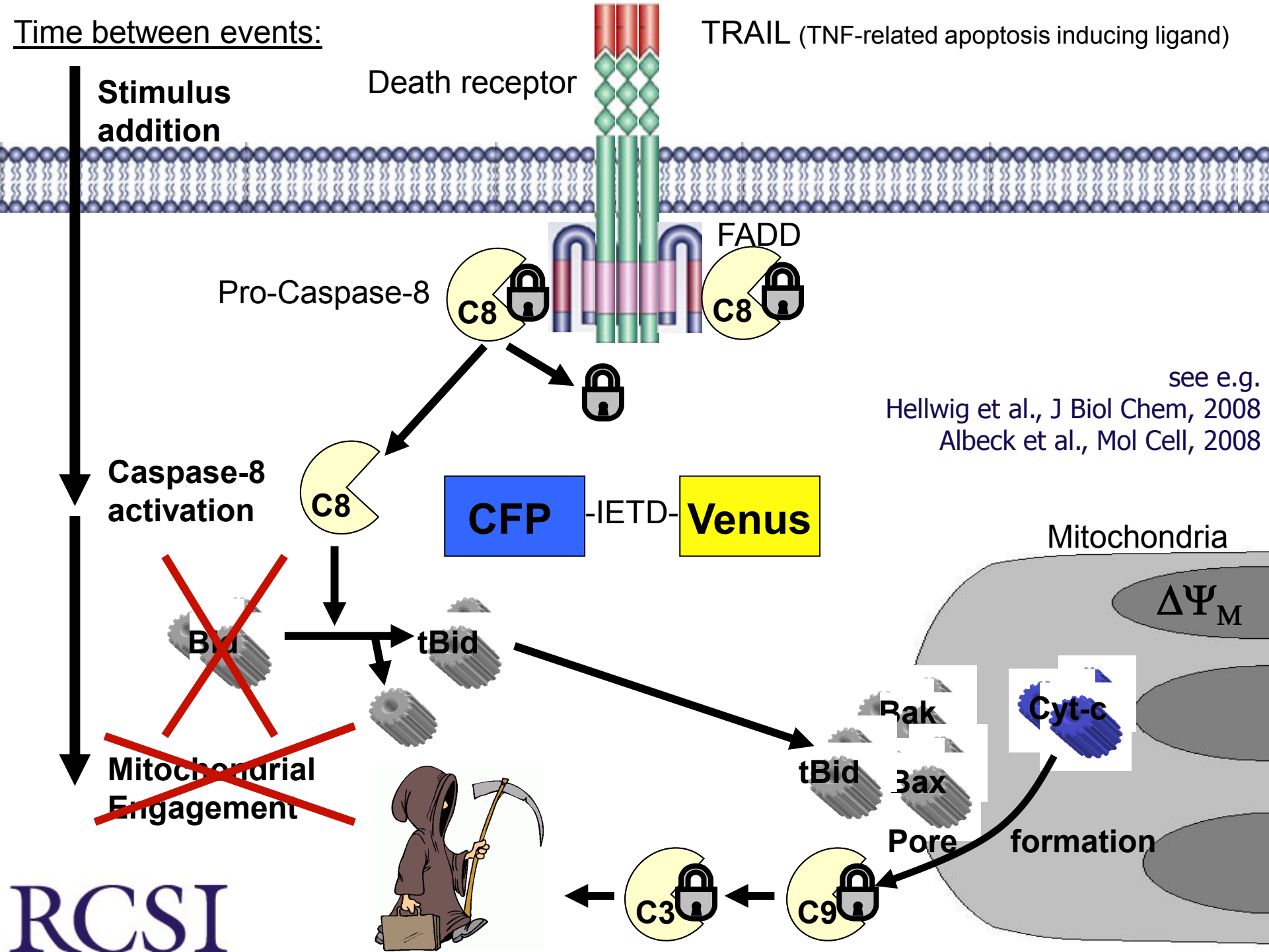
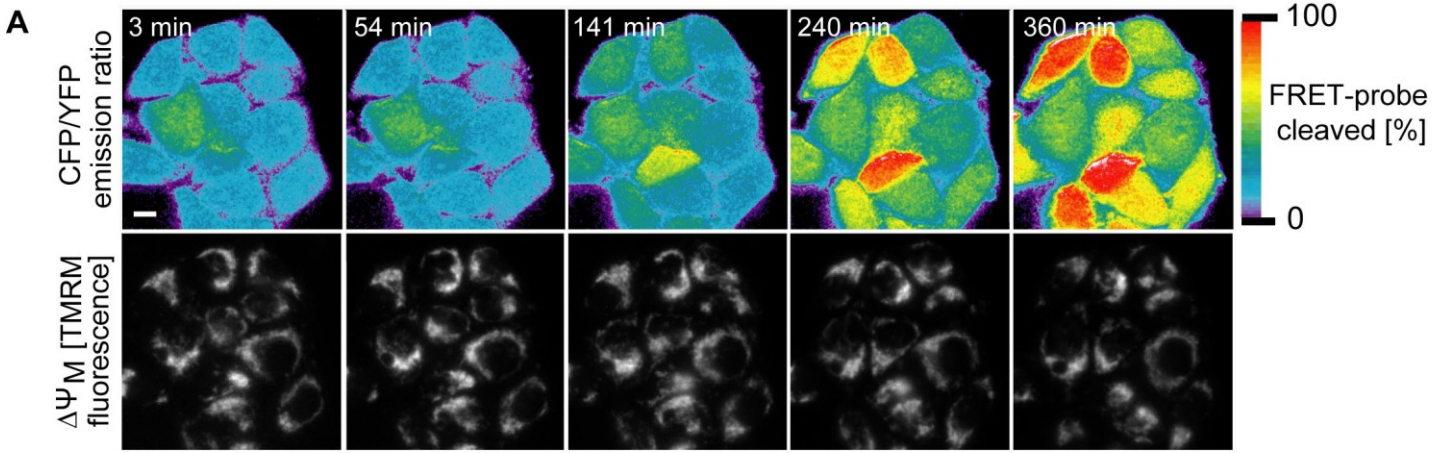
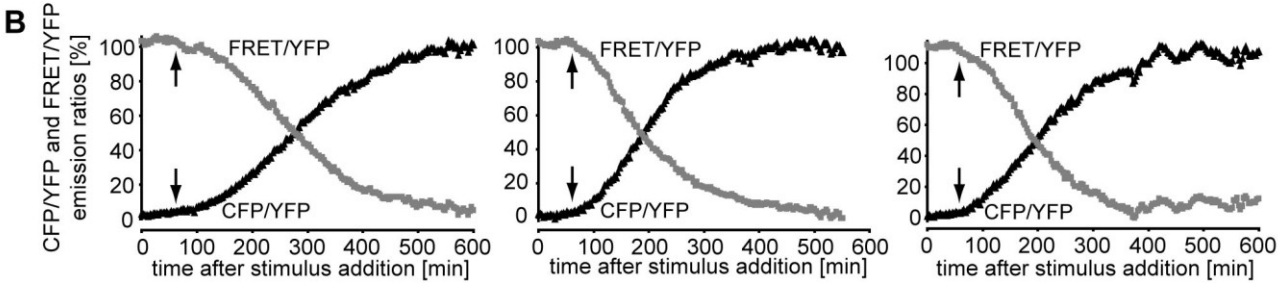

Time between events:
Stimulus addition
Death receptor
TRAIL (TNF-related apoptosis inducing ligand)
FADD
Pro-Caspase-8
C8
C8
C8
see e.g.
Hellwig et al., J Biol Chem, 2008
Albeck et al., Mol Cell, 2008
Caspase-8 activation
CFP
-IETD-
Venus
Mitochondria
ΔΨM
Bid
tBid
Bak
Cyt-c
tBid
Bax
Mitochondrial Engagement
Pore formation
C3
C9
RCSI

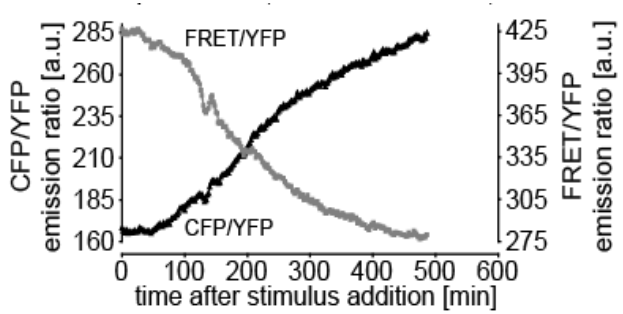
Caspase-8/-10 activity in living cells

Bid k/d scenario

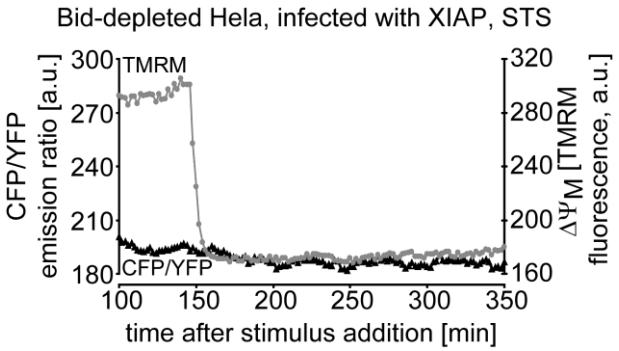


Caspase-8/-10 activity can persist for hours





XIAP over-expression does not inhibit the measured activity, but blocks activity during intrinsic apoptosis



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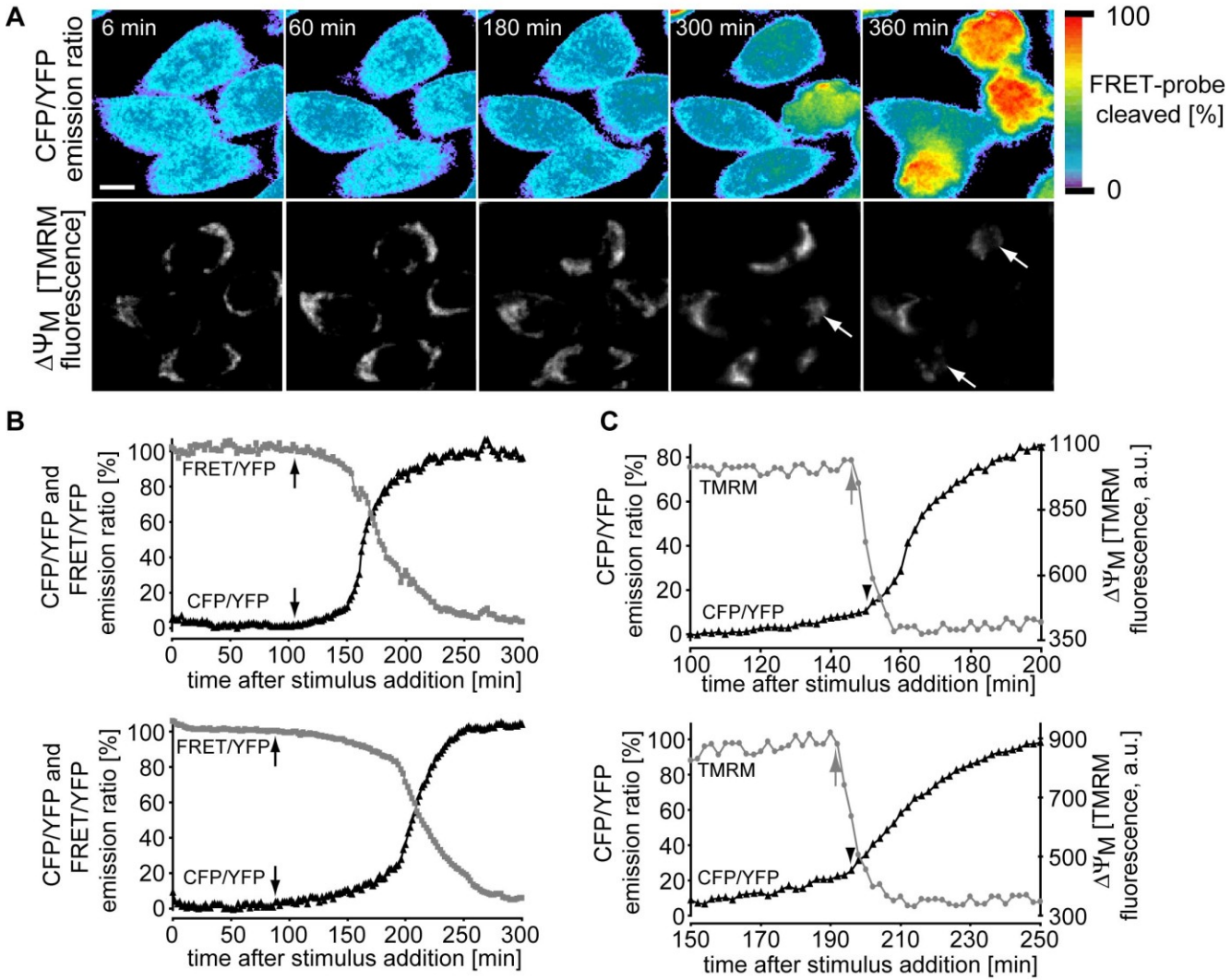
Caspase-8/-10 activity in living cells

Parental cells



Biphasic activity in parental cells

Activity increases following mito. engagement suggesting a contribution by effector caspases

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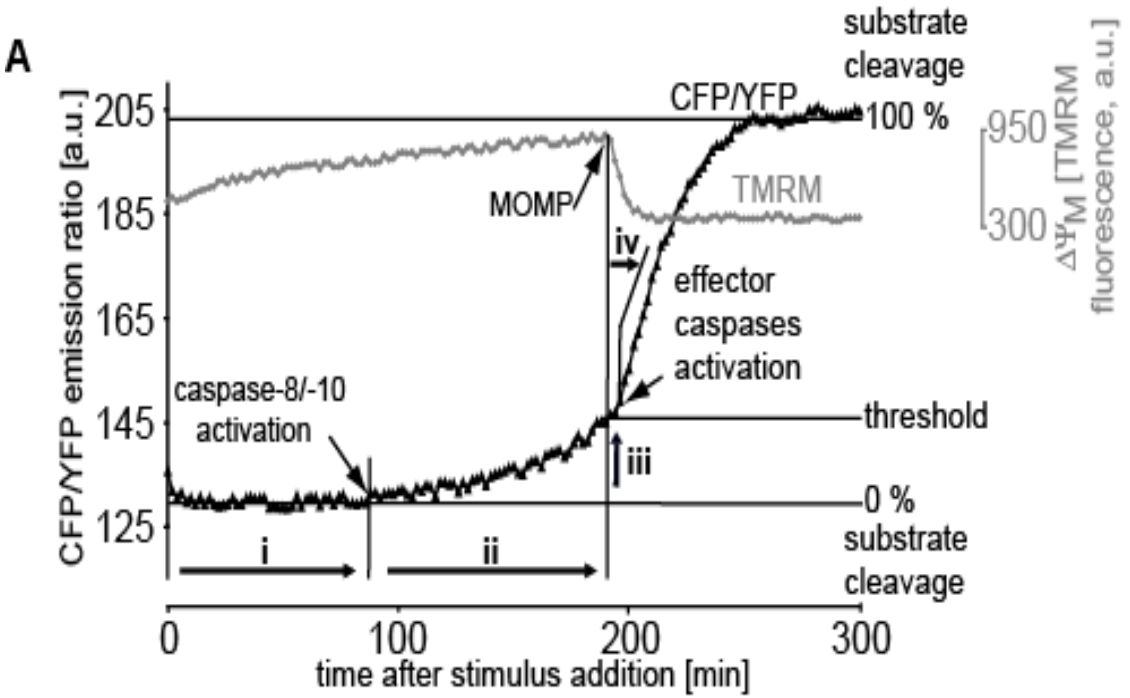
Multiple parameters from one probe

How is variability in TRAIL stimulation translated into a strict death decision of MOMP?



Hellwig et al., J Biol Chem, 2008
Albeck J et al., Mol Cell, 2008
Hellwig et al., J Cell Sci 2010

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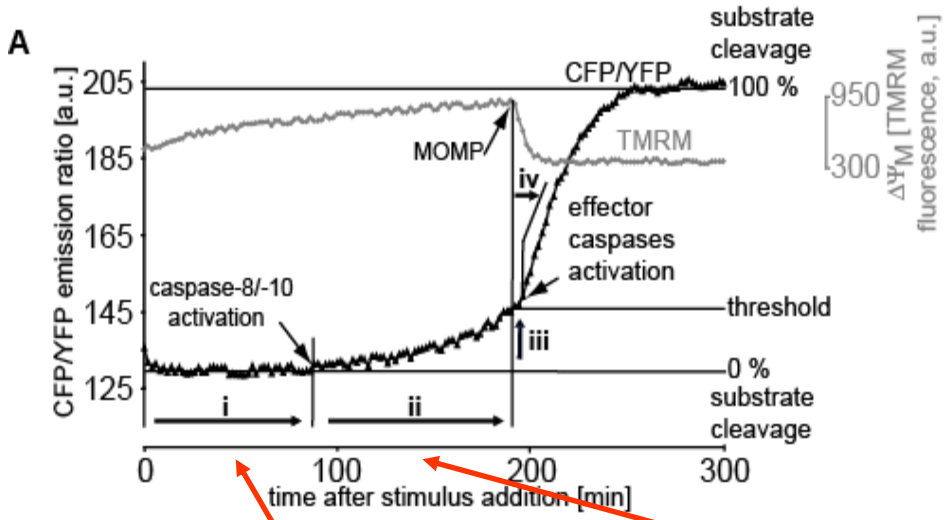
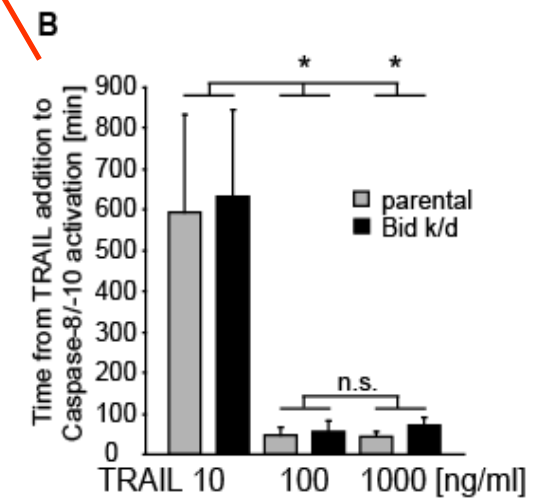
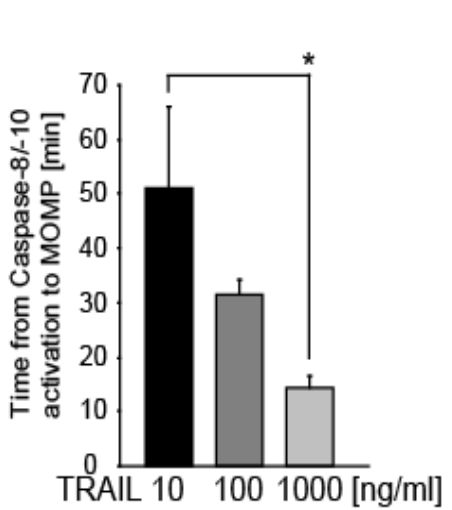
Parental cells - TRAIL







-Time from TRAIL addition to caspase-8/-10 activation is dose dependent

-Time for caspases-8/-10 to induce MOMP is dose dependent

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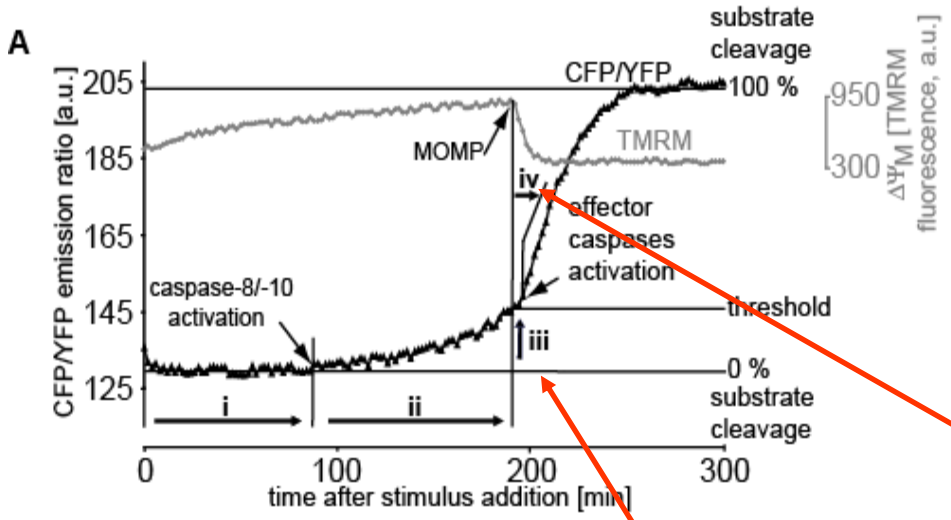
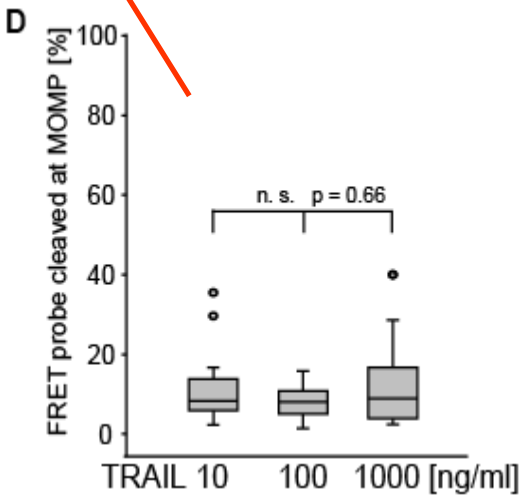
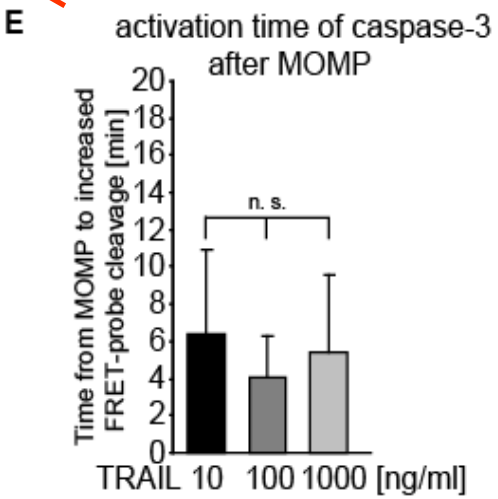
Parental cells - TRAIL



- Mitochondrial response occurs at a conserved threshold of cleaved FRET substrate. Threshold is dose independent!

- Subsequent lag time to effector caspase activation is also dose independent





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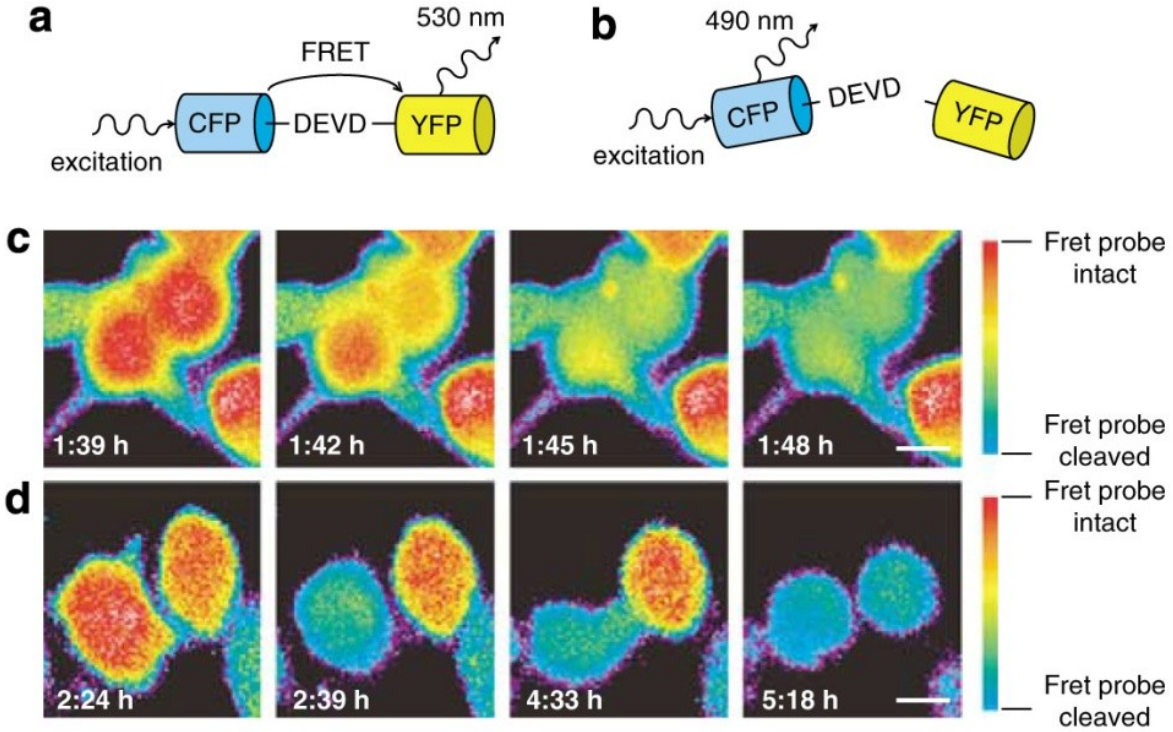
Cell to Cell variability



- Sibling cells

- Random cell pair

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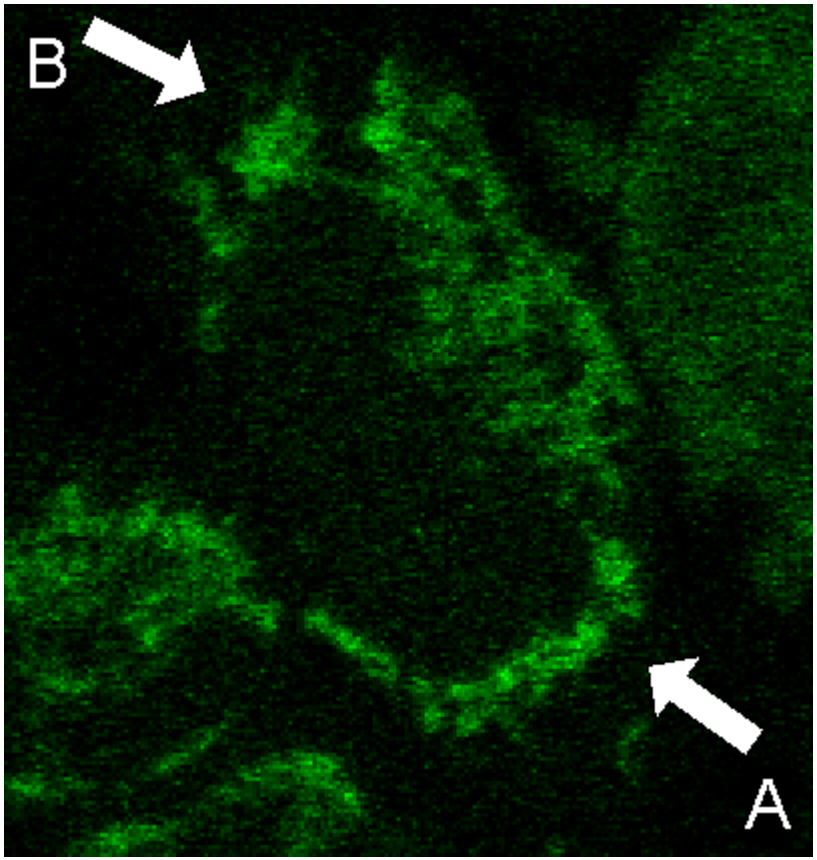
Rehm et al., Cell Death Differ, 2009



Cyt-c release can proceed as a wave

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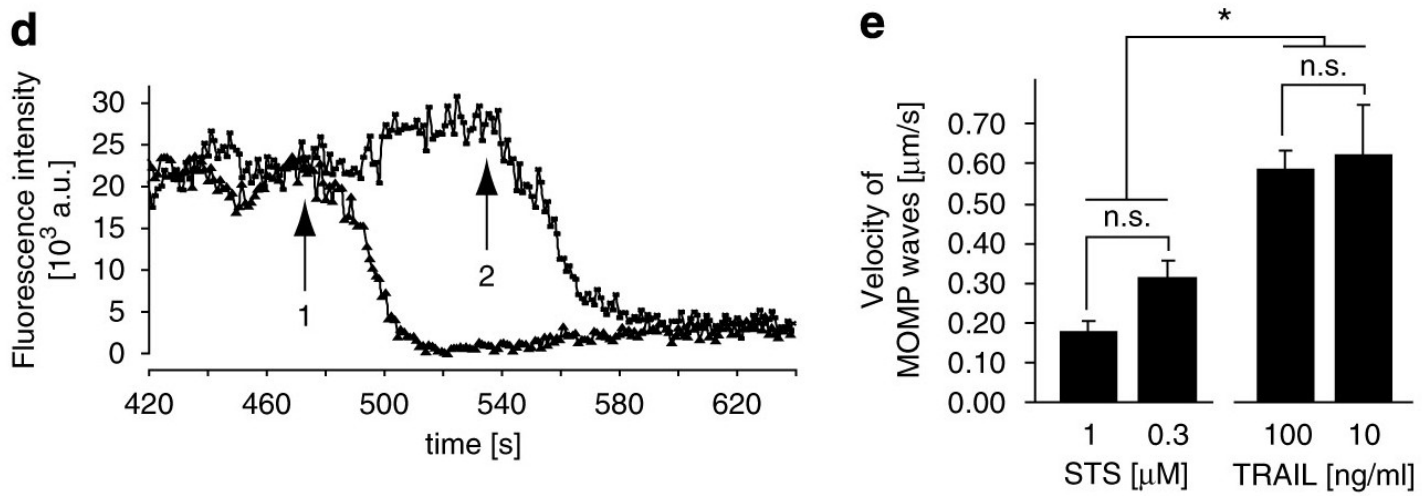
Rehm et al., Cell Death Differ, 2009

End-to-end velocities of mitochondrial permeabilisation waves



- Waves of permeabilisation spread faster during extrinsic vs. intrinsic signalling

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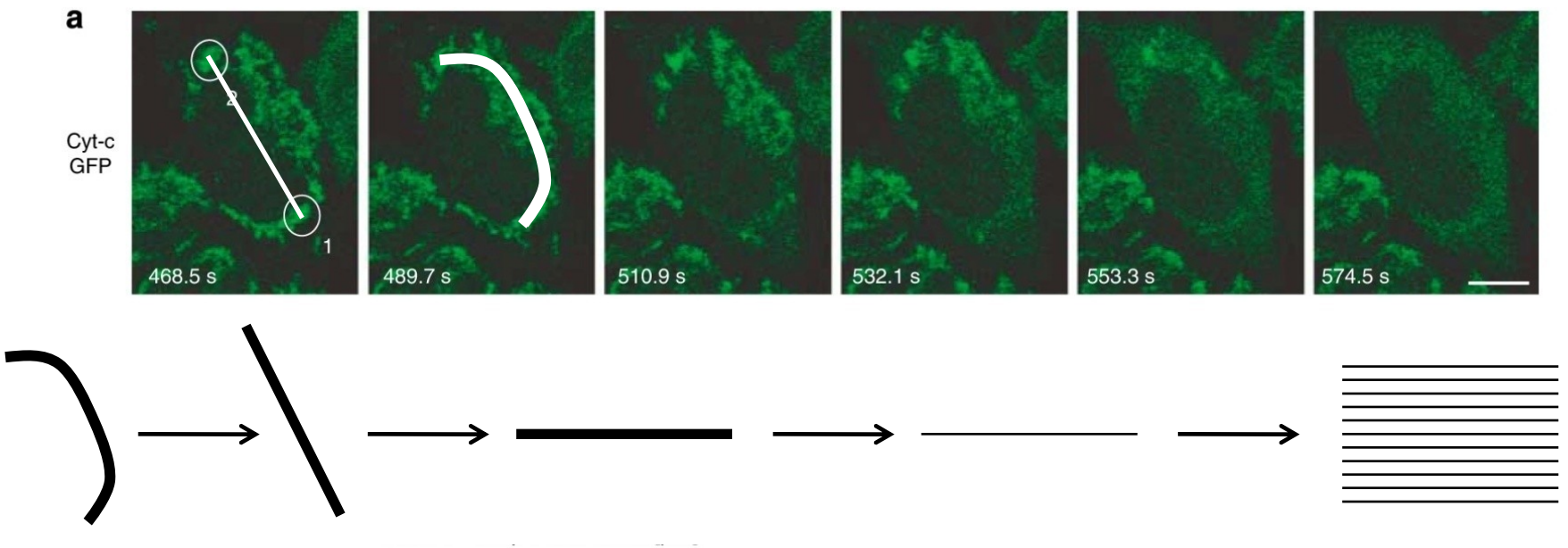
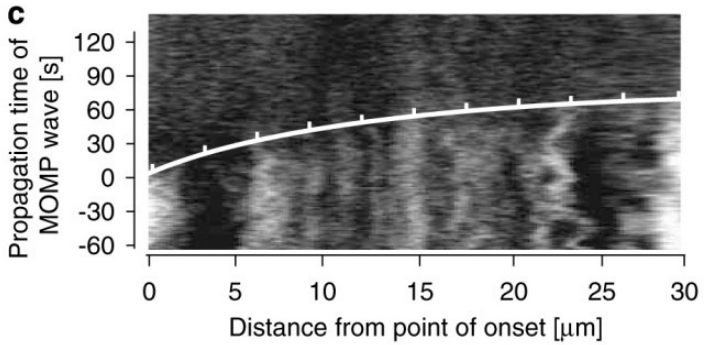
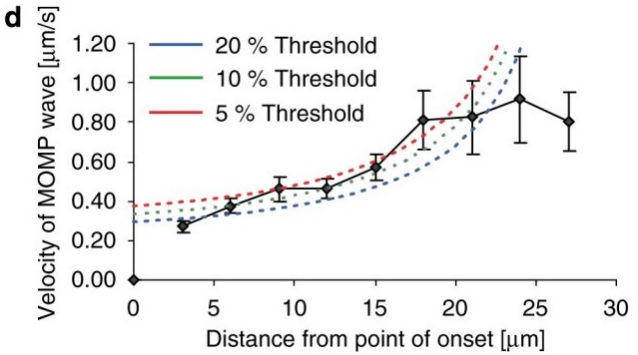
Rehm et al. (Cell Death Differ., 2009)

Characteristics of subcellular spread of the signal







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Rehm et al. (Cell Death Differ., 2009)

- Added value and benefits from (timelapse) imaging

- Image acquisition and processing

- Examples for imaging applications in cell death research

Web search: Confocal Microscopy List

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EU FP7 APO-SYS

National Development Plan/HEA

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