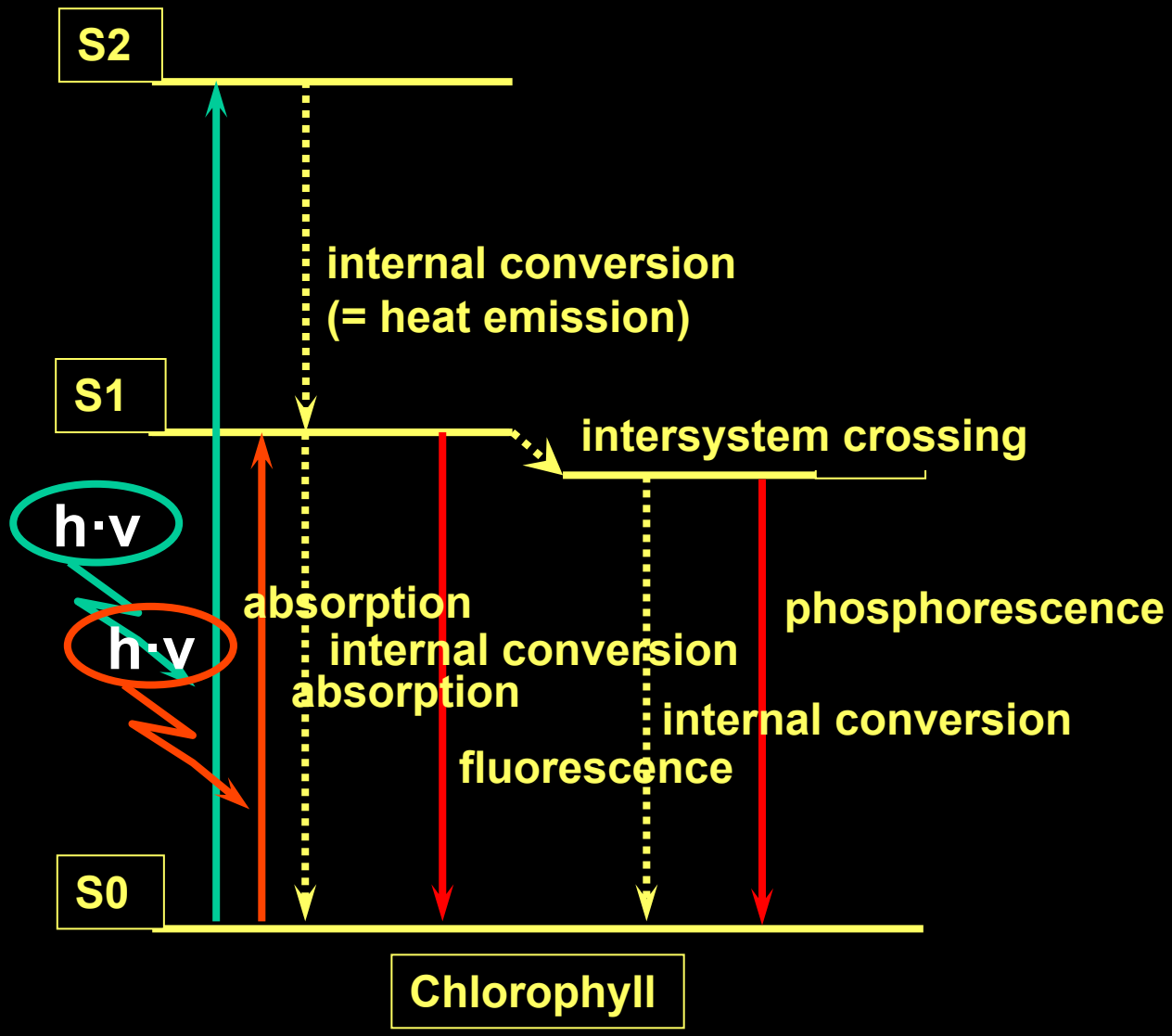
Biophysical and physicochemical methods for analyzing plants *in vivo* and *in situ*: UV/VIS-Spectroscopy from pigment analysis to quantification of mRNA

(3) UV/VIS fluorescence
→ Principle, example: Chlorophyll

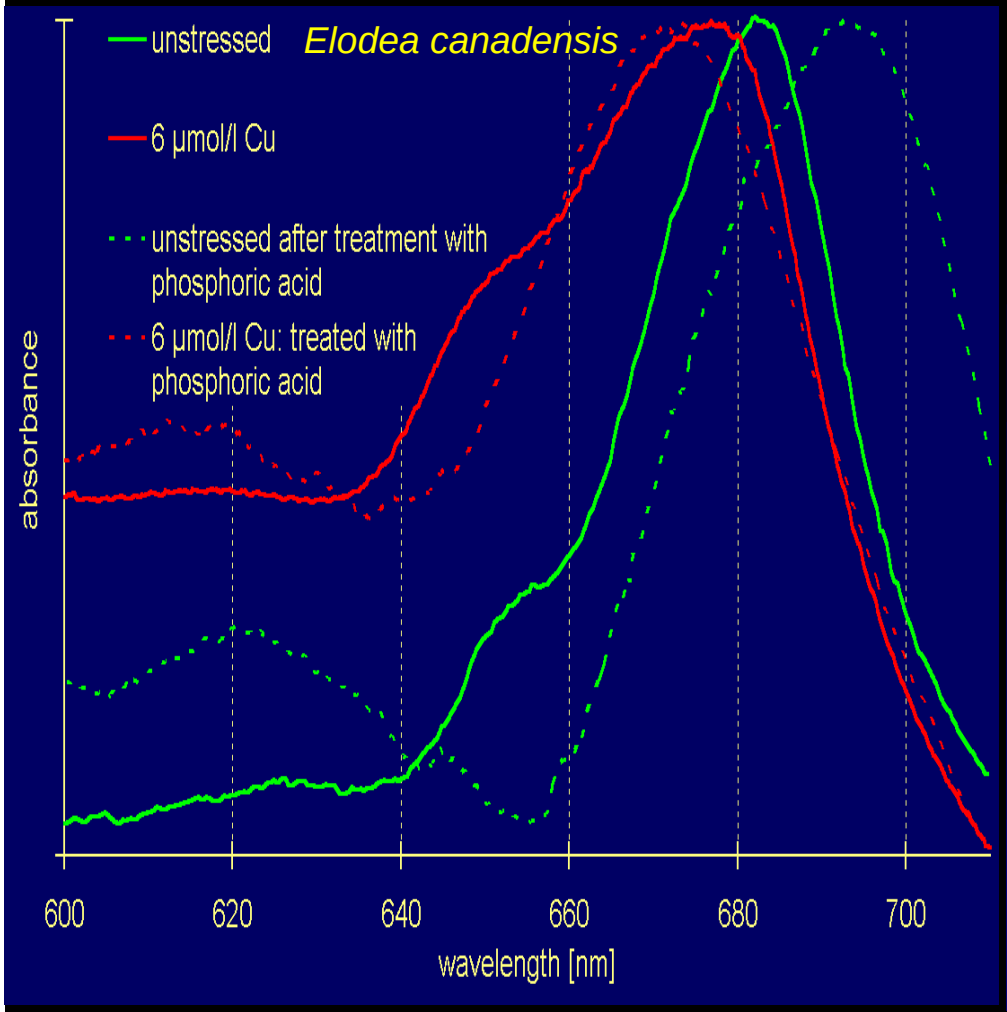


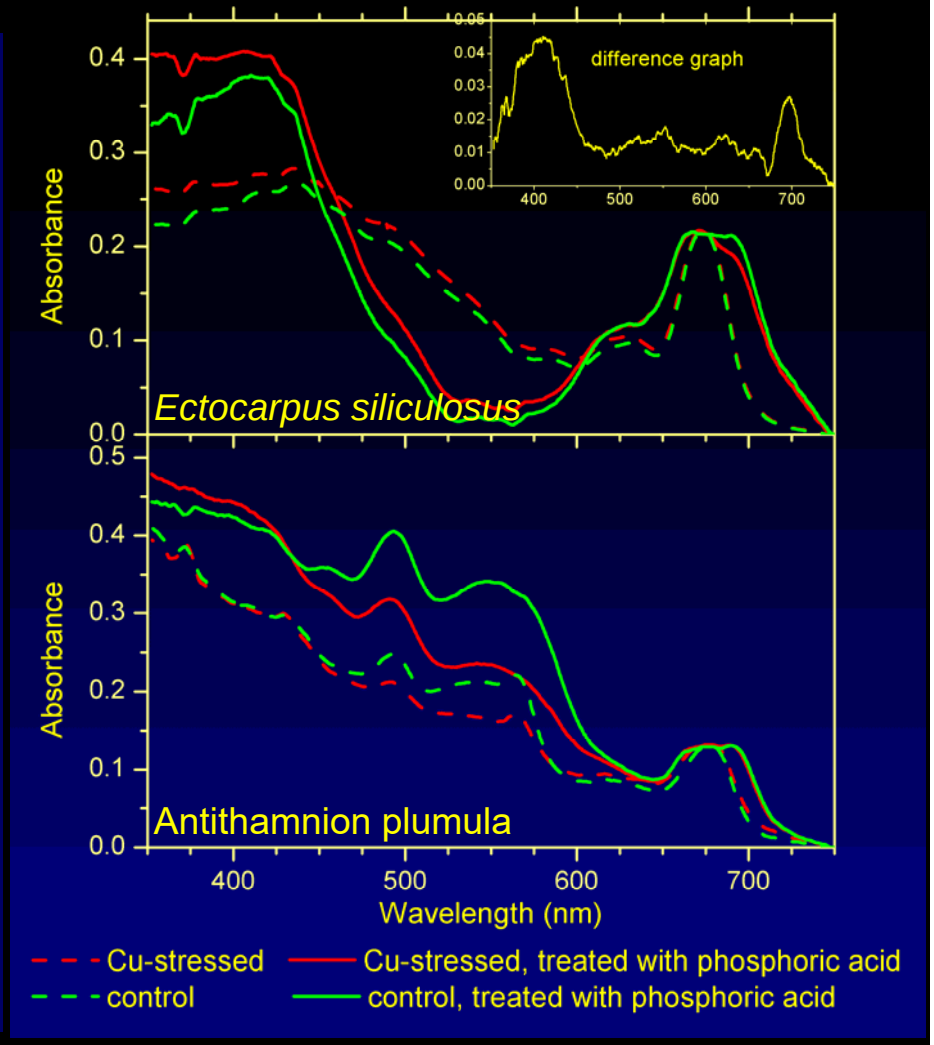
Measurement of *in vivo* / *in situ*-UV/VIS-Spectra (non-imaging)

Why *in vivo* / *in situ*?
--> direct correlation with physiological parameters possible
--> no extraction artefacts
--> measurement on single cells possible
--> high time resolution when measuring kinetics

Disadvantages compared to measurements of extracts:
--> many overlapping bands of the same pigment due to protein binding
--> bands very broad
--> extinctions coefficients *in vivo* usually unknown --> usually no absolute quantification

Example of the Application of *in vivo*-Absorption Spectra:
Formation of Cu-Chl during Cu- stress

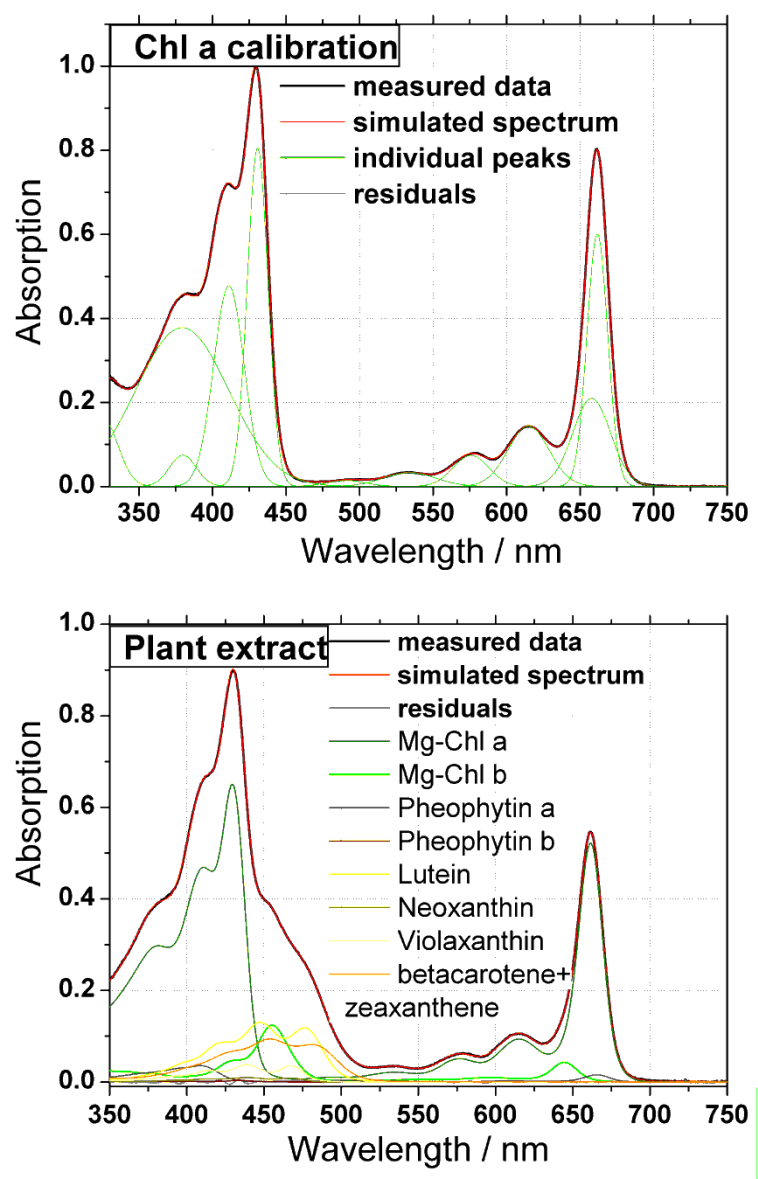




Küpper H, Küpper F, Spiller M (1998) Photosynthesis Research 58, 125-33

Küpper H, Šetlík I, Spiller M, Küpper FC, Prášil O (2002) Journal of Phycology 38(3), 429-441

Pigment Quantification in Extracts: Modern UV/VIS-Spectroscopic Method



Principle:

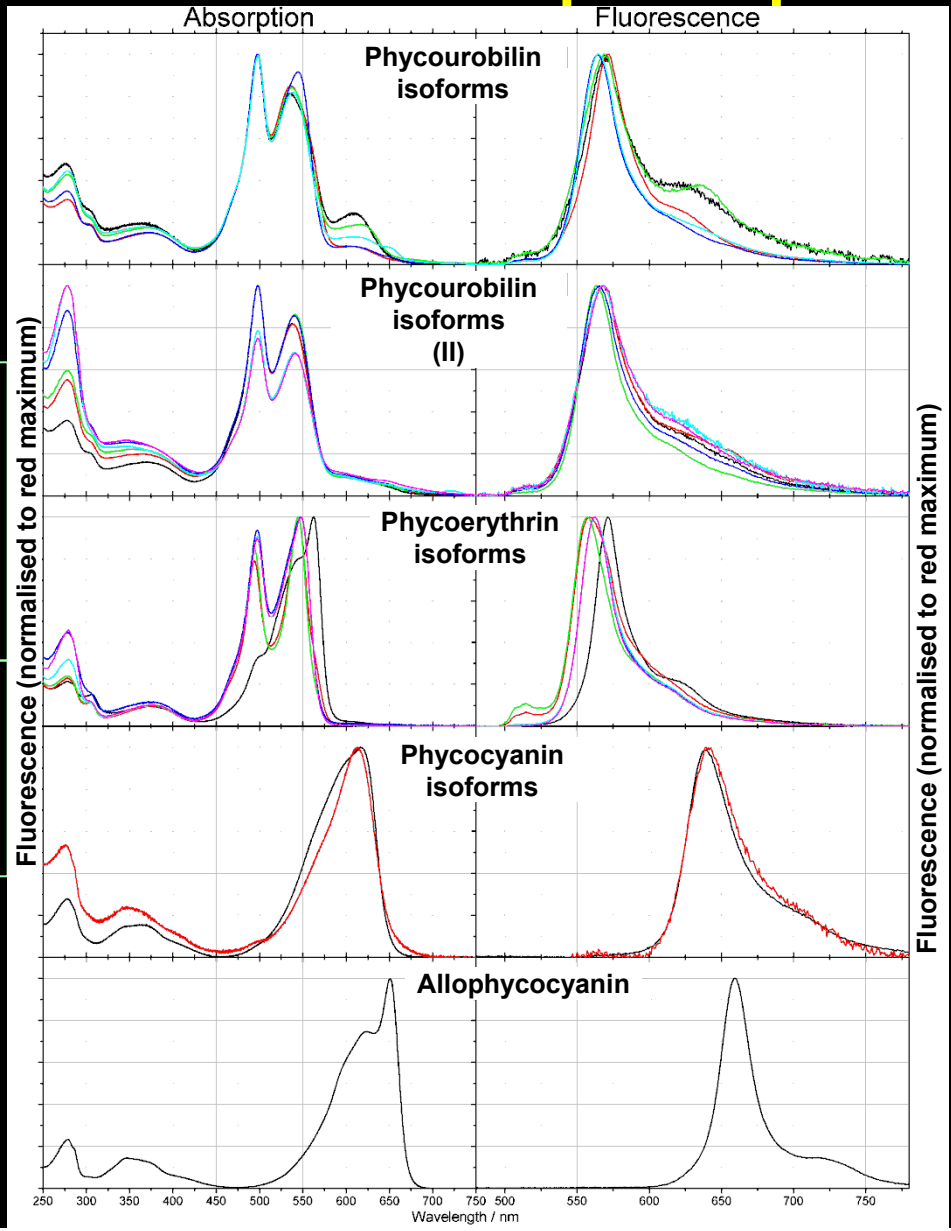
1) UV/VIS-Spectra are transferred into mathematic equations, so-called “GPS spectra” (published database currently contains 54 absorption spectra and 16 fluorescence spectra).
2) Before extraction, tissues/cells are frozen in liquid nitrogen and then freeze-dried. Afterwards, pigments are extracted in 100% acetone (for phycobiliprotein extraction from cyanobacteria, this step is followed by re-drying and extraction in 1x PBS).
3) A sum of the GPS spectra is then fitted to the measured spectrum of the extract. This fitting includes an automatic correction of base line drift and wavelength inaccuracy of the spectrometer as well a residual turbidity and water content of the sample.

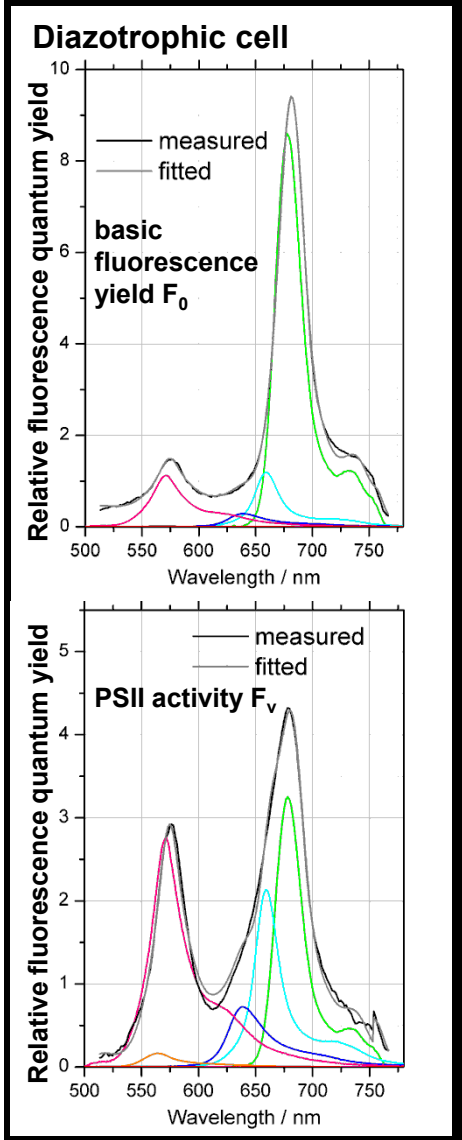
Method of deconvolution: Küpper H, Seibert S, Aravind P (2007) Analytical Chemistry 79, 7611-7627

Purification of *Trichodesmium* phycobiliproteins for deconvoluting spectrally resolved *in vivo* fluorescence kinetics and absorption spectra

Phycobiliprotein purification + characterisation: Küpper H, Andresen E, Wiegert S, Šimek M, Leitenmaier B, Šetlík I (2009) Biochim. Biophys. Acta (Bioenergetics) 1787, 155-167

Method of deconvolution: Küpper H, Seibert S, Aravind P (2007) Analytical Chemistry 79, 7611-7627



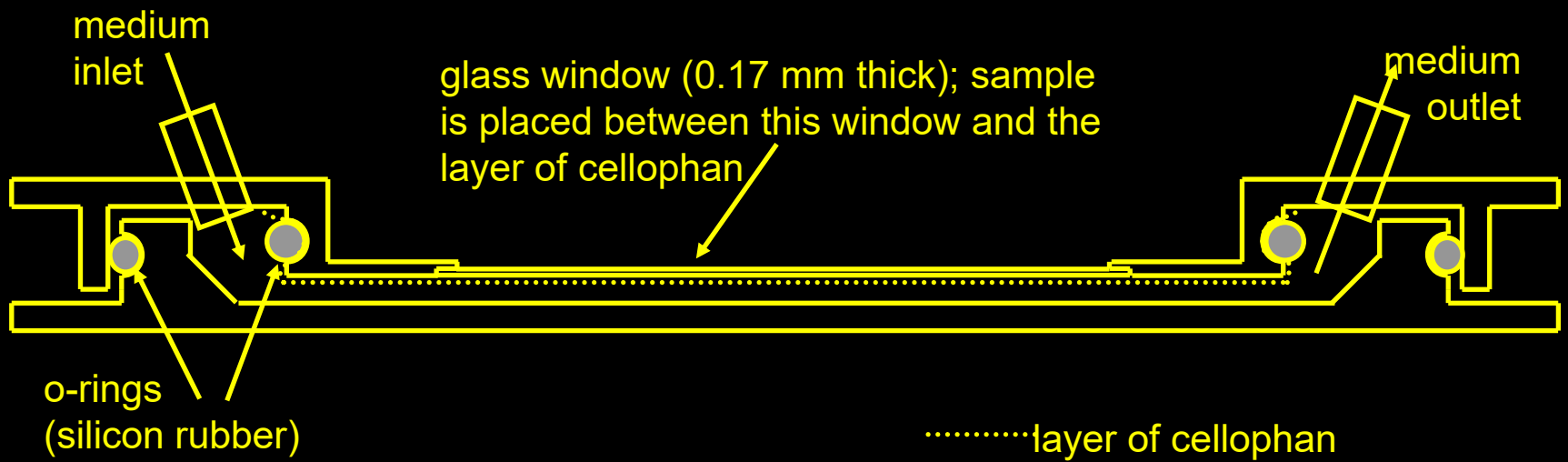


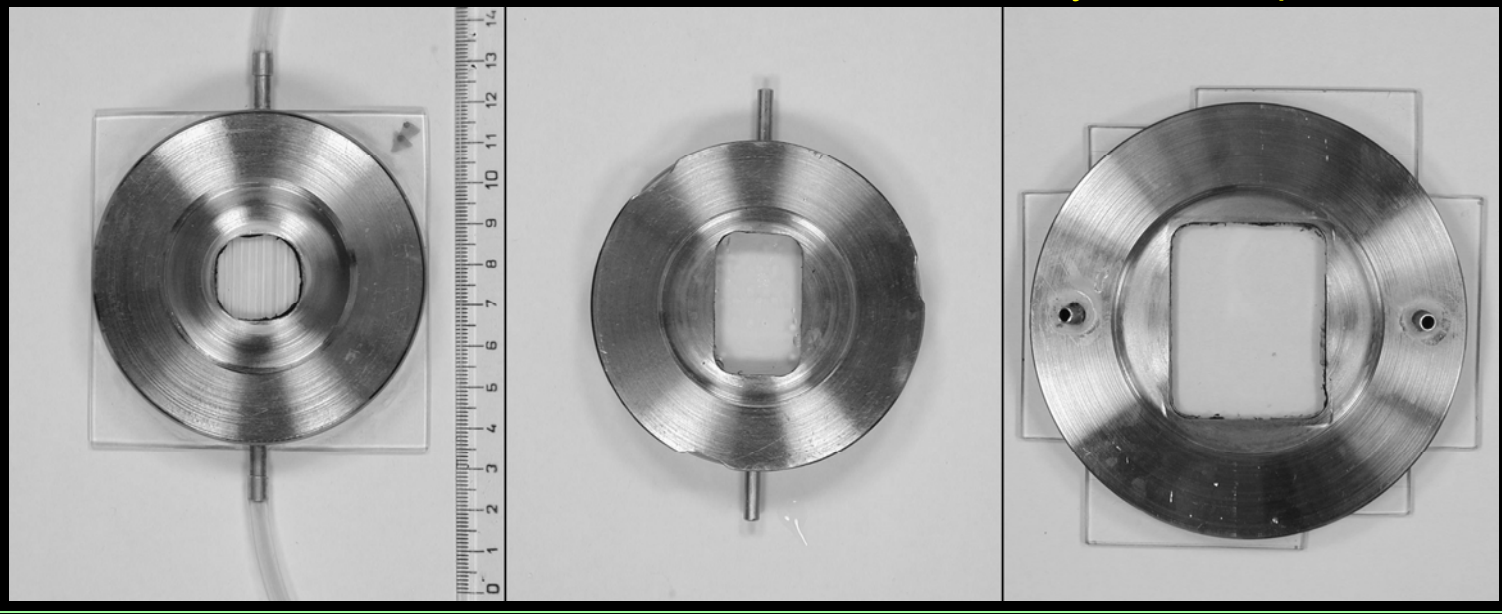
Imaging *in vivo*-VIS-Spectroscopy:
Modern Methods of Fluorescence Microscopy

Important prereqisites and facts
→ how to keep your cells alive while being measured
→ aperture vs. light capture efficiency
→ correct measurement
→ overlap / interference of signals

Methods
--> separation of chromophores
--> FRET
--> measurement of physiological parameters with fluorescent dyes
--> FRAP
--> FCS
--> QISH
--> fluorescent proteins

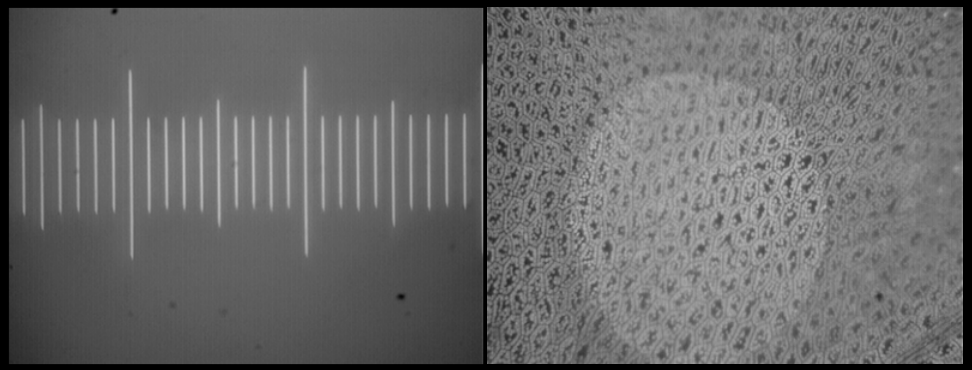
Decisive for measuring LIVING cells: keep the sample in physiological conditions!





Küpper H, Šetlík I, Trtilek M, Nedbal L (2000) Photosynthetica 38(4), 553-570

Decisive for measuring LIVING cells: don't apply too much light!



$$\text{irr} = (\text{irradiance of } 6.3\times\text{objective}) \cdot \frac{\dfrac{(\text{radiation throughput of tested objective})}{(\text{irrad. field of tested objective})}}{\dfrac{(\text{radiation throughput of } 6.3\times\text{objective})}{(\text{irrad. field of } 6.3\times\text{objective})}}$$

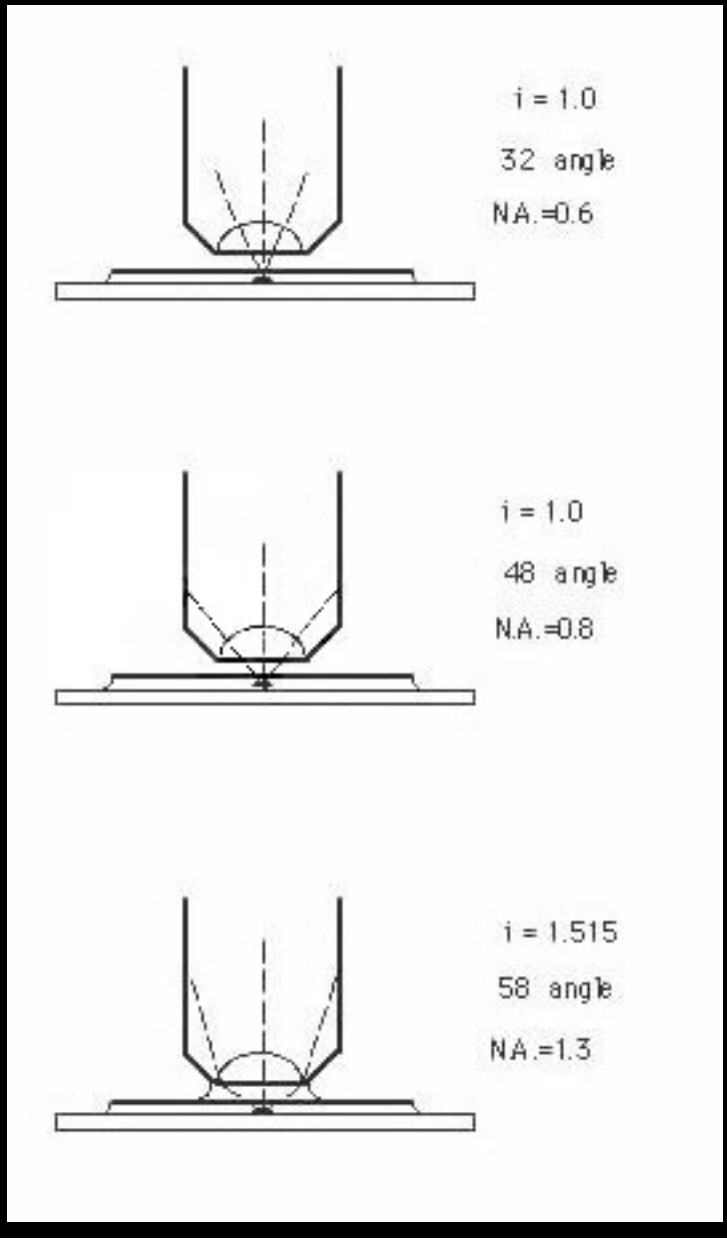
Lens	Light field diameter [mm]	Measuring irradiance [µmol m^{-2} s^{-1}]	Actinic irradiance [µmol m^{-2} s^{-1}]	Saturating irradiance [µmol m^{-2} s^{-1}]
6.3×/0.20	2.90	0.006	686	524
16×/0.40	1.06	0.026	2835	2167
25×/0.63	0.67	0.075	8295	6332
40×/0.95	0.38	0.200	22058	16904
63×/0.95	0.23	0.270	30311	23218
100×/1.30	0.16	0.270	29441	22546

Küpper H, Šetlík I, Trtilek M, Nedbal L (2000) Photosynthetica 38(4), 553-570

Aperture

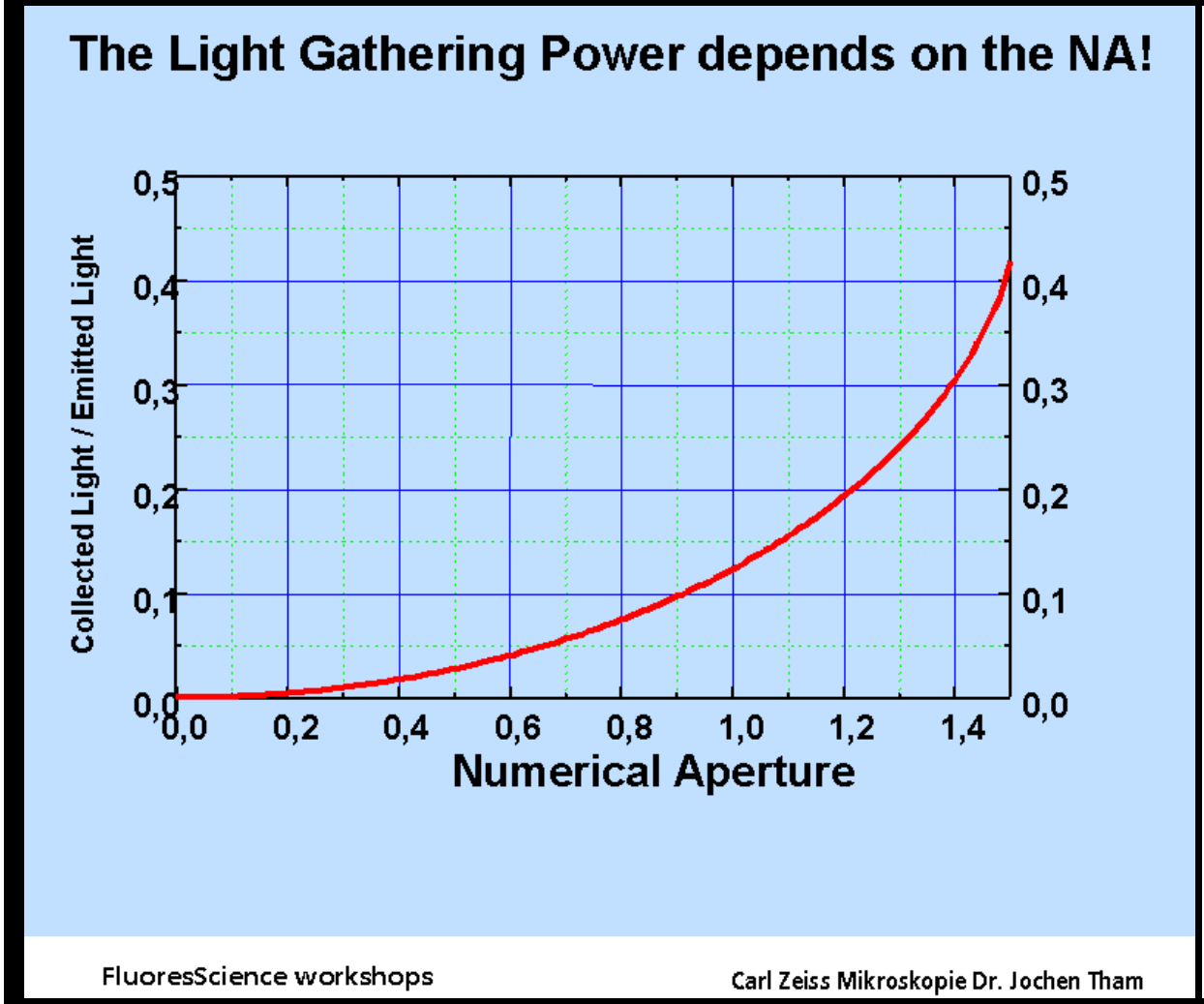
numerical Aperture NA = I * sinus q

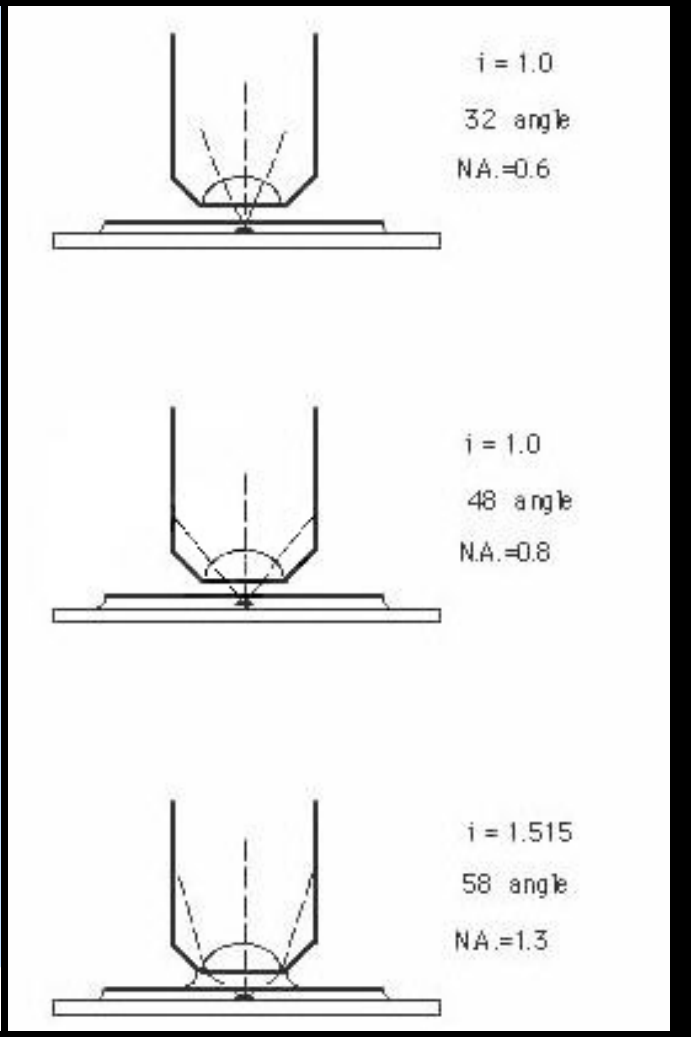
I = refractive index of the medium
q = half opening angle of the objective



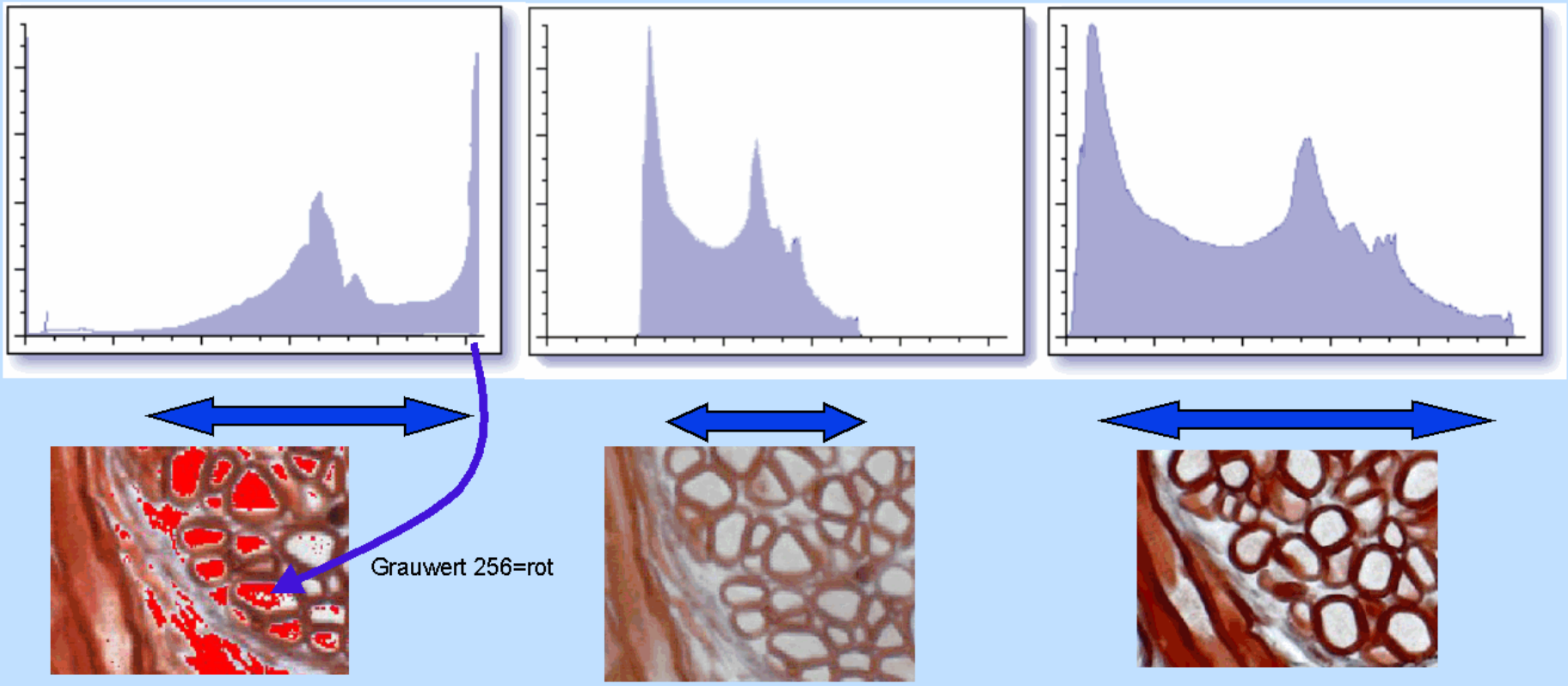
From: Zeiss workshop

Decisive for measuring LIVING cells: in order to be able to work with low light: choose a suitable objective

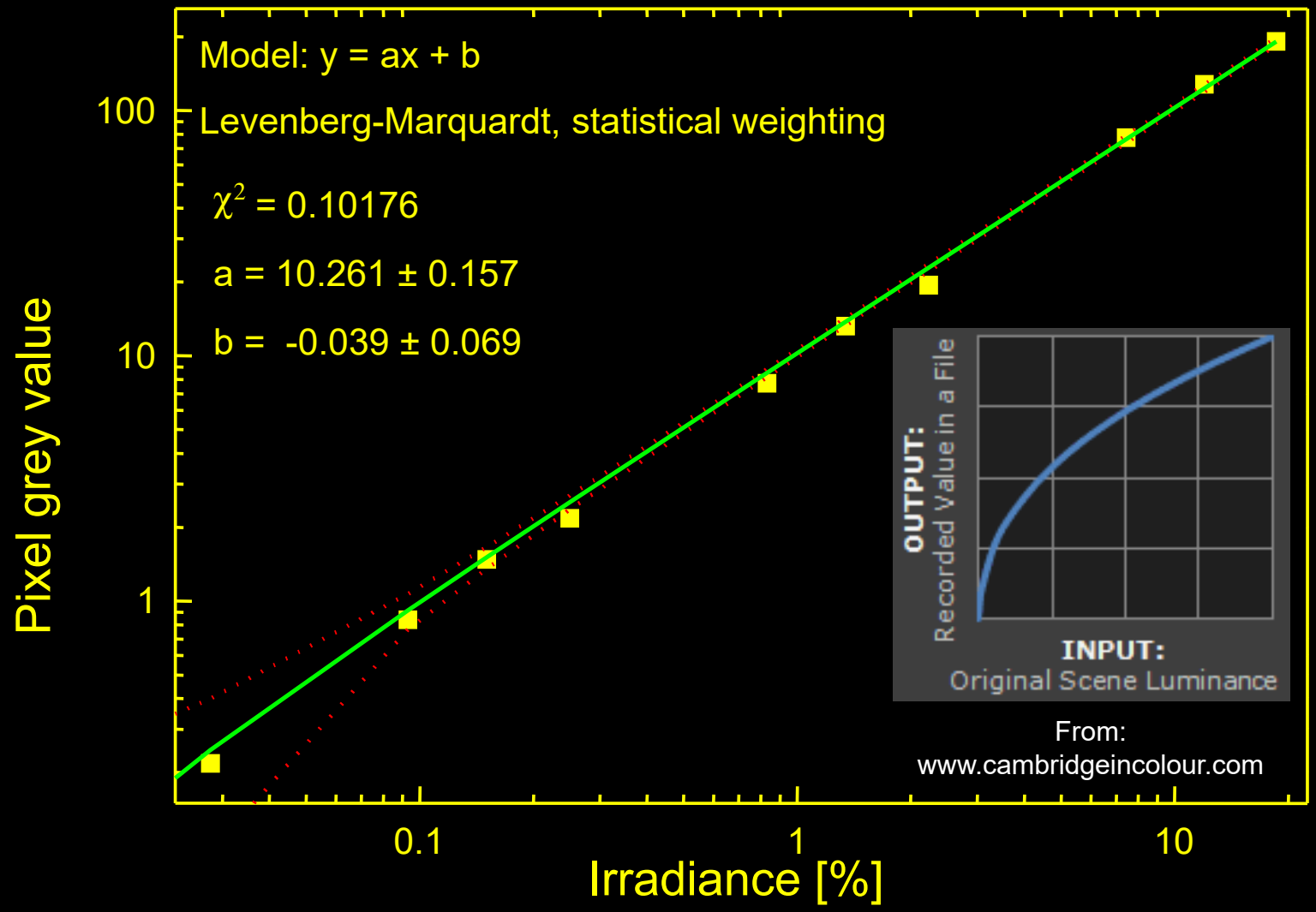




Decisive for quantification: don’t overexpose!

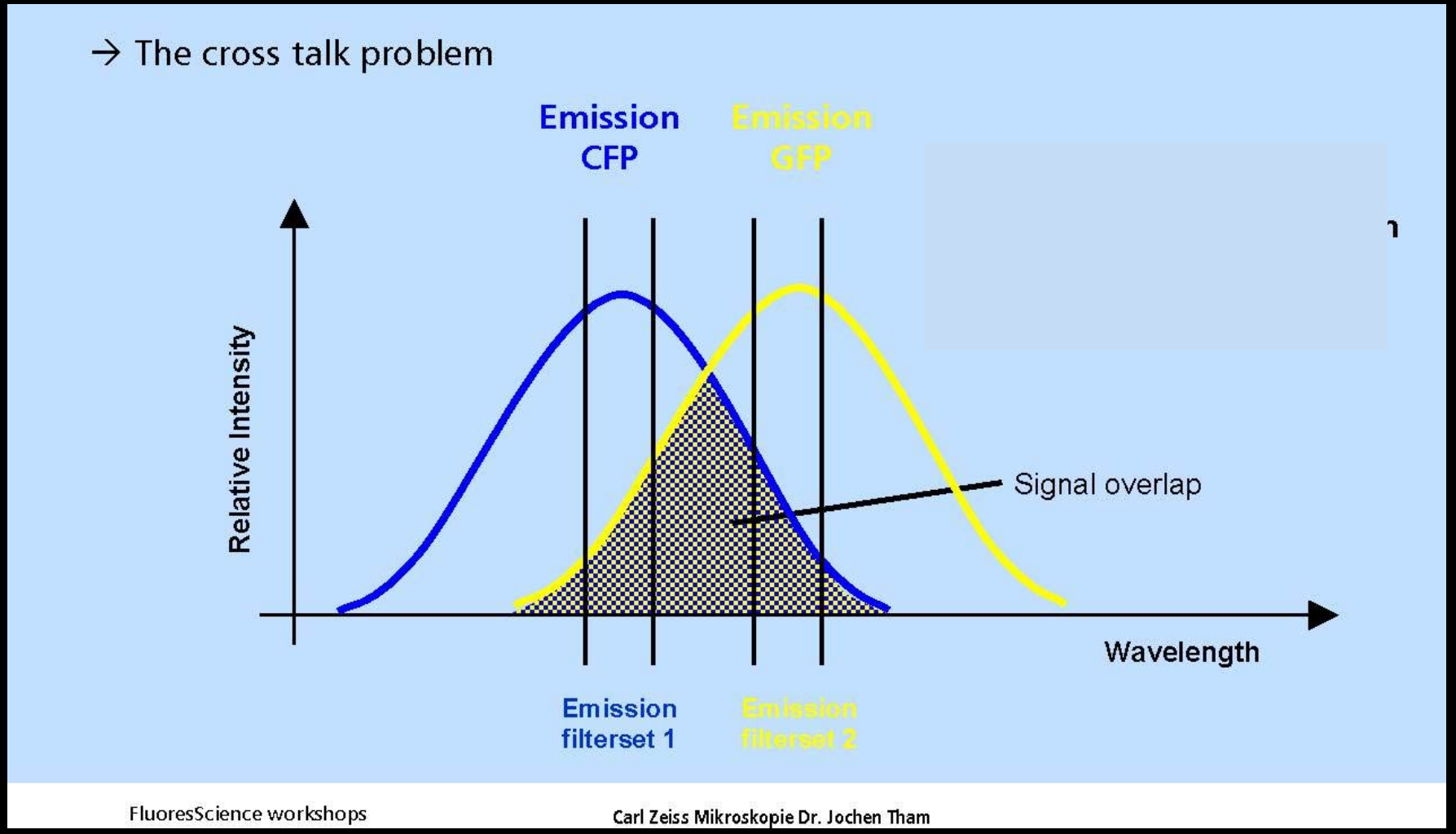


Decisive for quantification: correct calibration of the detector

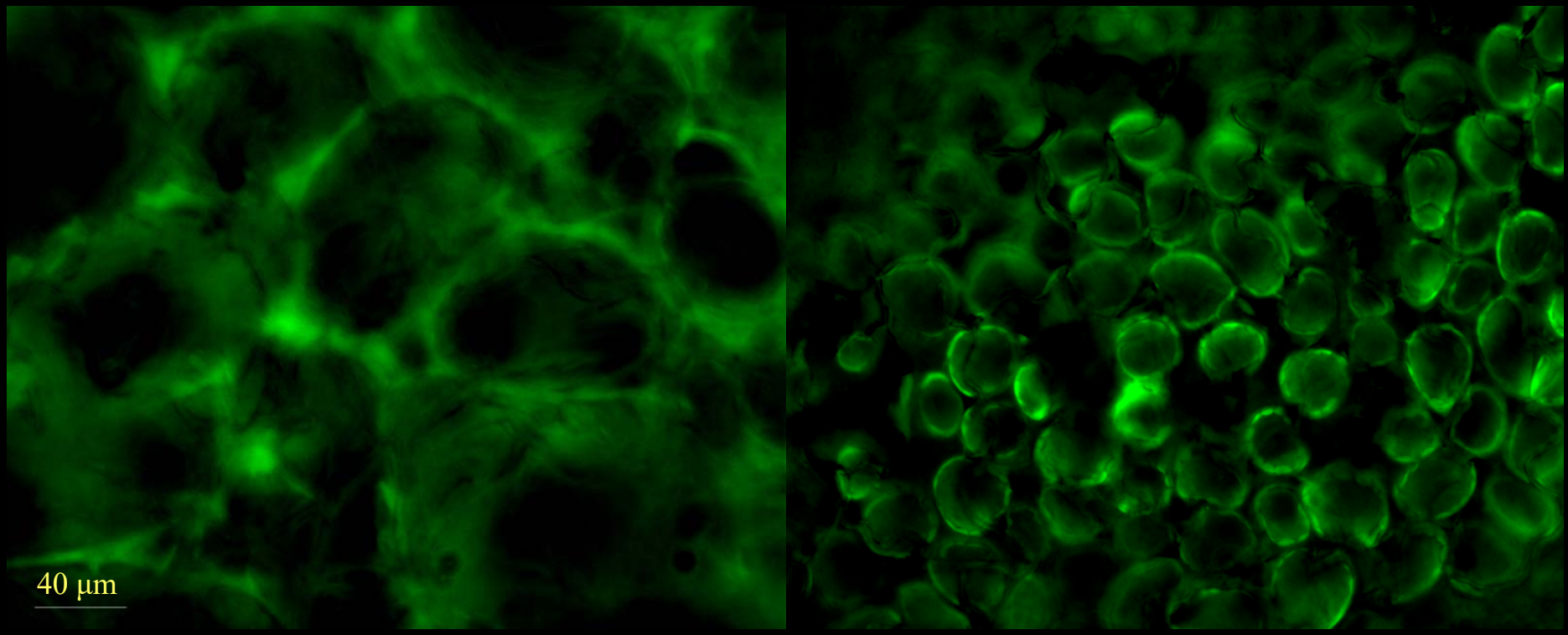


Küpper H, Šetlík I, Trtilek M, Nedbal L (2000) Photosynthetica 38(4), 553-570

Important for interpreting fluorescence signals: Overlap of absorption / emission bands



Preliminary tests with GFP in young leaves of *Arabidopsis thaliana*



Epidermis

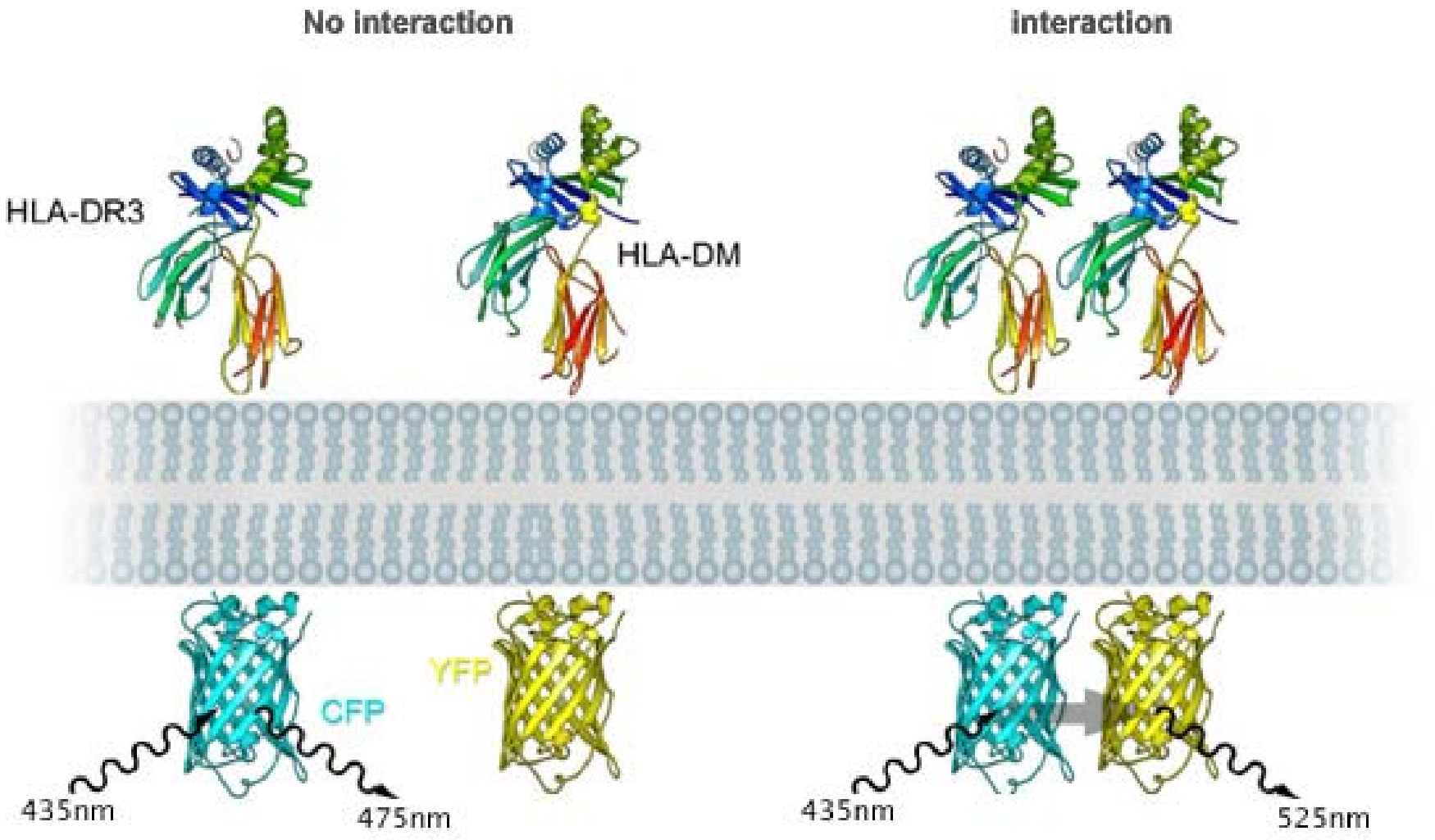
Mesophyll

Fluorescence observed through GFP filterset

NON-transformed plant...

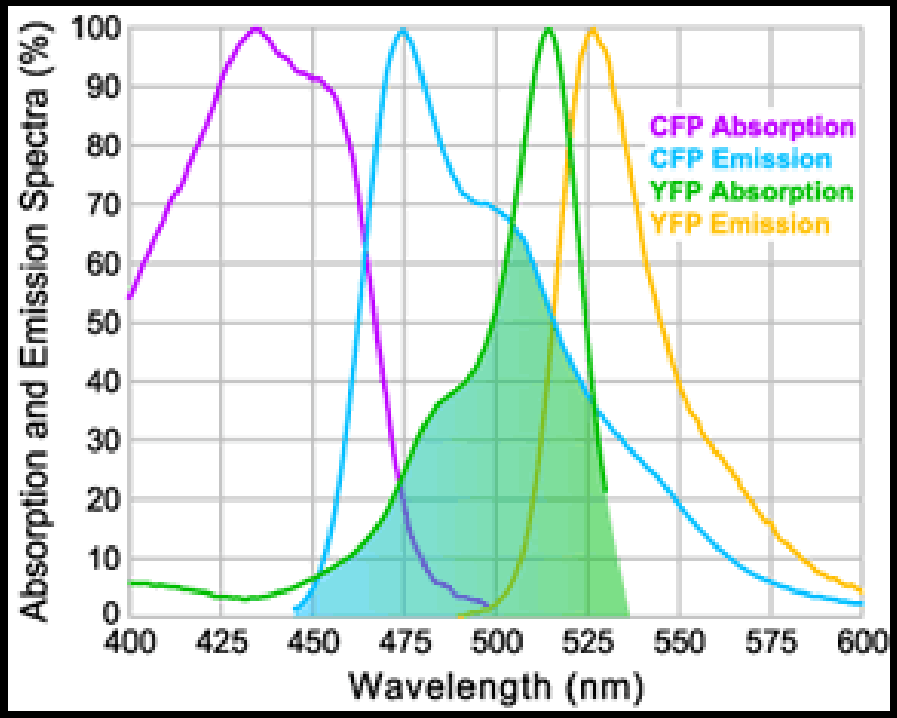
All the signal was AUTOFLUORESCENCE

Use of overlapping Abs/Em-Bands for FluorescenceResonanceEnergyTransfer (FRET)

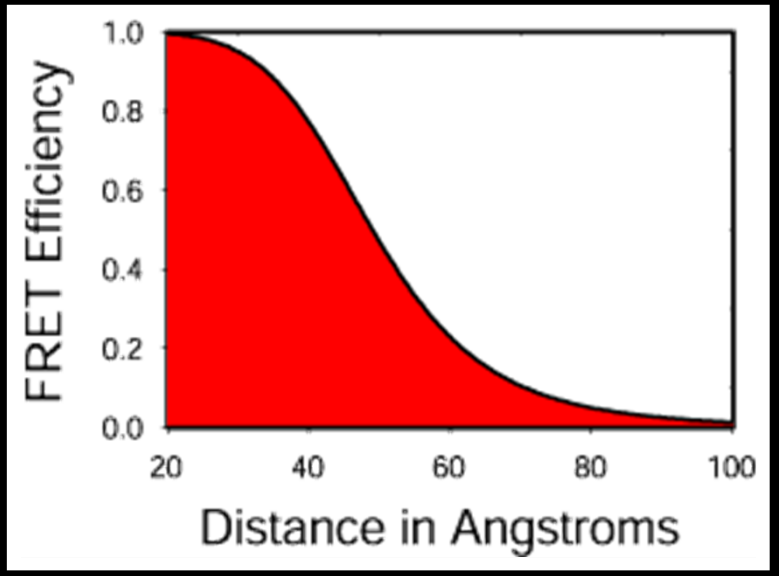


From: Zeiss workshop

Prerequisites for FluorescenceResonanceEnergyTransfer (FRET)

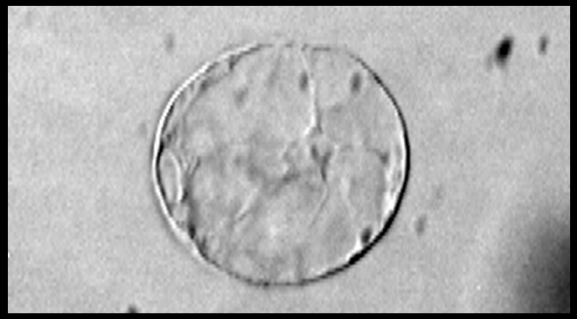


$$Eff = \frac{R_o^6}{\left(R_o^6 + r^6\right)}$$



From: Zeiss workshop

(3) UV/VIS fluorescence of metal specific fluorescent dyes → Principle

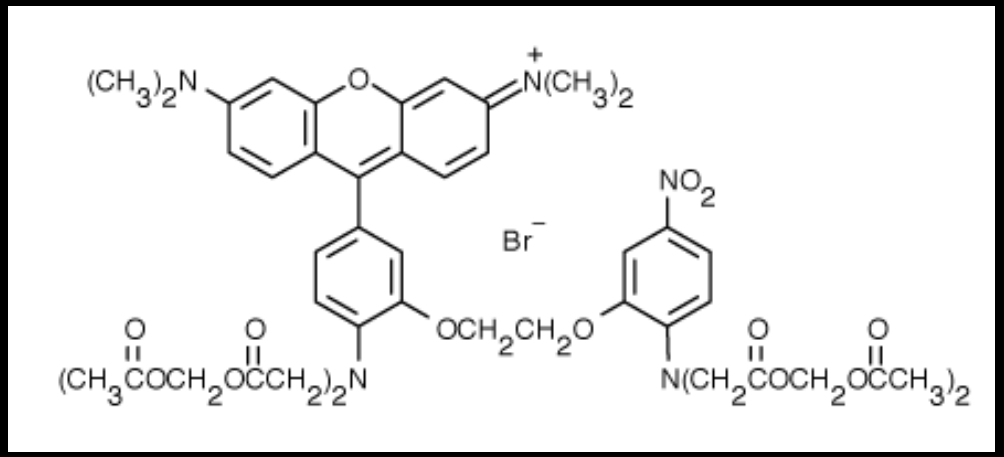


Transmitted light:

information about structure and cell type

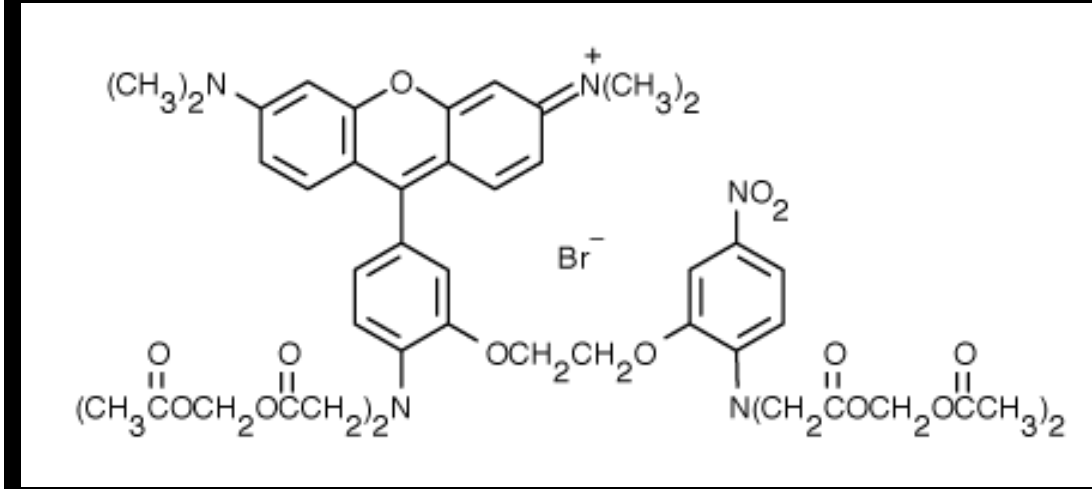
dye fluorescence:

metal measurement



Leitenmaier B, Küpper H, (2011) Plant Cell and Environment 34, 208-219

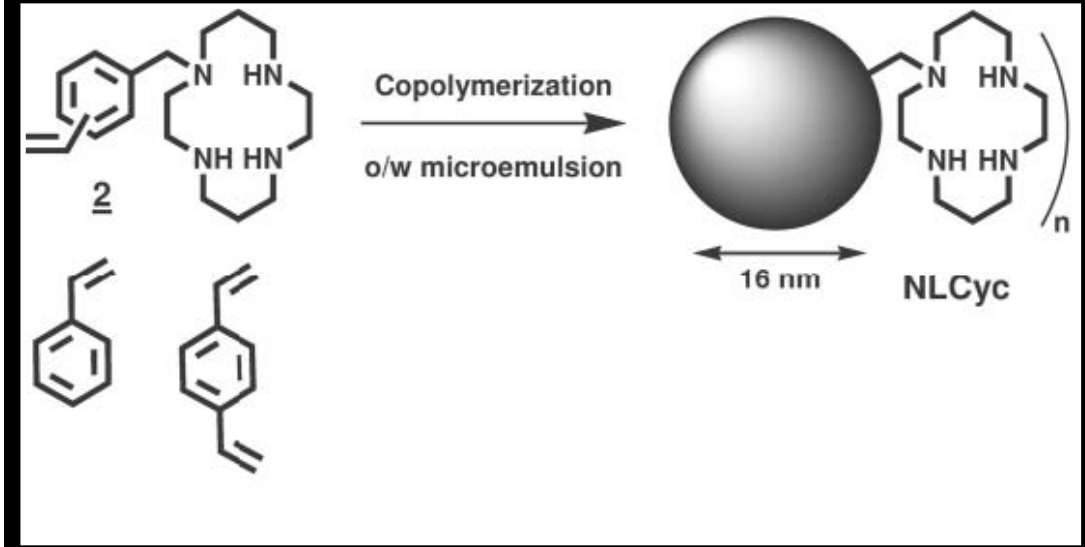
(3) UV/VIS fluorescence of metal specific fluorescent dyes → Types of dyes



From: www.Invitrogen.com

organic dyes

- Already available for many metals with many different binding and fluorescence characteristics
- Many dyes cell permeable

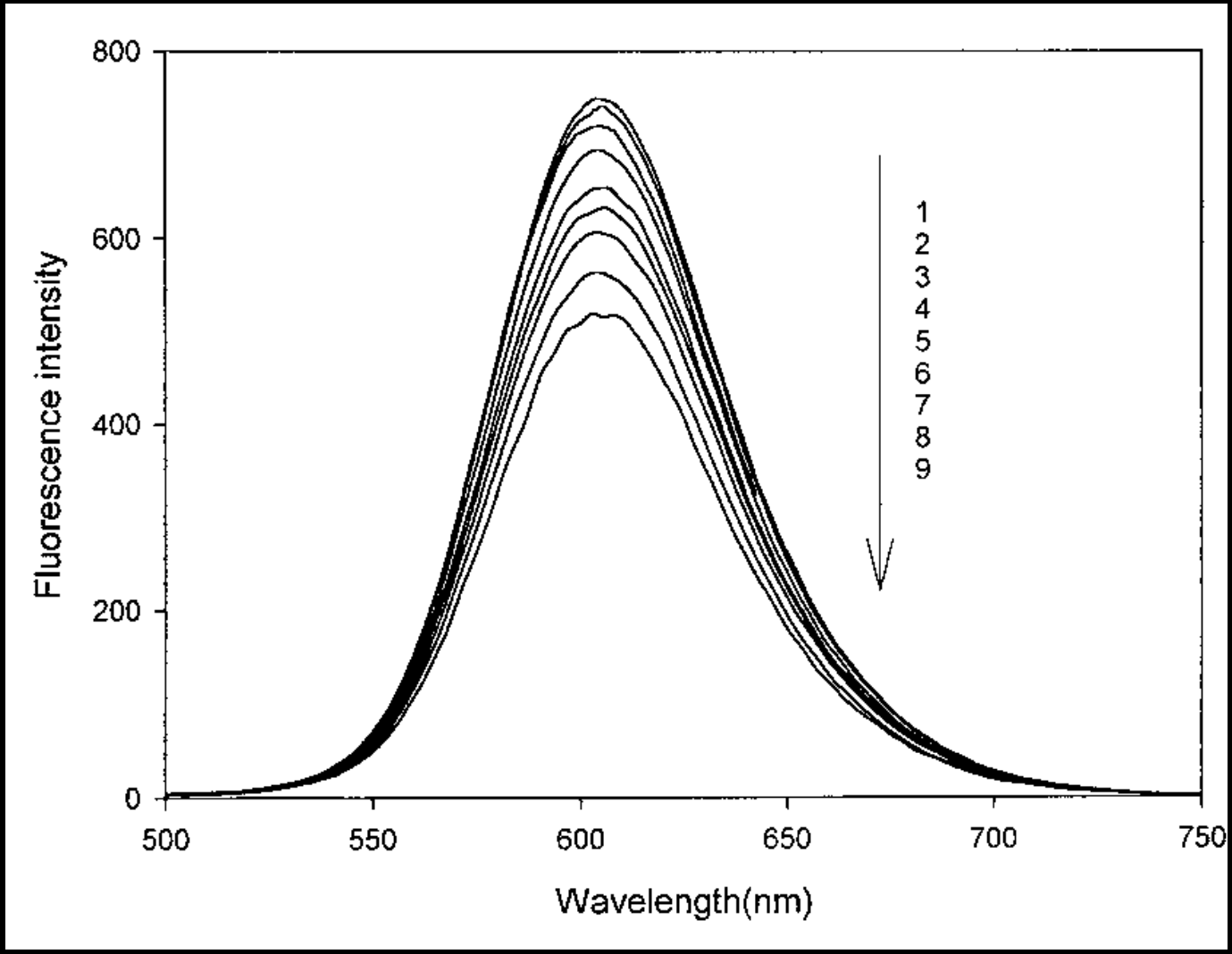


From: Méallet-Renault R, Hérault A, Vachon JJ, Pansu RB, Amigoni-Gerbier S, Larpent C, 2006, PhotochemPhotobiolSci 5, 300 - 310

nanoparticles

- new development, reliability and applicability not yet shown
- So far not cell permeable

(3) UV/VIS fluorescence of metal specific fluorescent dyes → types of response

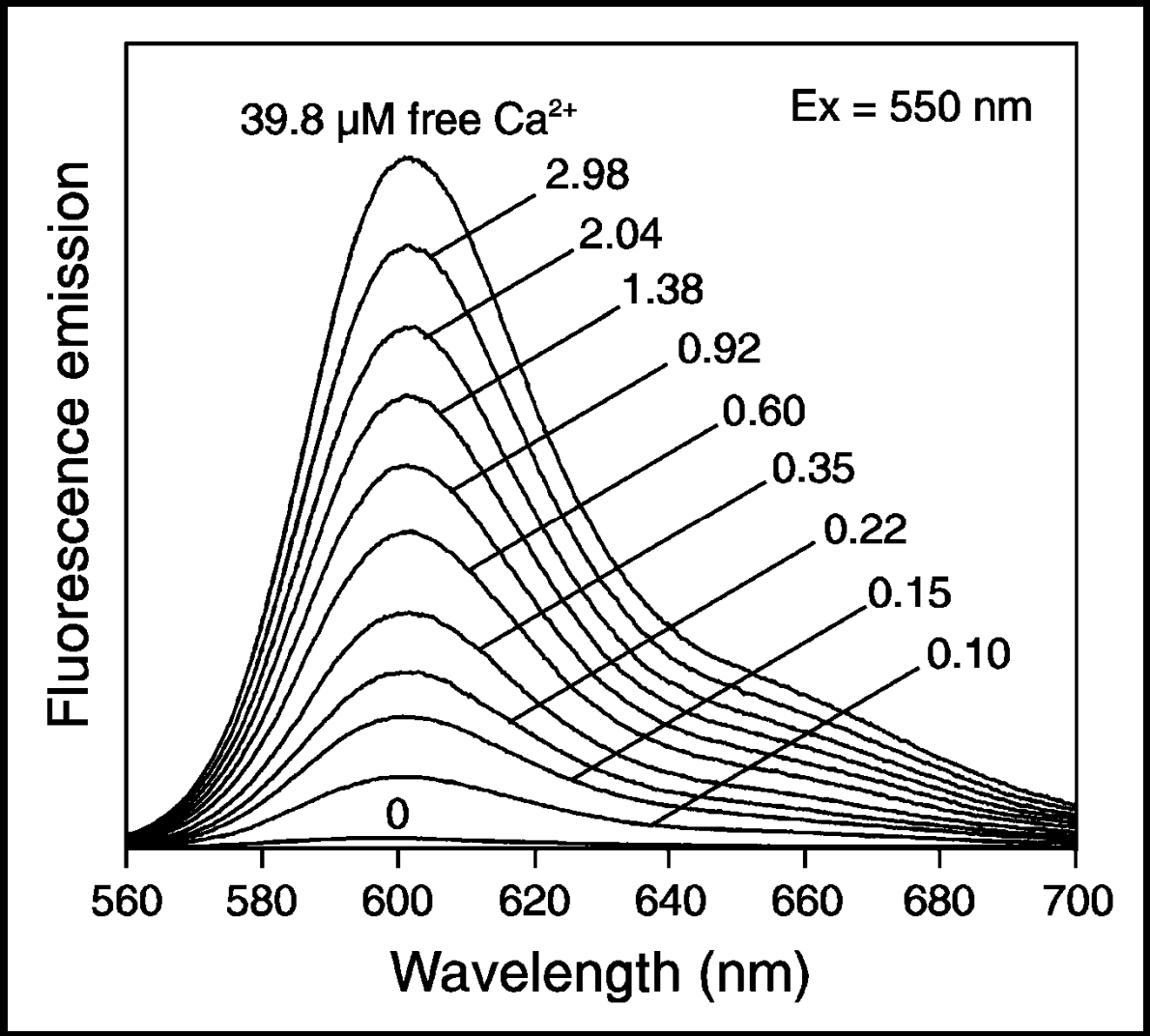


From: He CL et al., 2006, AnalytSci 22, 1547-

fluorescence quenching
constant absorption

From: www.Invitrogen.com

(3) UV/VIS fluorescence of metal specific fluorescent dyes → types of response

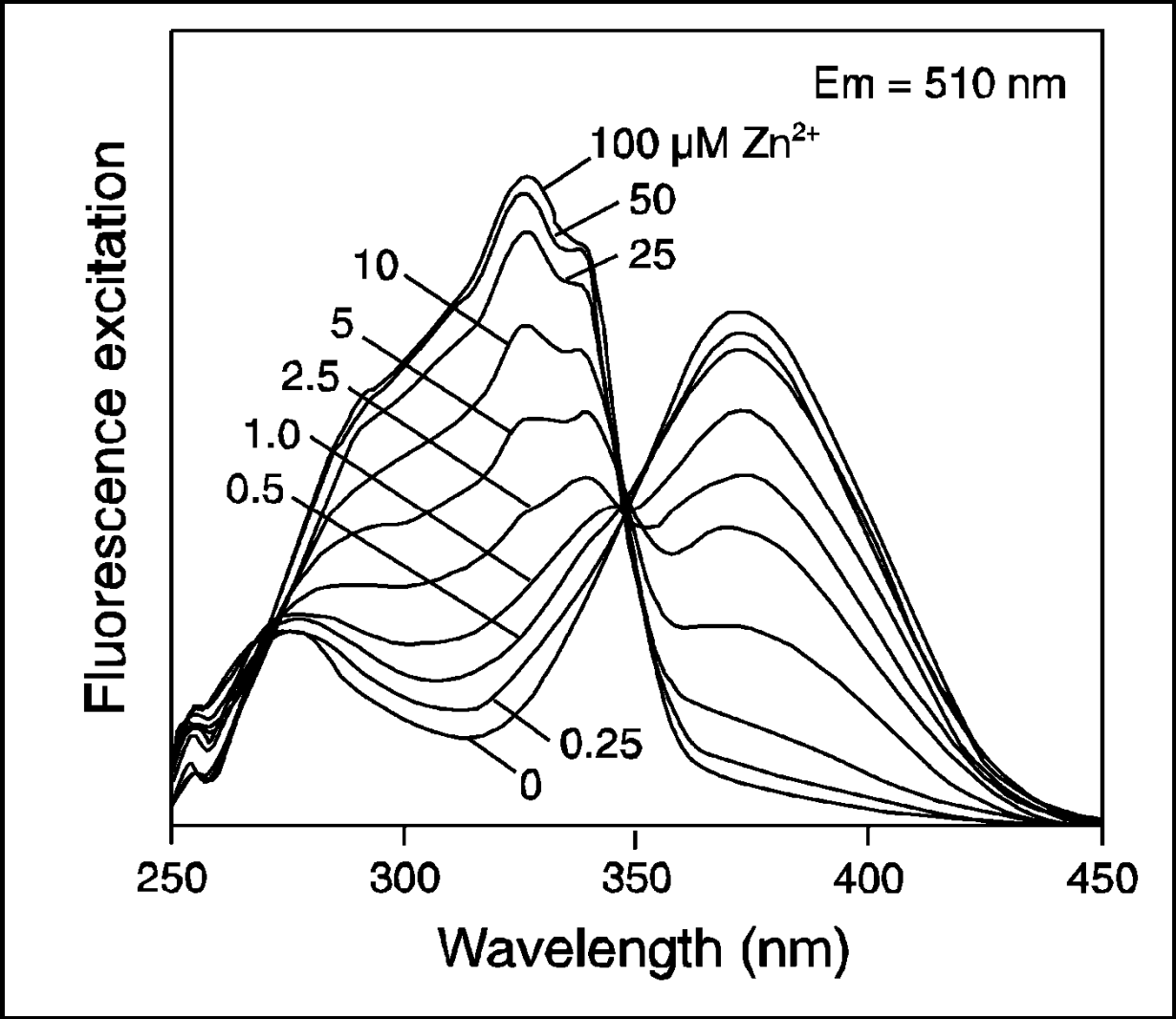


From: www.Invitrogen.com

fluorescence turn – on
constant absorption

From: www.Invitrogen.com

(3) UV/VIS fluorescence of metal specific fluorescent dyes → types of response

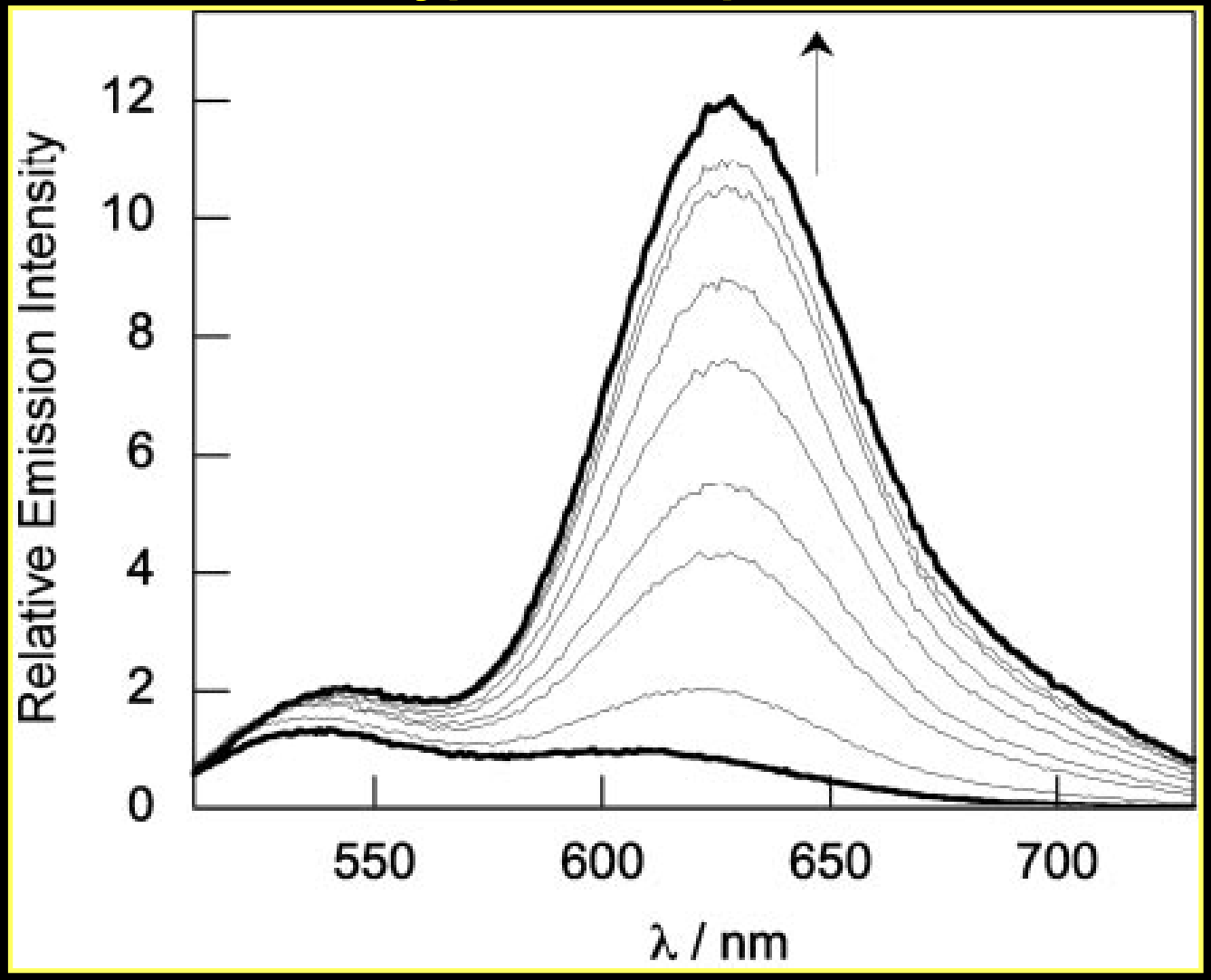


From: www.Invitrogen.com

fluorescence constant
ratiometric absorption

From: www.Invitrogen.com

(3) UV/VIS fluorescence of metal specific fluorescent dyes → types of response

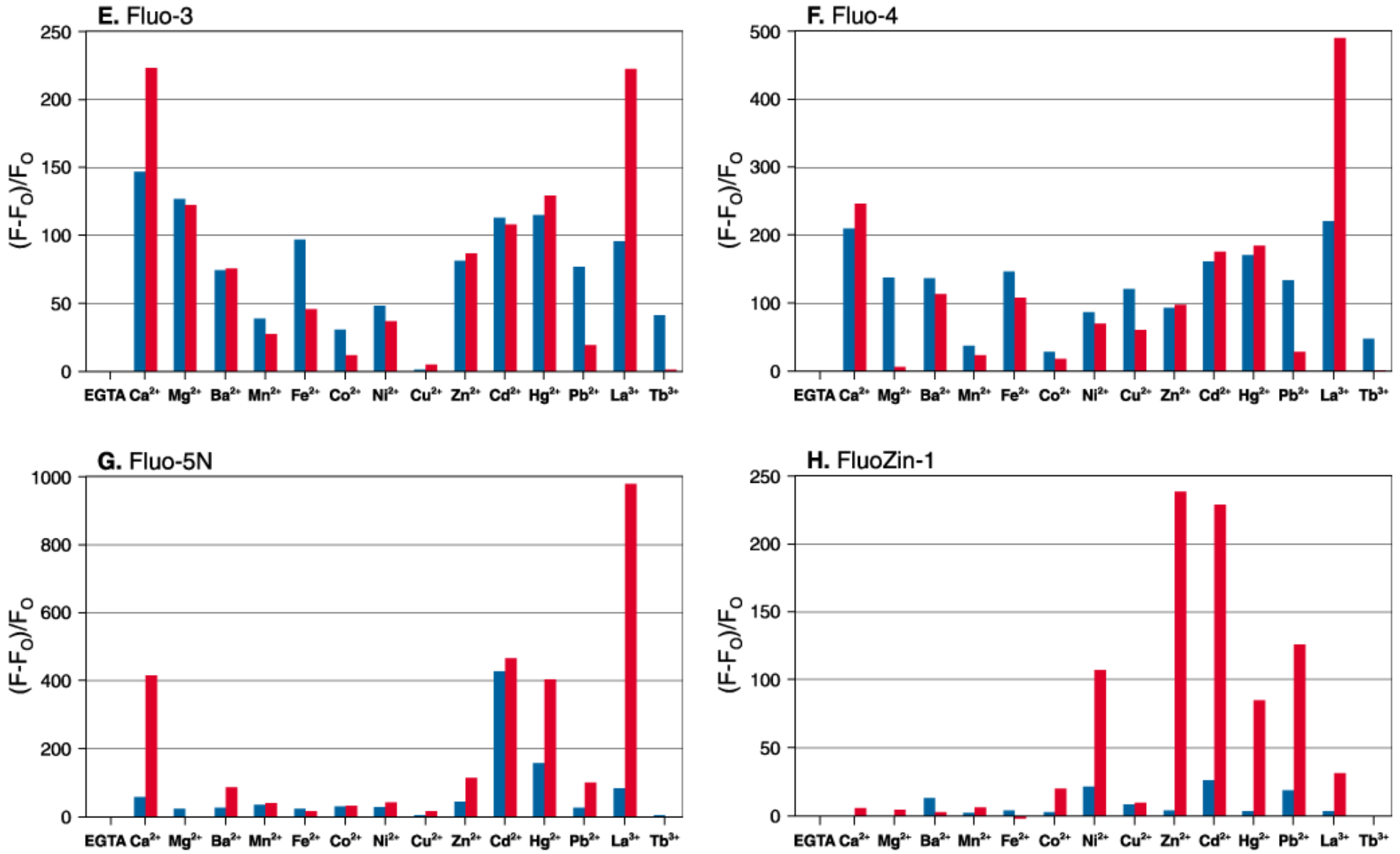


From: Chang CJ, Jaworski J, Nolan EM, Sheng M, Lippard SJ, 2004, PNAS101, 1129-34

ratiometric fluorescence
constant absorption

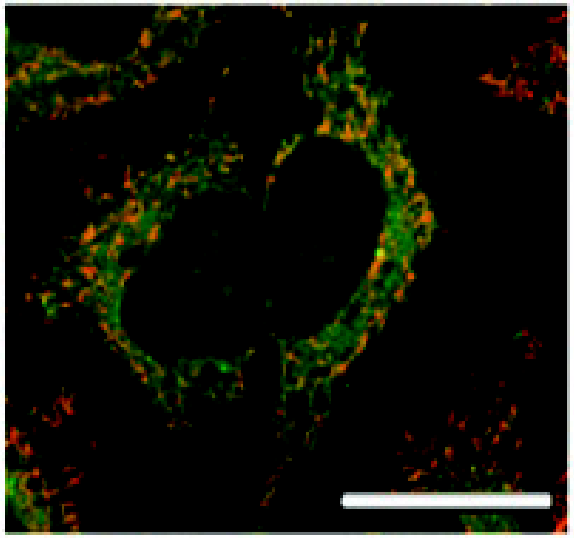
From: www.Invitrogen.com

(3) UV/VIS fluorescence of metal specific fluorescent dyes → specificity

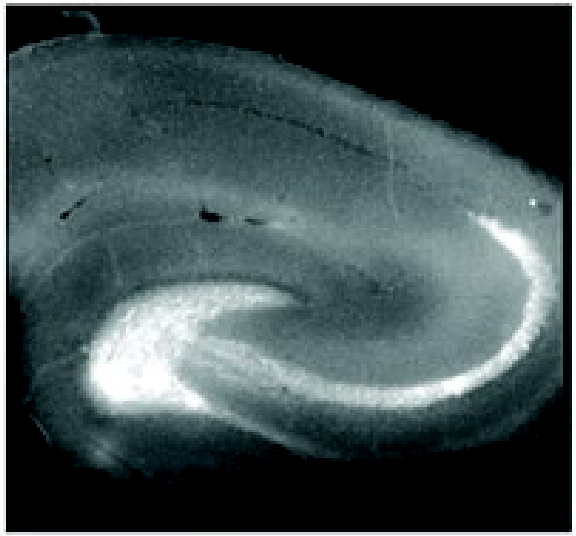


From: www.Invitrogen.com

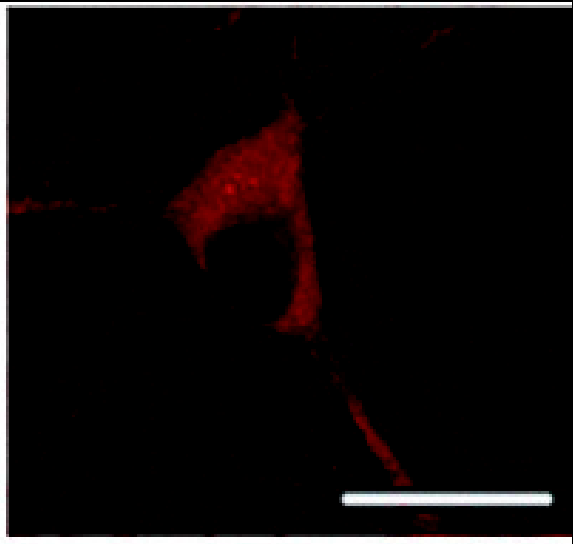
Examples of non-quantitative applications: Animal cells



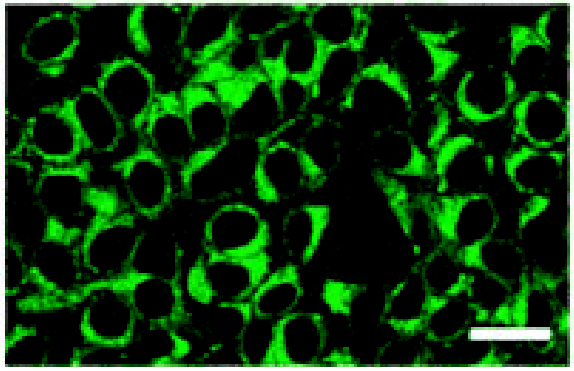
HeLa cells loaded with 50 μM Zn^{2+}/pyrithione and 10 μM ZS5



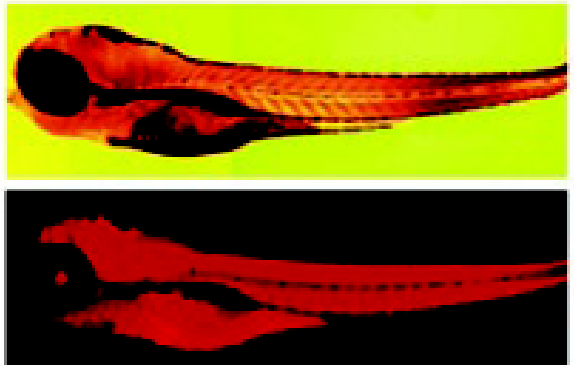
10 μM ZnAF-2F DA–loaded rat hippocampal slices



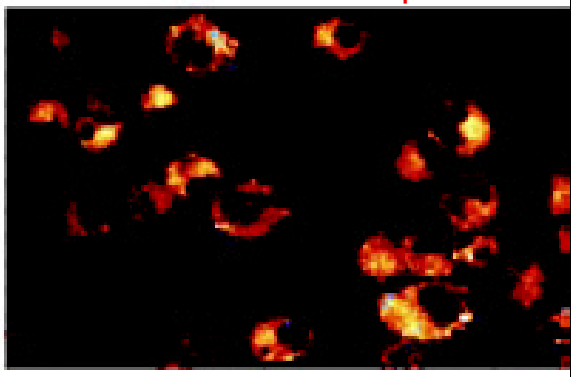
rat neurons loaded with 100 μM Cu^{2+} & stained with 5 μM CS1



HEK-293T cells treated with 1 μM MG1-AM and exposed to 20 μM Hg^{2+}



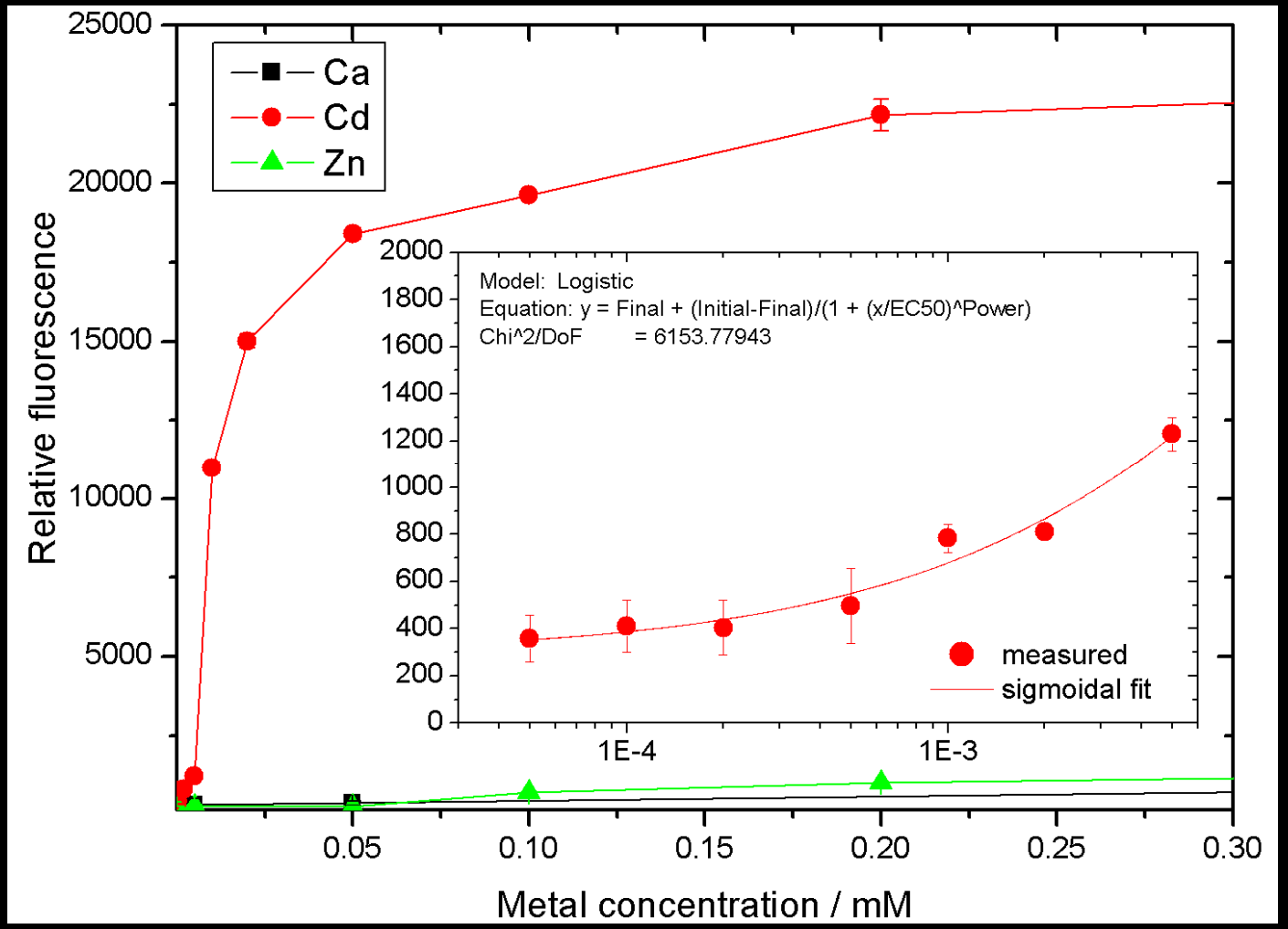
5-day-old zebrafish treated with 50 μM of a Hg^{2+}-selective dye and 50 μM Hg^{2+}



DC cells treated with a Cd^{2+}-selective fluorophore (5 μM) and 5 μM Cd^{2+}

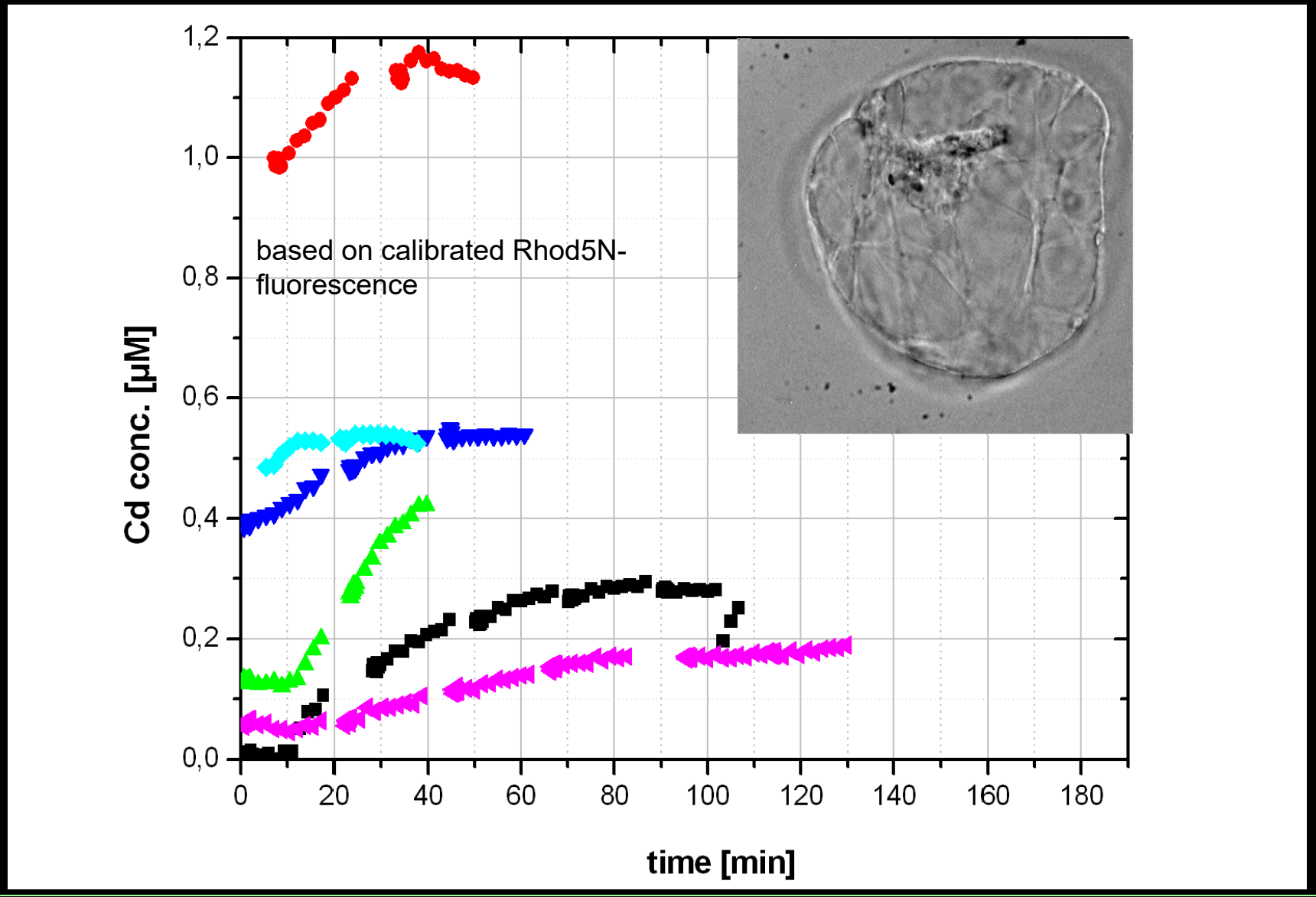
From: Chang CJ, Jaworski J, Nolan EM, Sheng M, Lippard SJ, 2004, PNAS101, 1129-34

(3) UV/VIS fluorescence
(a) Metal specific fluorescent dyes
→ calibration



Leitenmaier B, Küpper H, (2011) Plant Cell and Environment 34, 208-219

Quantitative measurement using metal-selective fluorescent dyes: Cd-uptake kinetics in *Thlaspi caerulescens* protoplasts



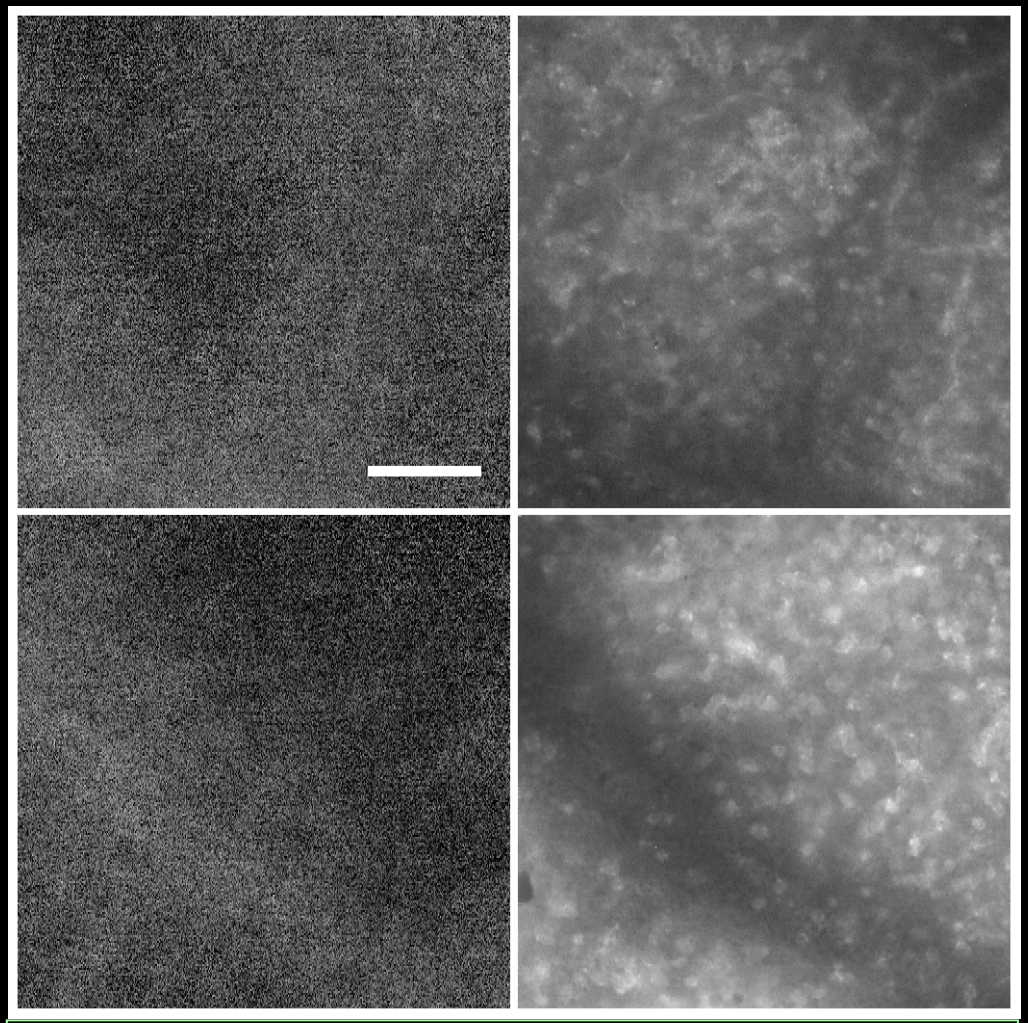
Leitenmaier B, Küpper H (2011) Plant Cell & Environment 34, 208-219

Advantage of metal dyes under physiological conditions: correlation between metabolic activity and metal accumulation

→ transient heterogeneity of mesophyll activity during period of Cd-induced stress

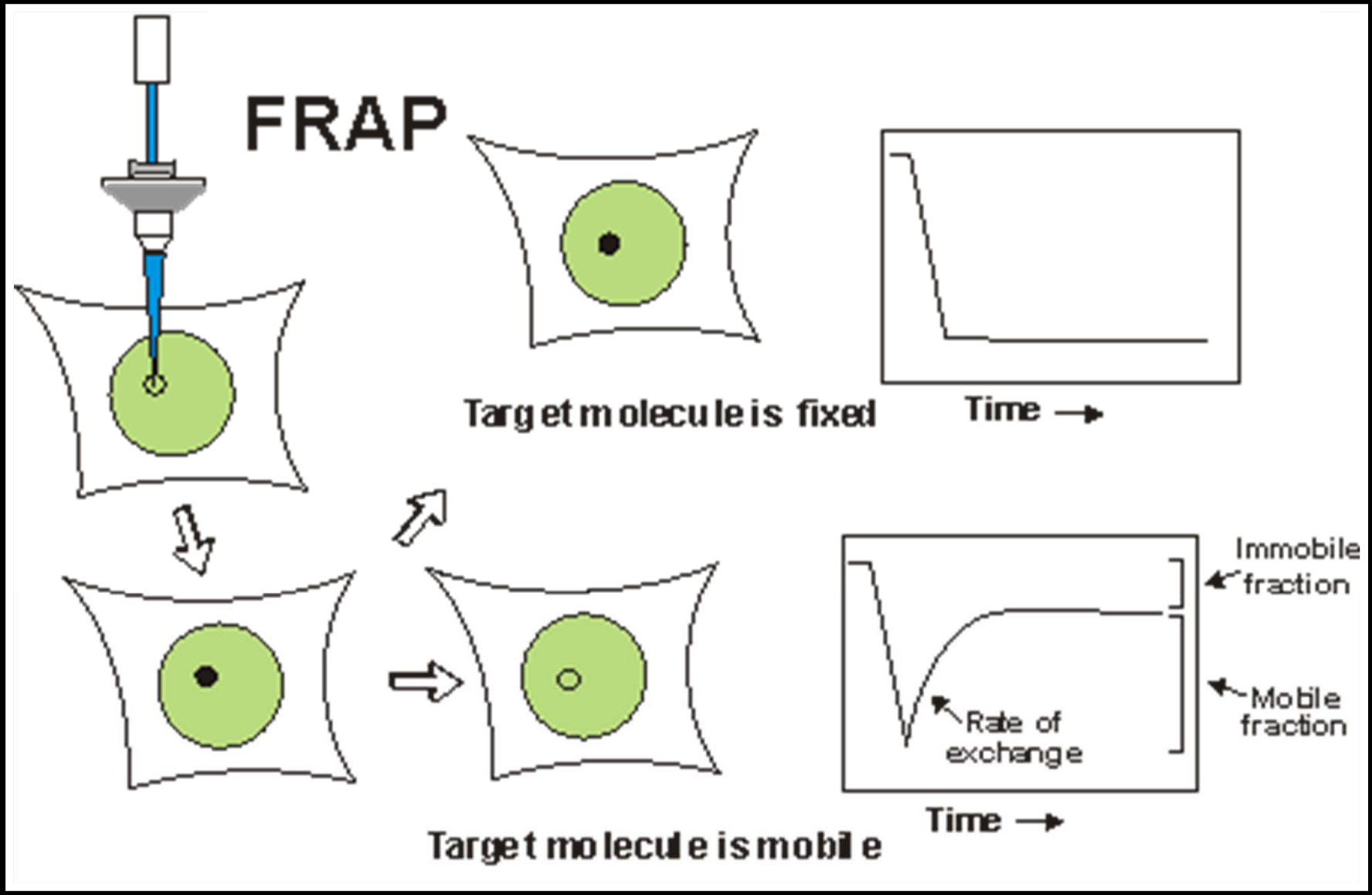
correlates with transient heterogeneity of Cd-accumulation

in *Thlaspi caerulescens*!



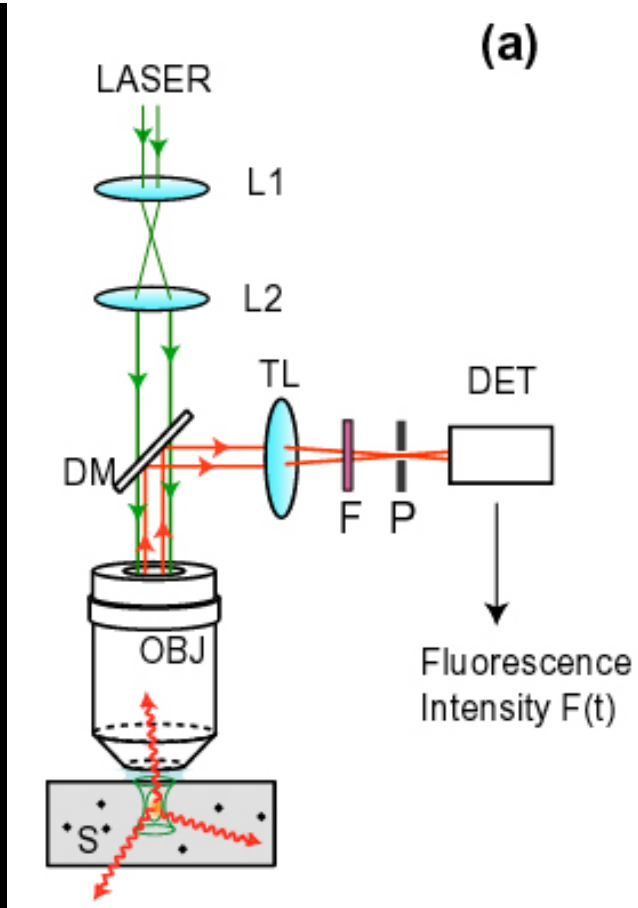
Küpper H, Aravind P, Leitenmaier B, Trtilek M, Šetlík I (2007)
New Phytol175, 655-74

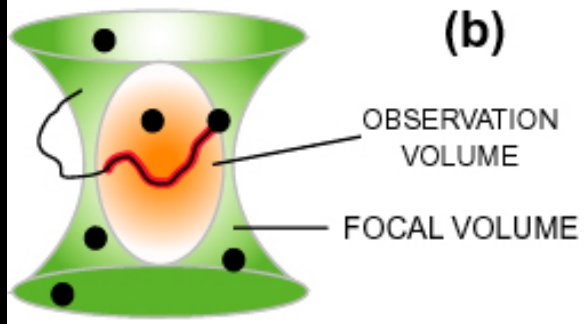
Analysis of molecule mobility:
FluorescenceRecoveryAfterPhotobleaching (FRAP)

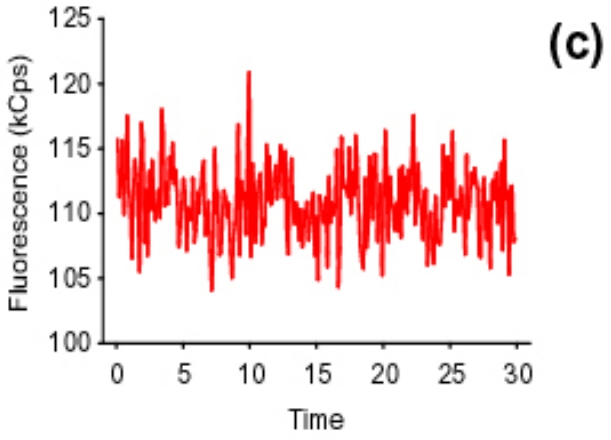


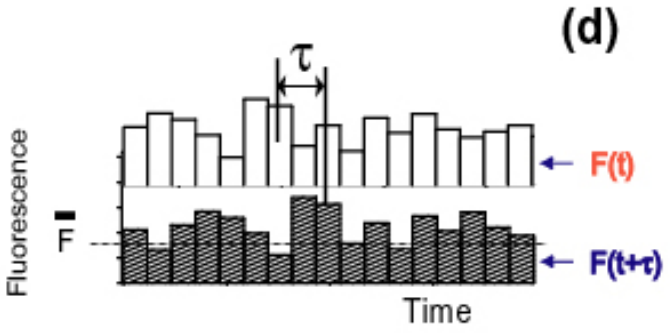
From: Zeiss workshop

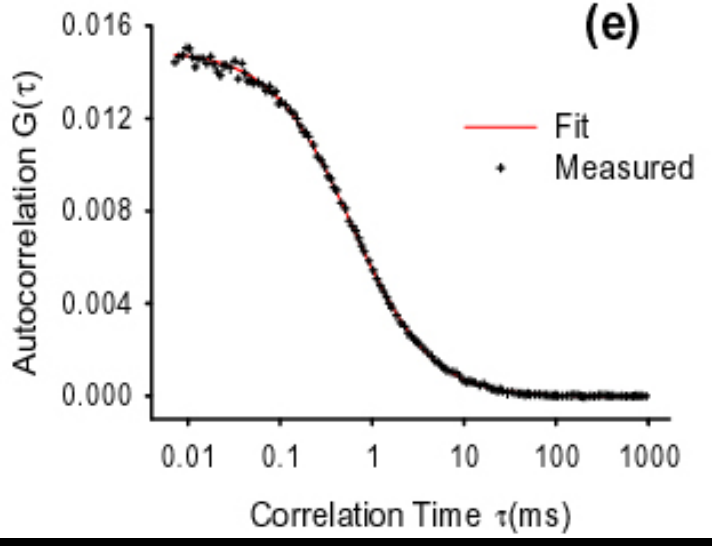
Fluorescence Correlation Spectroscopy (FCS)





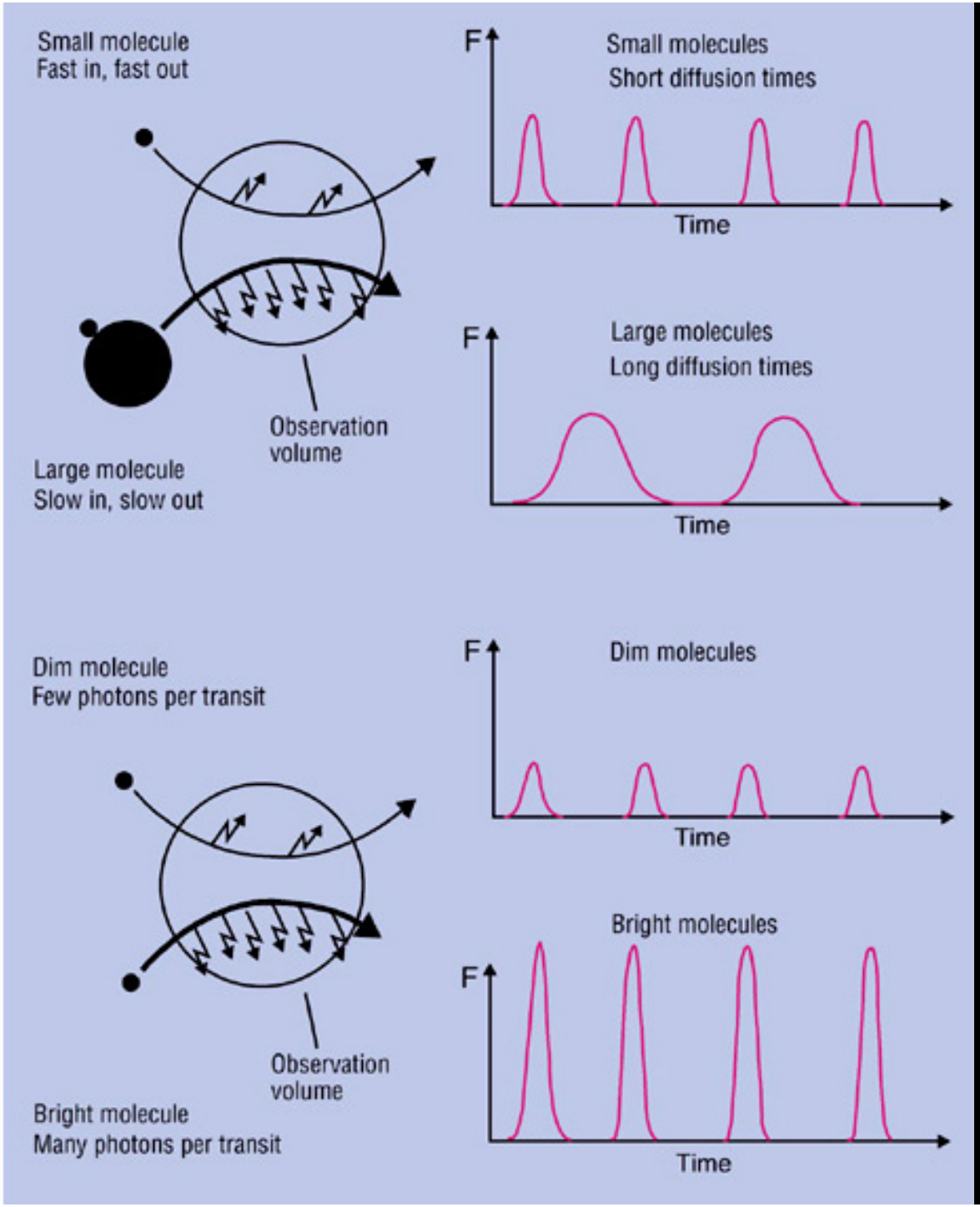






From: Zeiss workshop

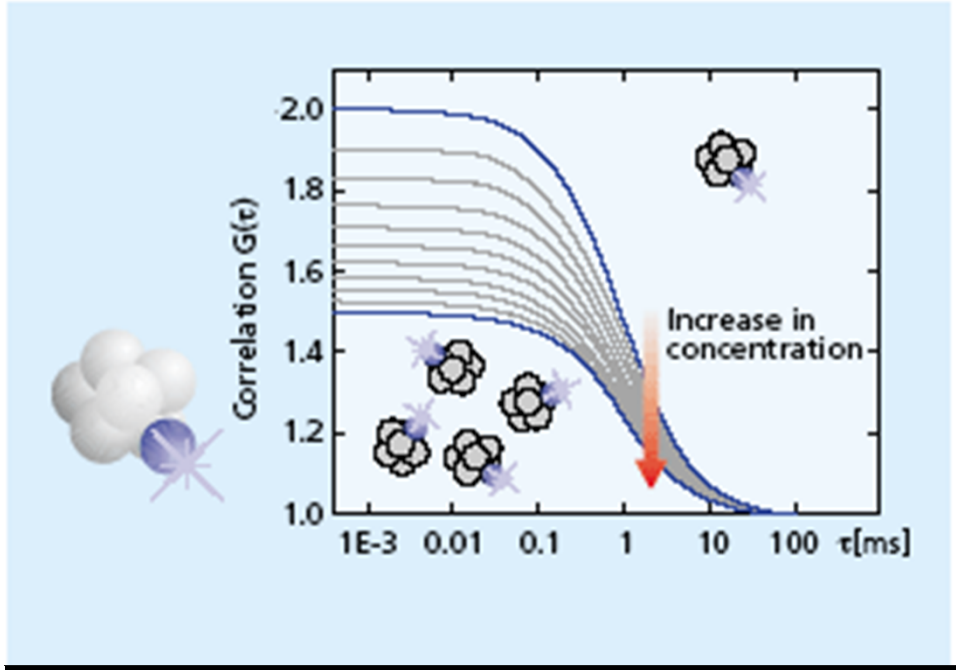
SmithKline Beecham

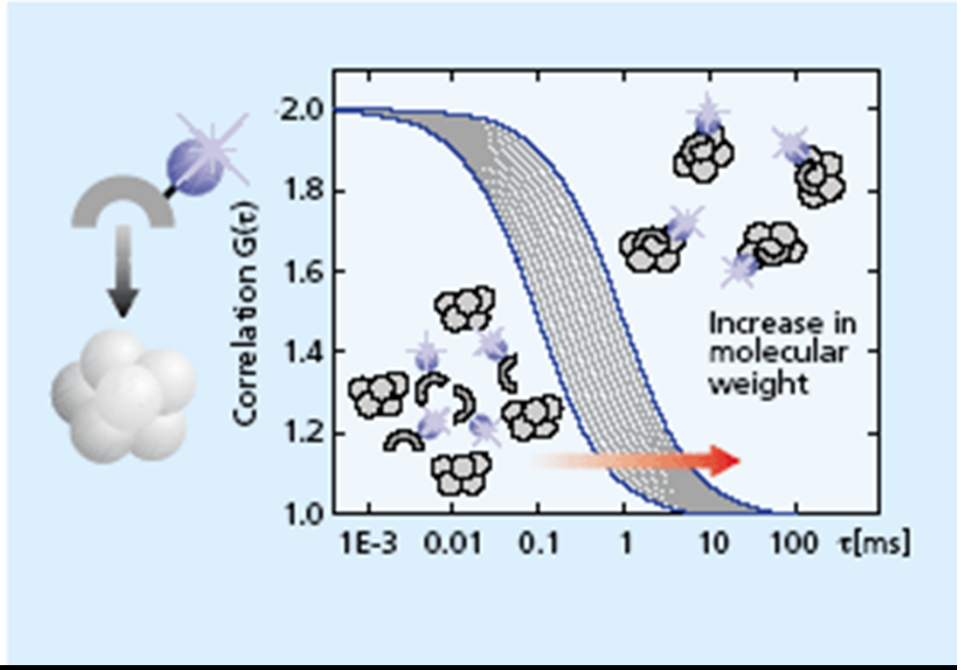


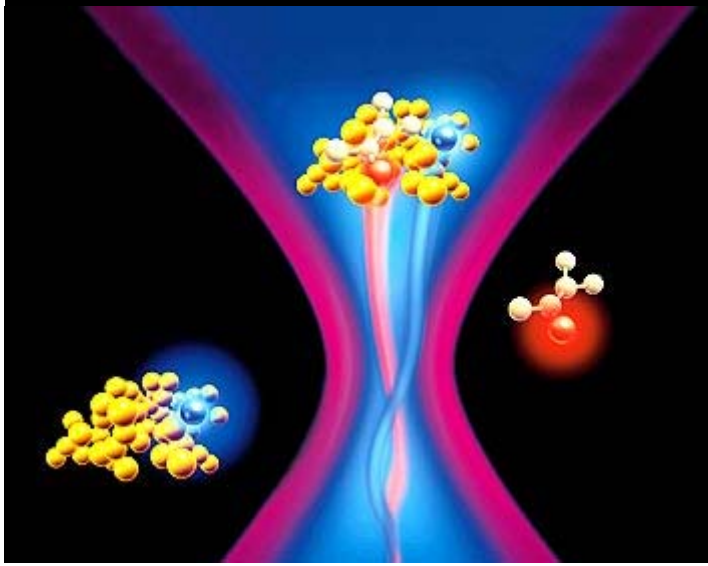
Fluorescence Correlation Spectroscopy (FCS) II

From: Zeiss workshop

Fluorescence Correlation Spectroscopy (FCS) III



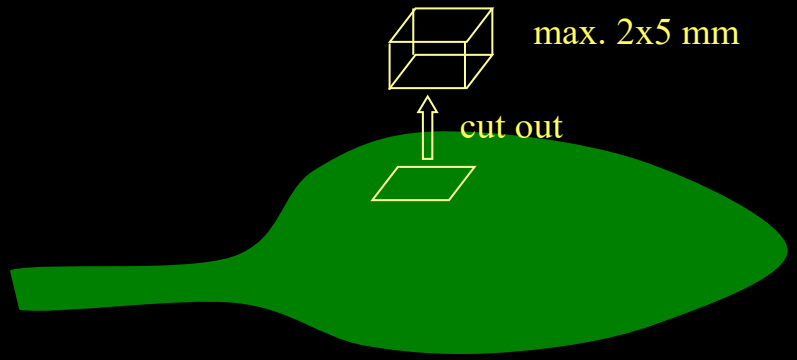




--> info about molecular concentration, brightness, diffusion, and chemical kinetics

From: Zeiss workshop

Quantitative mRNA *in situ* hybridisation (QISH): overview of the method



vacuum infiltrate with alkaline fixation solution

extract pigments and dehydrate

extract hydrophobic compounds

rehydrate

digest proteins

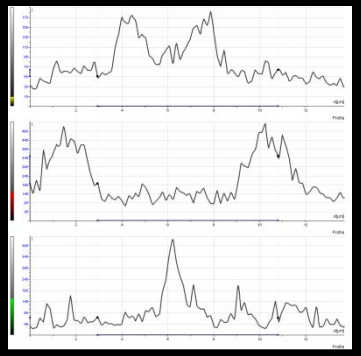
postfixate

hybridise with fluorescent oligonucleotides

extract and quench background

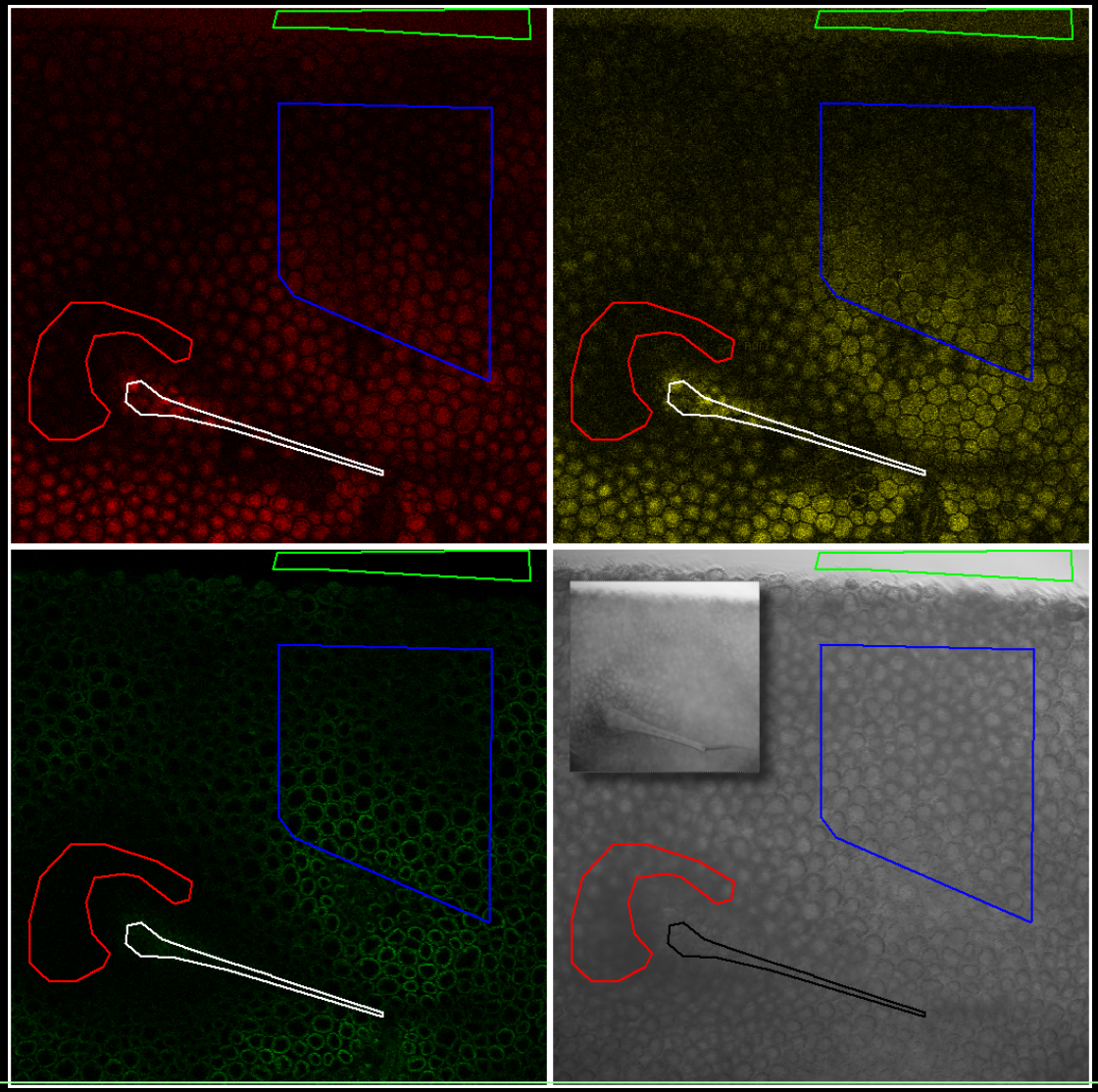
record images in CLSM

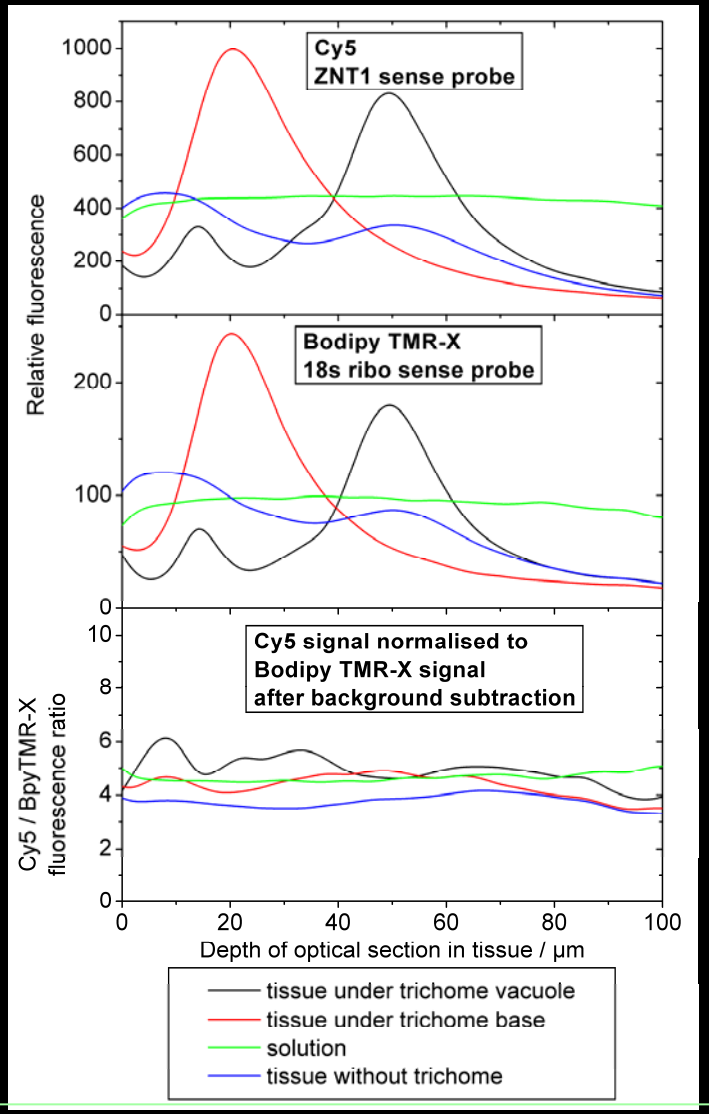
quantify



Analysis of metal transporter gene expression via a novel method for quantitative *in situ* hybridisation

Characteristics of the method: effects of tissue optics

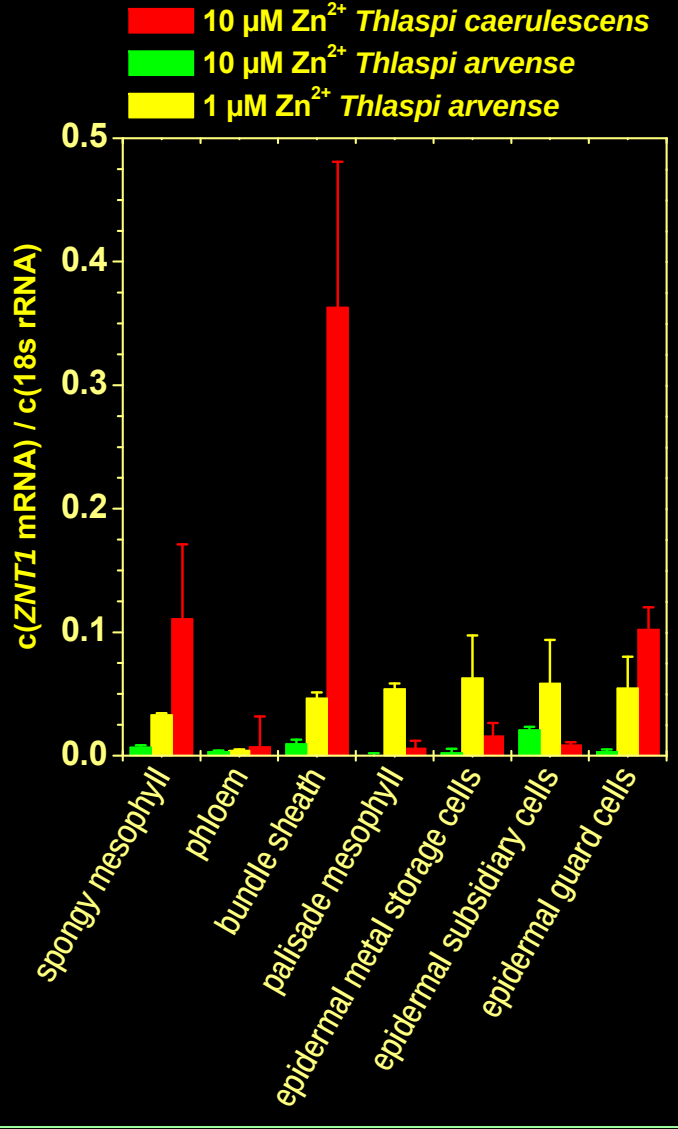


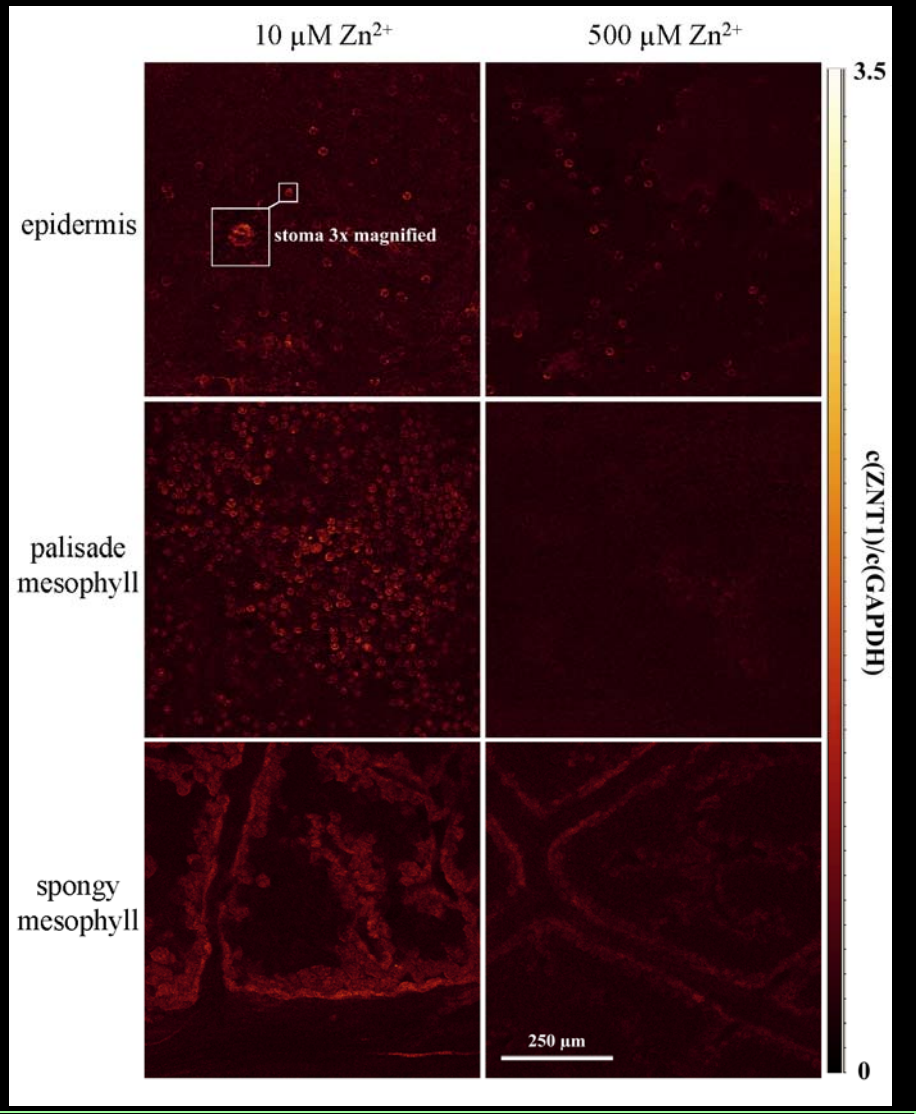


Küpper H, Seib LO, Sivaguru M, Hoekenga OA, Kochian LV (2007) The Plant Journal 50(1), 159-187

Regulation of ZNT1 transcription analysed by quantitative mRNA *in situ* hybridisation (QISH)

in a non-hyperaccumulating and a hyperaccumulating *Thlaspi* species





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Qualitative Observation of Transcription&Translation ***in vivo*** via Fluorescent Proteins

Construct vectors for plant transformation

promoter

EYFP

transform *Agrobacterium* with the constructs

transfrom plants by agrobacterium infection (floral dip with or without vacuum infiltration)

germinate seeds of transformed plants on selective medium (e.g. agar containing Kanamycin)

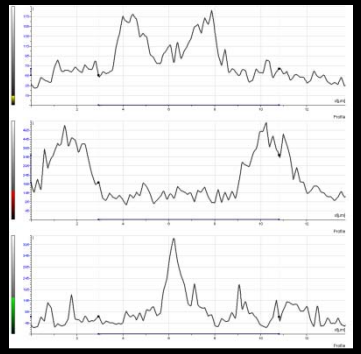
select healthy (resistant) seedlings

select for YFP expression

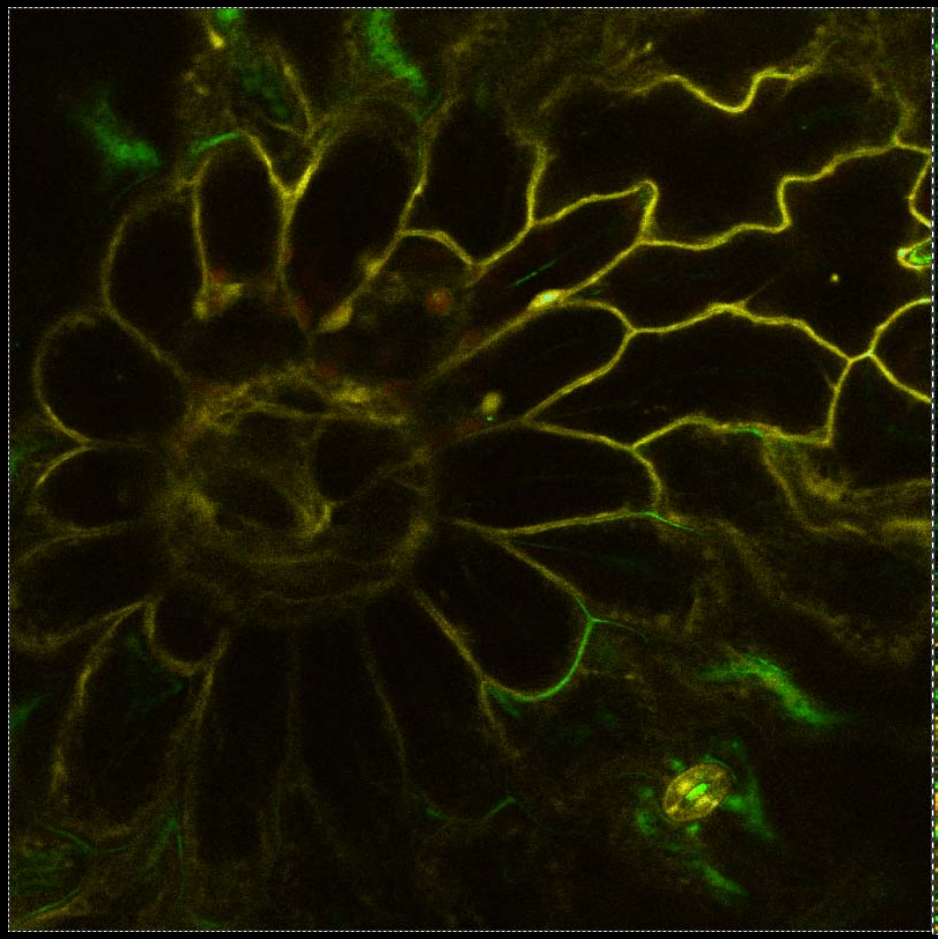
prepare tissue pieces or whole mounts

record images in CLSM

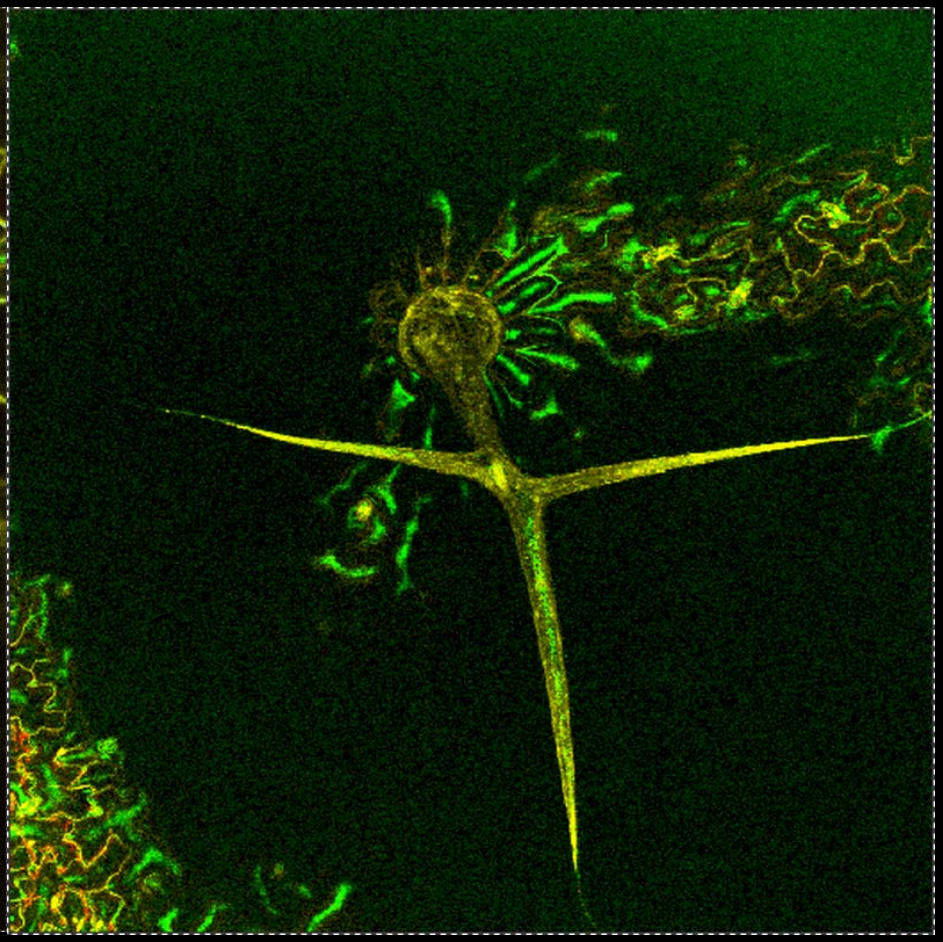
quantify



35S promoter in young leaves of *Arabidopsis thaliana*:
epidermis



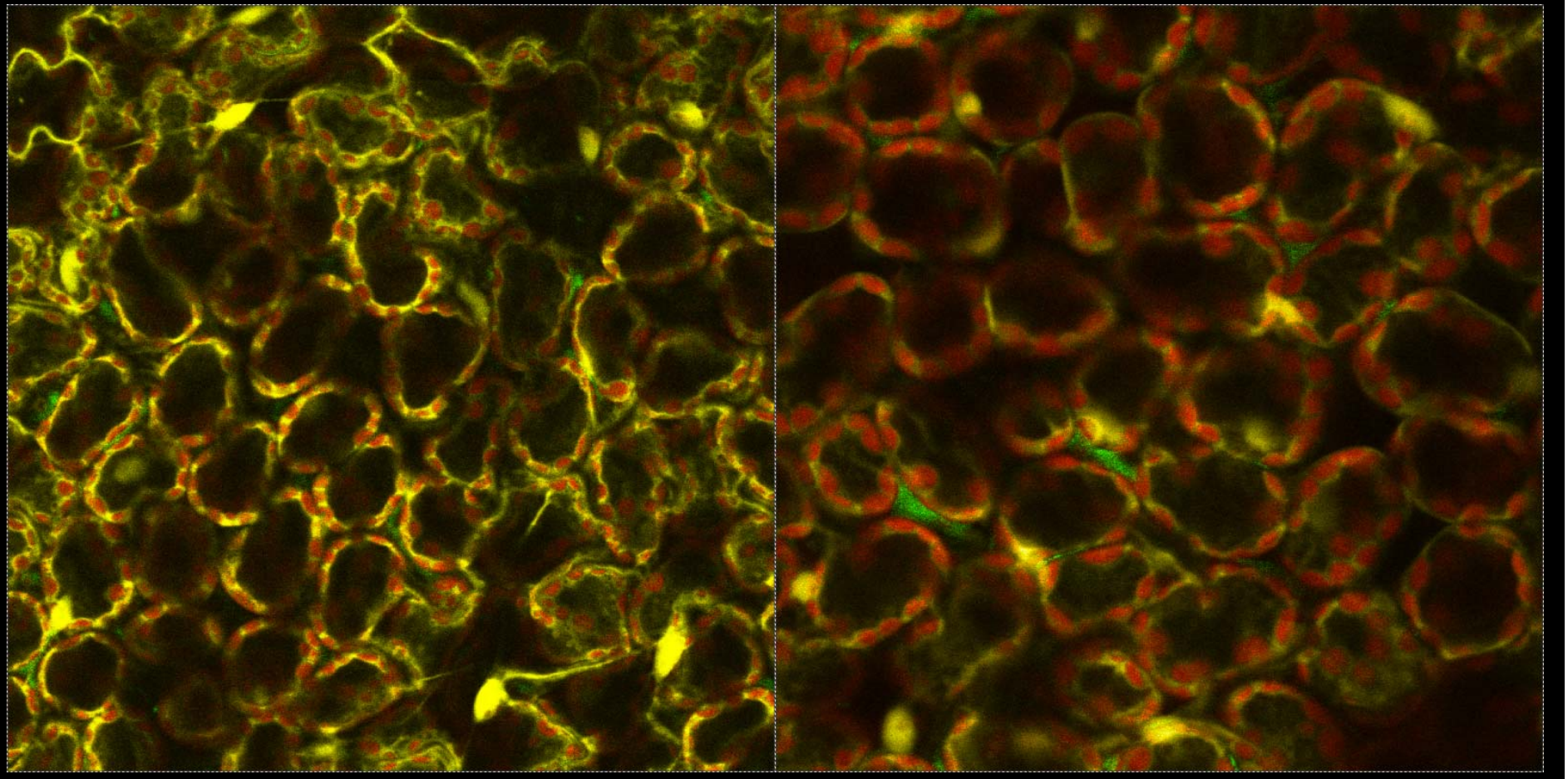
Trichome base, epidermal cells and stoma



Trichome

Overlays of green autofluorescence, red (chlorophyll) autofluorescence and yellow YFP fluorescence

35S promoter in young leaves of *Arabidopsis thaliana*:
mesophyll



Clone with high YFP expression

Clone with medium YFP expression

Overlays of green autofluorescence, red (chlorophyll) autofluorescence and yellow YFP fluorescence

Comparison of our *in situ* hybridisation method with promoter-GFP/YFP/DsRed/... constructs

In situ hybridisation

- Easy cellular quantification because whole cells are labelled

- No macroscopic (whole plant) quantification possible because of diffusion limits

- Low background fluorescence because chlorophyll, carotenoids, flavonoids and many further fluorescent compounds are extracted

- No direct comparison of gene expression with physiology because samples are fixed (dead)

- Very fast: Ordering the fluorescently labelled oligonucleotides takes 1-2 weeks, the hybridisation procedure itself takes 3 days

- All plants can be analysed (→ *Thlaspi* work)

- The gene sequence has to be known

Fluorescent proteins

- Quantification on a cellular level difficult because only the narrow ring of cytoplasm is labelled

- Macroscopic (whole plant) observation and quantification easily possible with fluorescence measuring camera (so far only tested with GFP)

- High background fluorescence because all autofluorescent compounds are present in the samples

- Direct comparison of gene expression with physiological parameters (photosynthesis, electrophysiology) possible because samples are alive

- Very time-consuming because of the cloning, transformation and plant growth/selection steps;

- The plant has to be transformed (→ *Arabidopsis*)

- The promoter has to be cloned

Küpper H, Seib LO, Sivaguru M, Hoekenga OA, Kochian LV (2007) The Plant Journal 50(1), 159-187

All slides of my lectures can be downloaded from my workgroup homepage

Biology Centre CAS → Institute of Plant Molecular Biology → Departments → Department of Plant Biophysics and Biochemistry,

or directly

http://webserver.umbr.cas.cz/~kupper/AG_Kuepper_Homepage.html