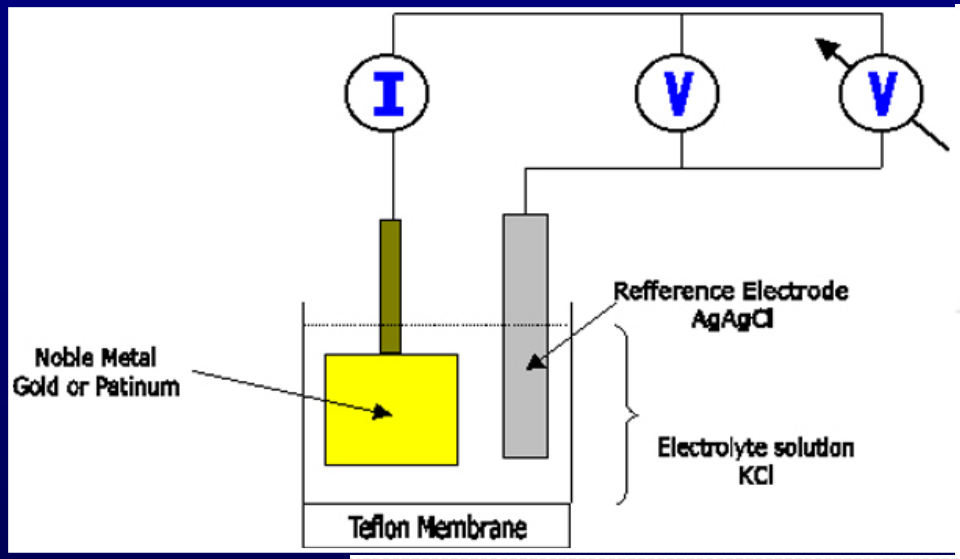
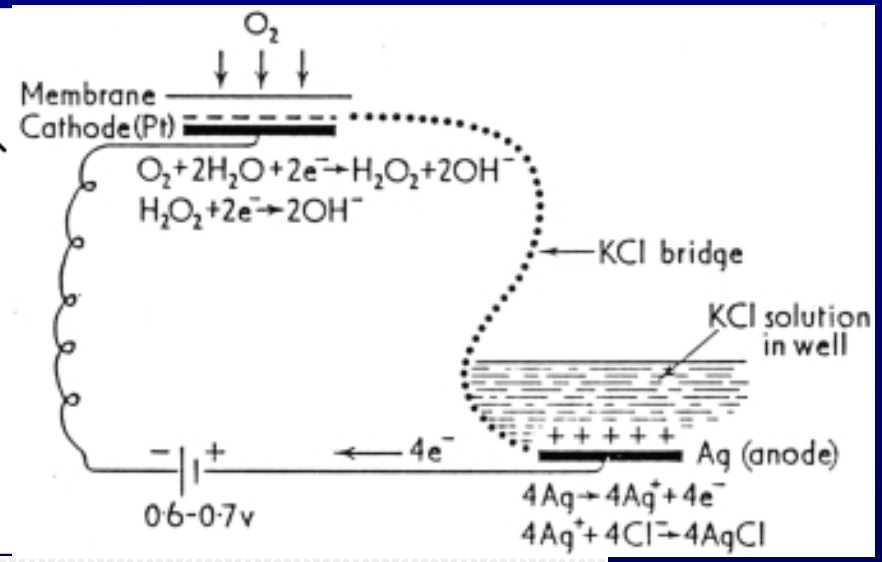
Biophysical & physicochemical methods
for analyzing plants *in vivo* and *in situ*:

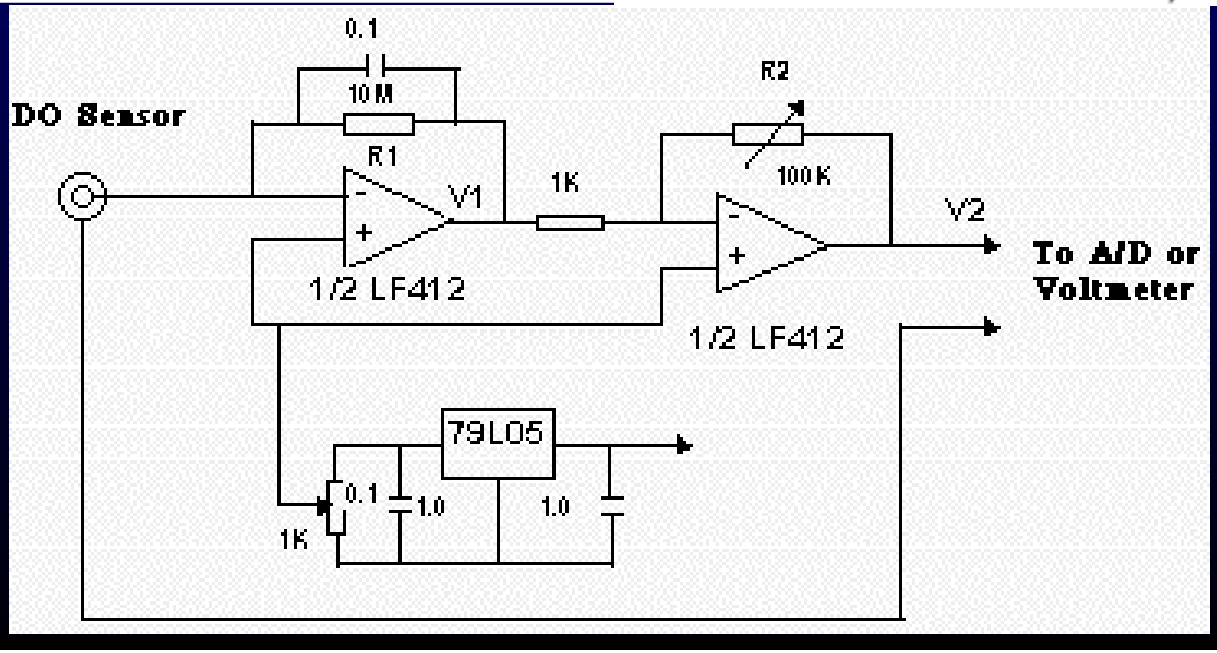
Methods for Analysis of Photosynthesis - a few important examples

Measurement of photosynthesis activity

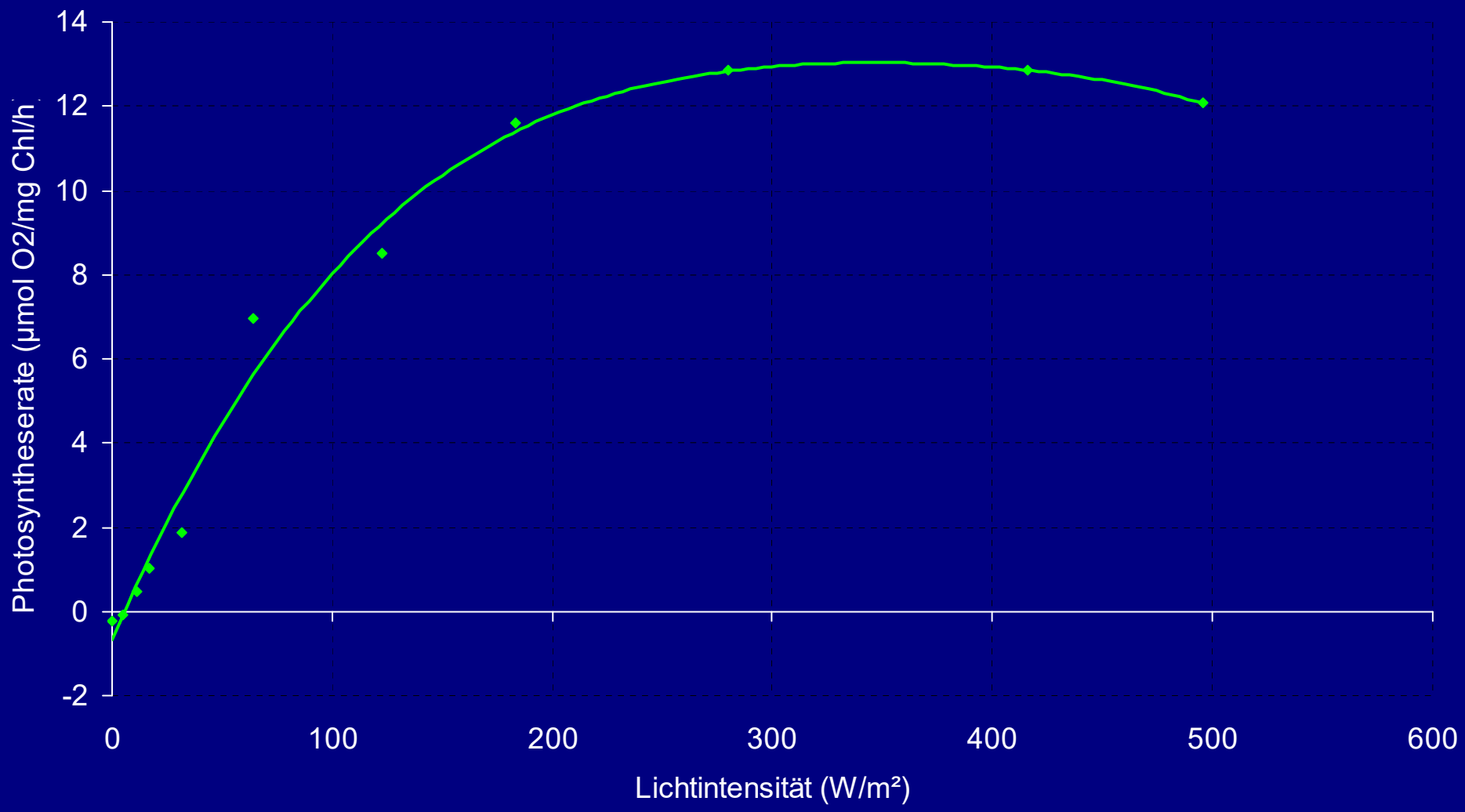
Polarographic measurement of O_2 exchange





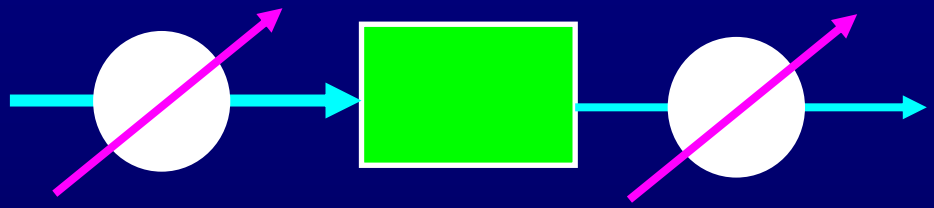


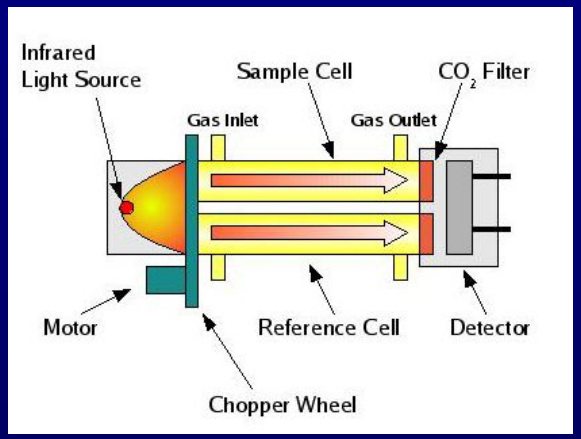
Lichtsättigungskurve der Photosynthese

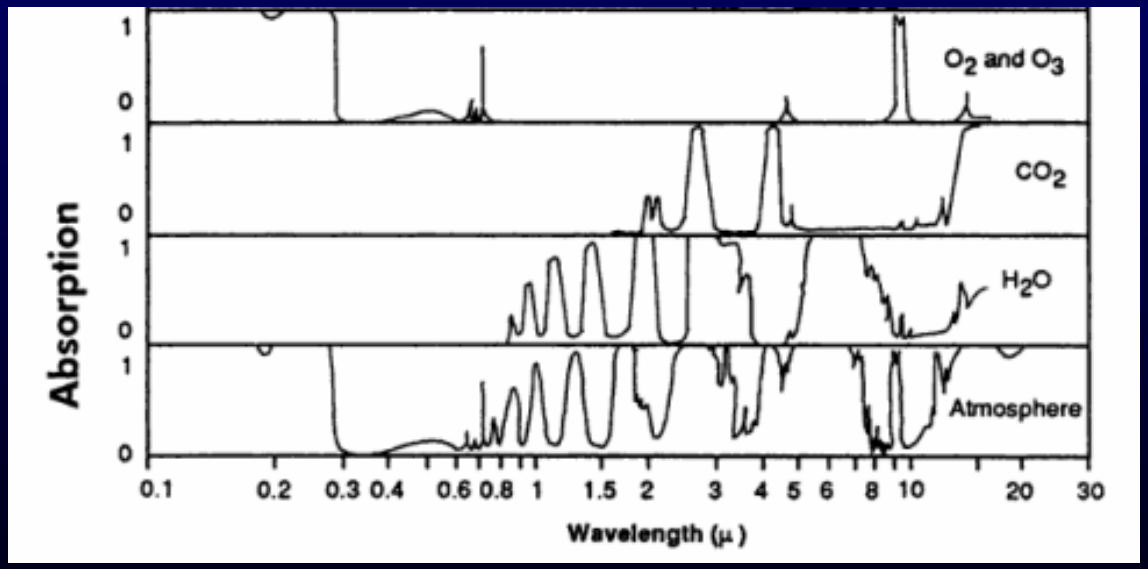


Measurement of photosynthesis activity

IR-Measurement of CO_2 assimilation

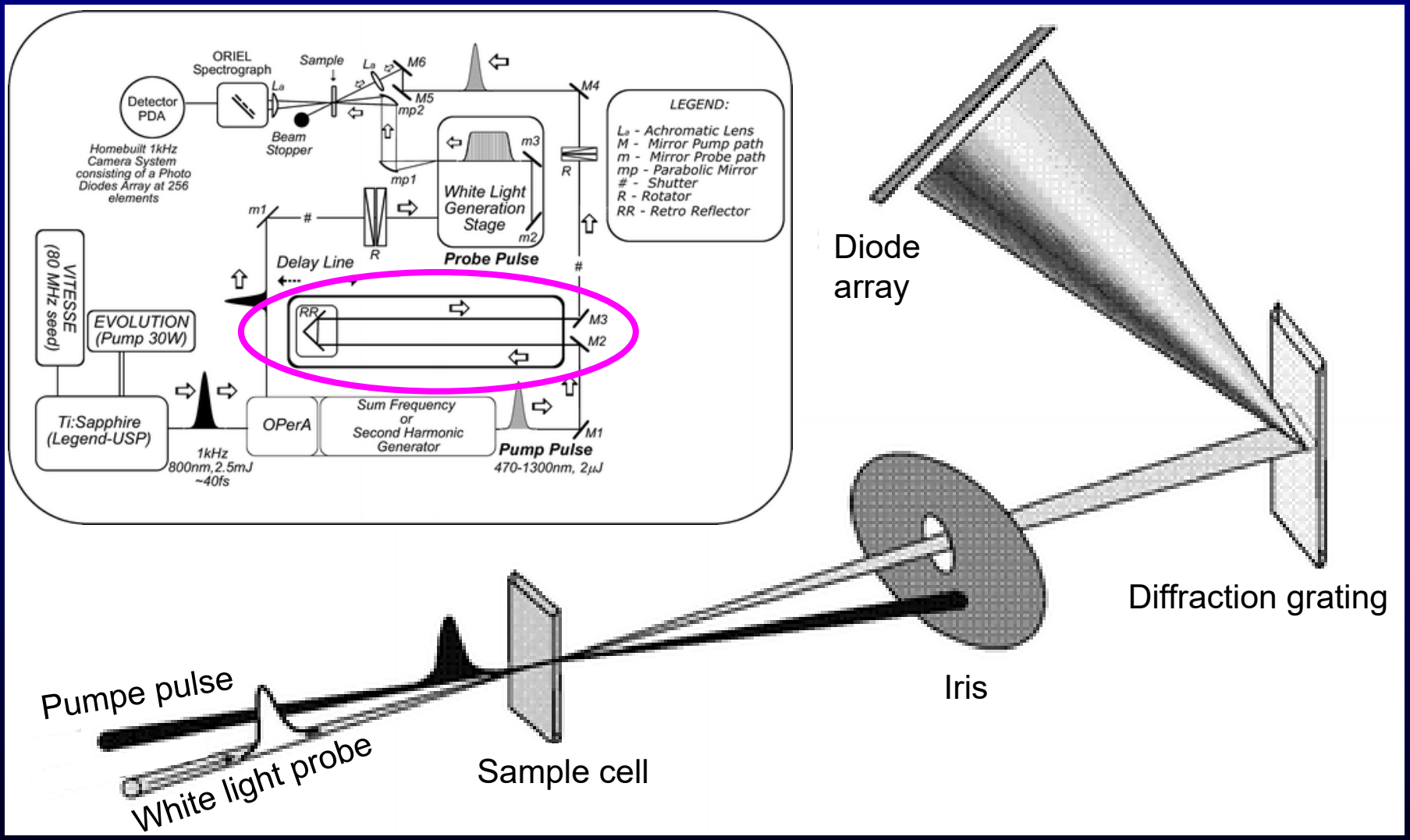






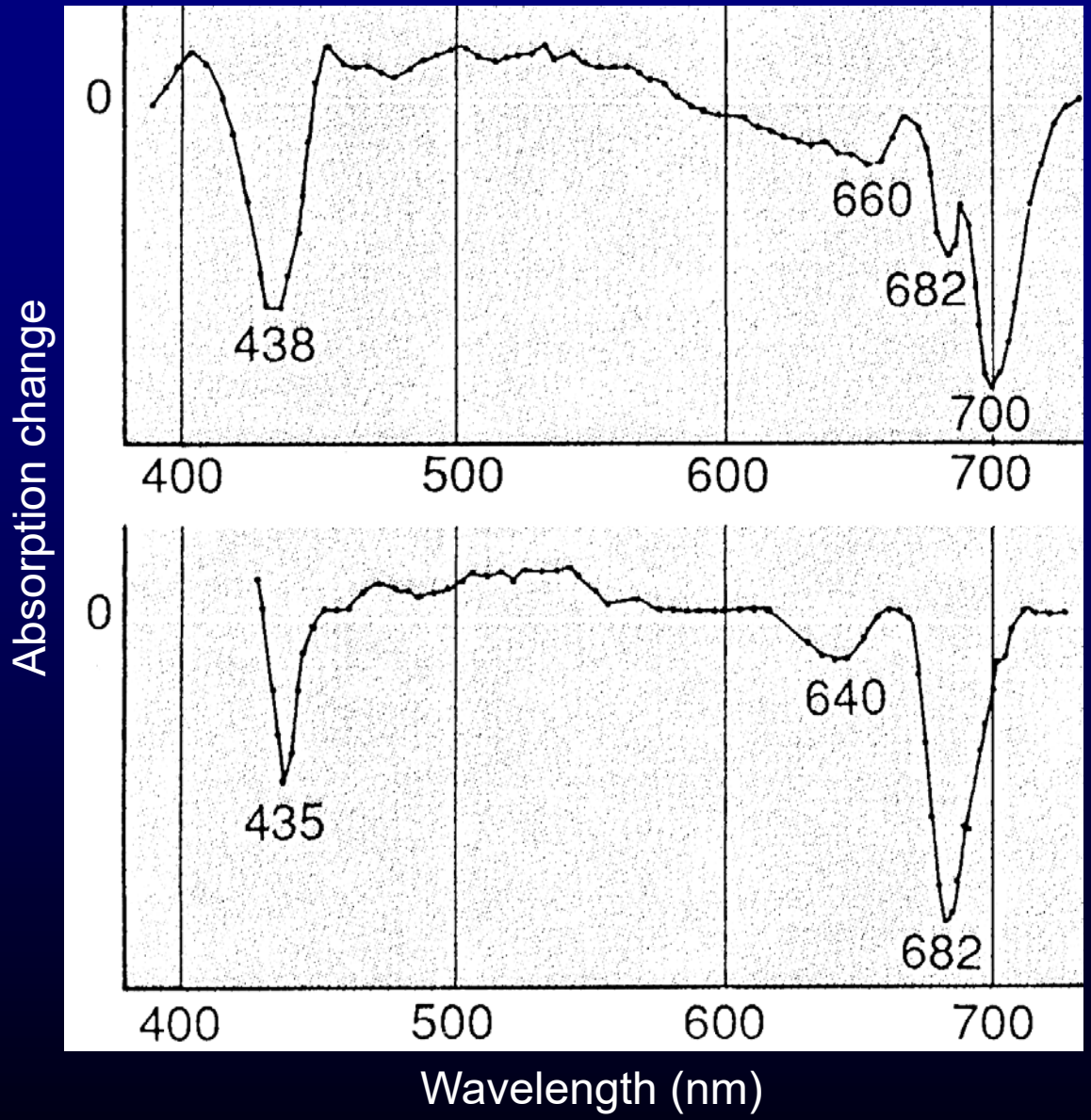
Pictures from: esrl.noaa.gov; Walz GmbH; Fleagle and Businger, 2006

Measurement of fast events, e.g. primary charge separation



From: Berera R_vanGrondelle R_Kennis JTM_Ultrafast transient absorption spectroscopy_PhotosynthRes101_105-118

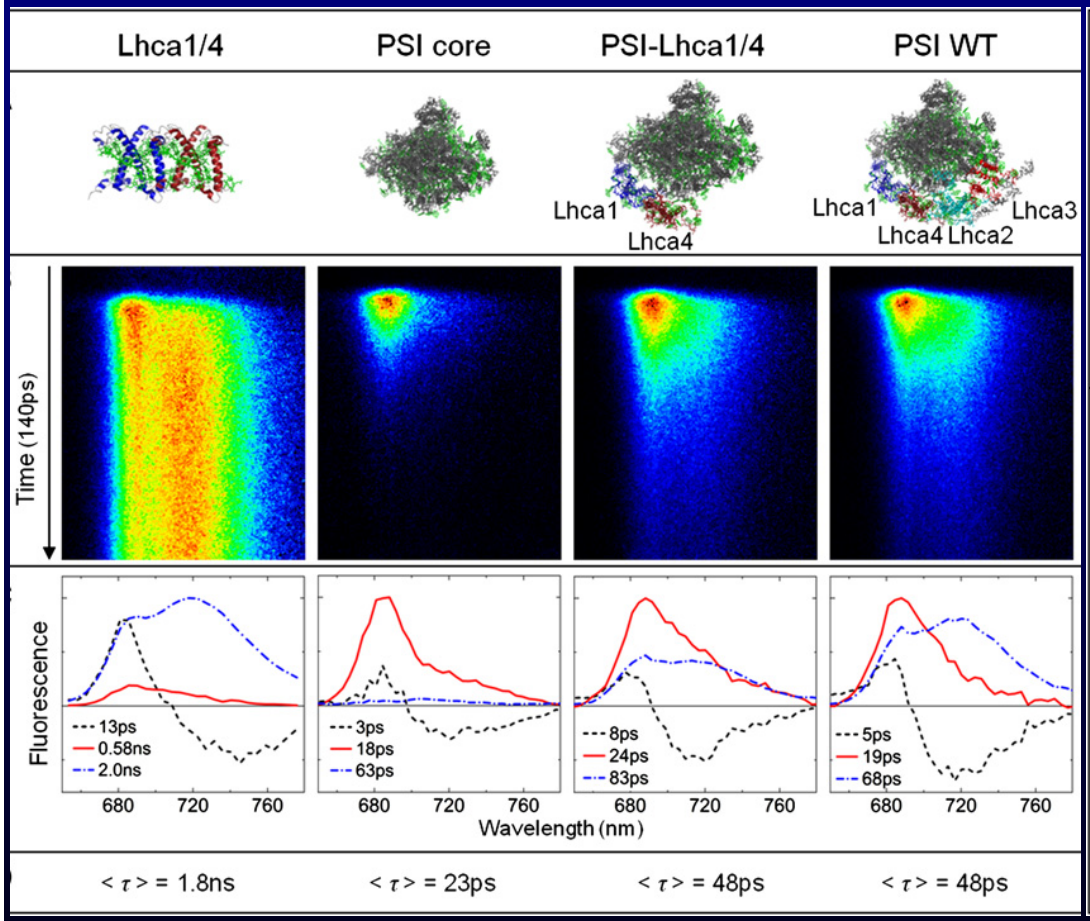
Measurement of primary charge separation



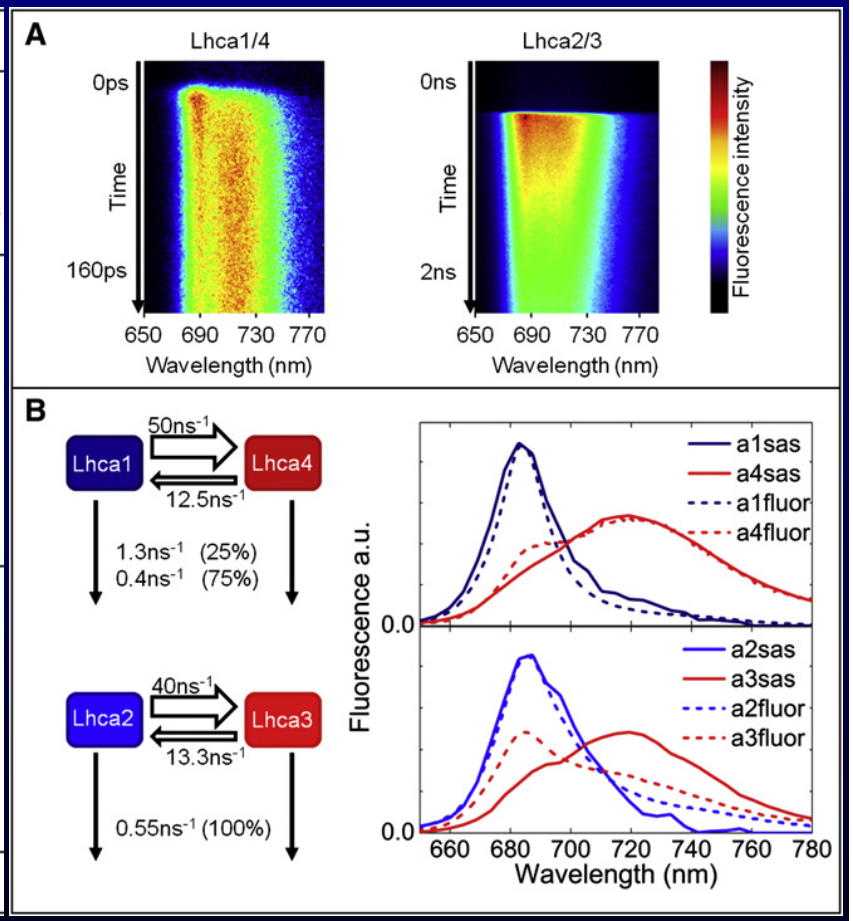
From: Lawlor DW (1990) Thieme, Stuttgart, 377S

Ultrafast UV/VIS spectroscopy

Excitation transfer times between light harvesting complexes

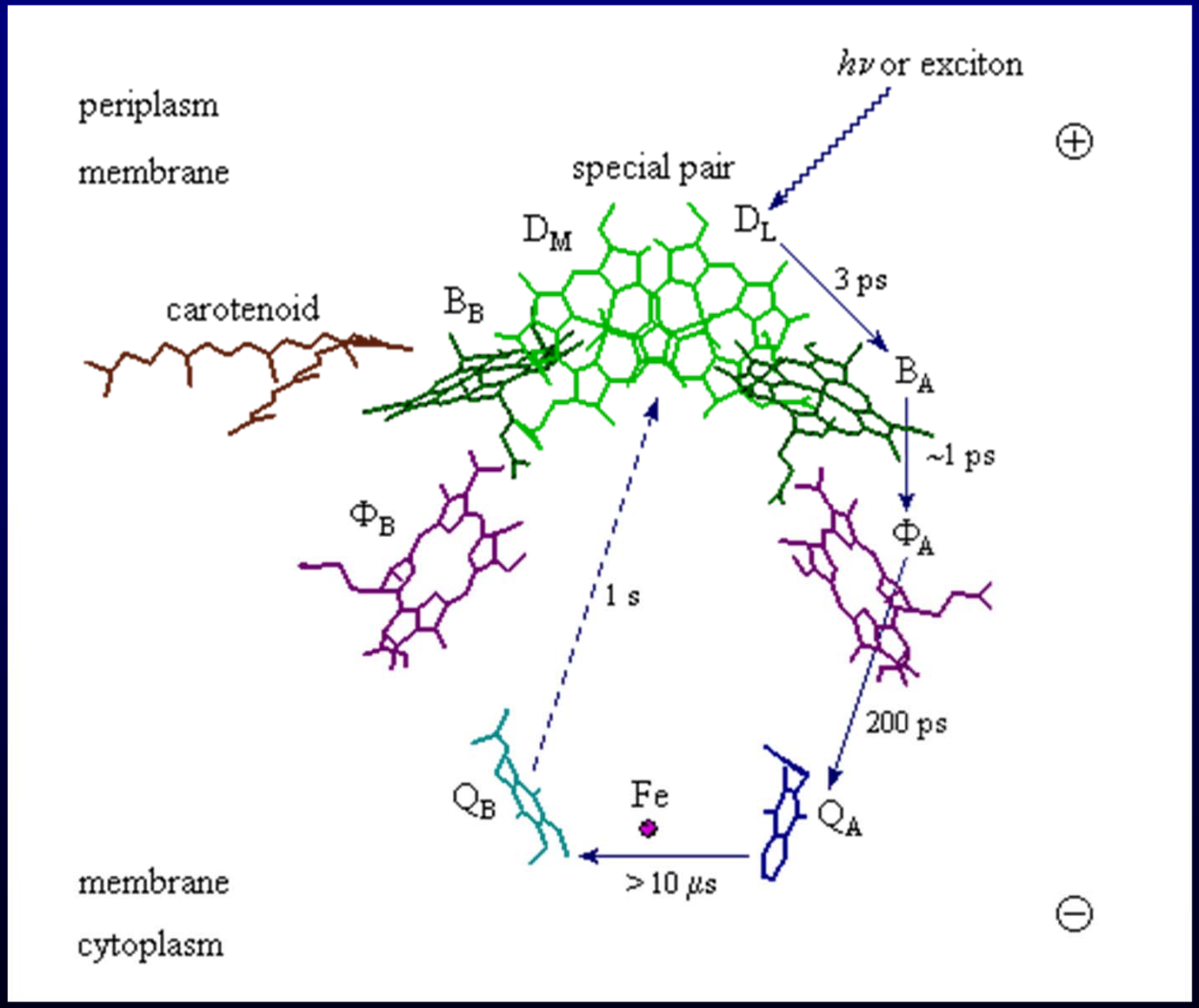


From: Wientjes E_et al (2011) BiophysJ101, 745-54

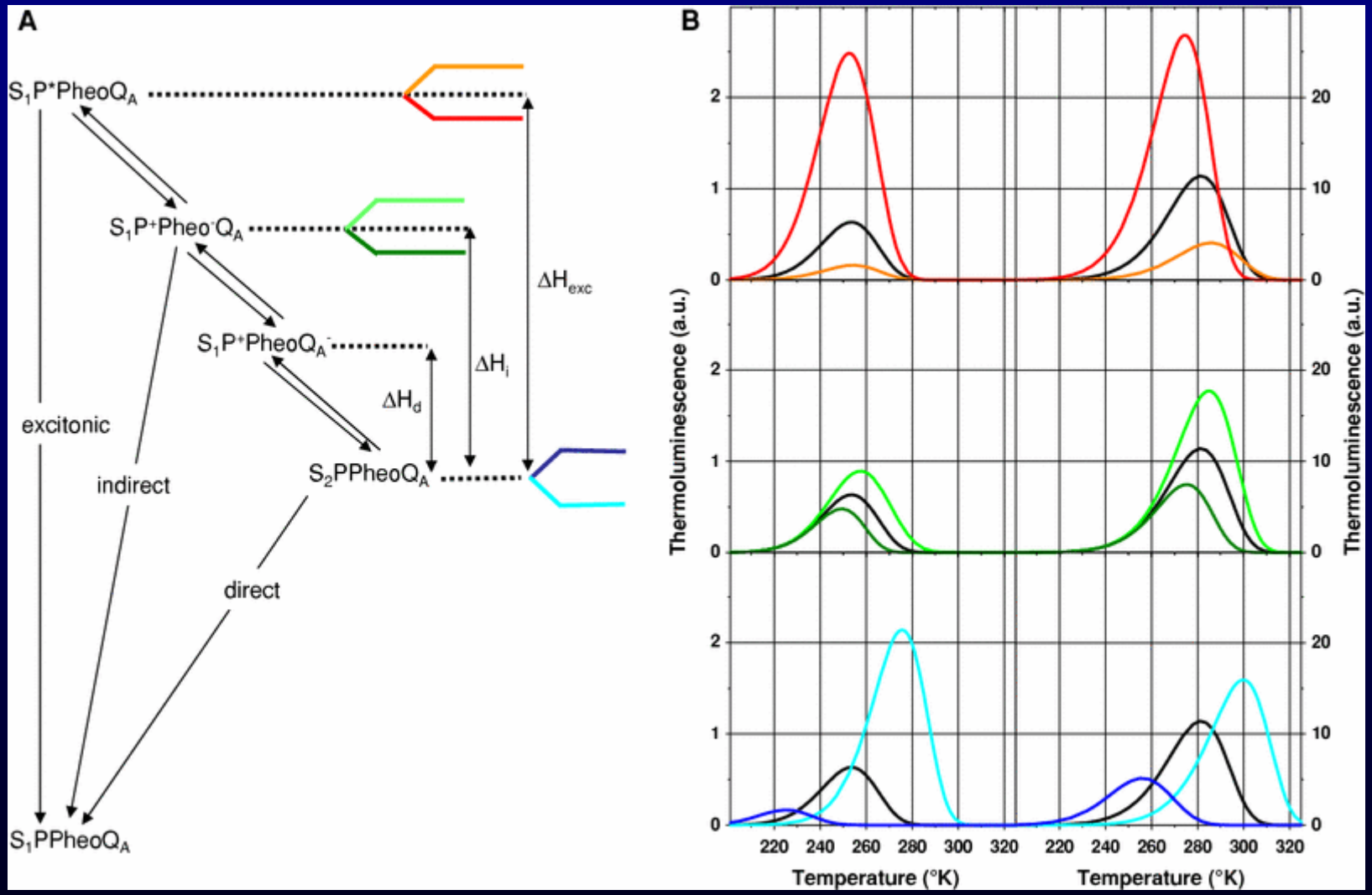


From: Wientjes E_et al (2011) BiophysJ 100, 1372-80

Photosystem II reaction centre: steps of electron transfer → to be analysed with thermoluminescence measurements

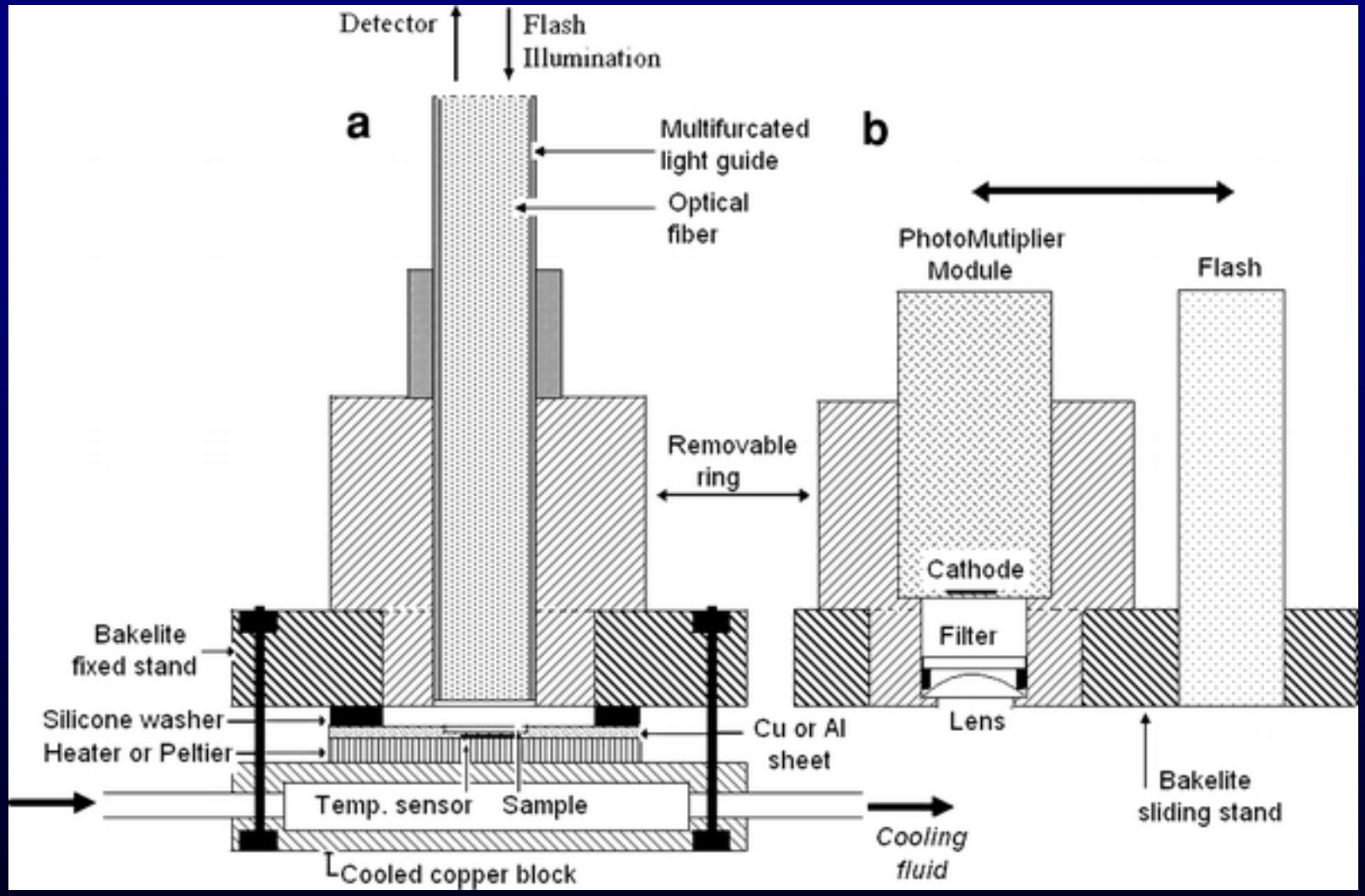


Thermoluminescence measurement



From: Rappaport F_Lavergne J_2009_Thermoluminescence theory_PhotosRes101_205-216

Thermoluminescence measurement



From: Ducruet JM_Vass I_2009_Thermoluminescence experimental_PhotosRes101_195-204

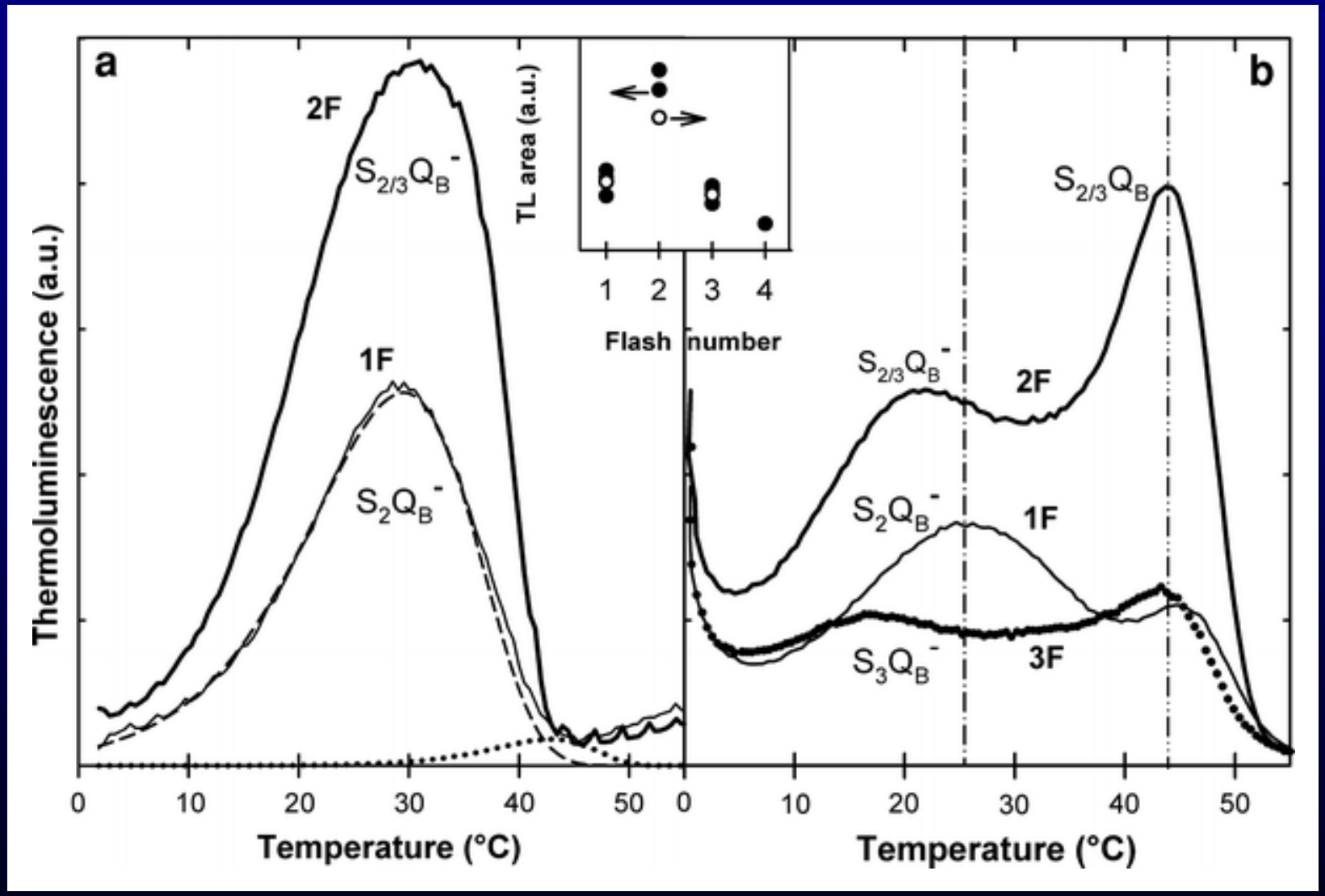
Thermoluminescence measurement: parameters

Name	T_M range	Origin	Origin PS II	Comments
Z	−160°C	Pigments	−	Low temperature pigment photochemistry
Zv	−70 to −100°C	(P_{680}^+ Q_A^-?)	+	Tm depends on illumination temperature
A_T	−10 to −20°C	$TyrZ^+Q_A^-$	+	Damage to Mn oxygen-evolving complex (TyrZ is the functional donor to PS II center)
A	~−15°C	$S_3Q_A^-$?	+	
Q	+2 to 10°C	$S_2Q_A^-$	+	Damage to secondary Q_B quinonic acceptor or inhibition by DCMU-like herbicides
B	30 to 38°C	$S_{2/3}Q_B^-$	+	Lumen pH > 7
B2	28 to 32°C	$S_2Q_B^-$	+	Lumen pH < 7
B1	20 to 30°C	$S_3Q_B^-$	+	Lumen pH < 7
AG	+45°C (→ +35°C)	$S_2/S_3Q_B + e^-$	(+)	Electron from stroma, in intact chloroplasts or cells
C	+52/55°C	$TyrD^+Q_A^-$	+	Minor band, increased by DCMU or damage (TyrD is the non functional donor to PS II center)
HTL1	60 to 85°C	?	−	Different bands of unknown origin, without illumination
HTL2	120 to 140°C	Lipid peroxides	−	Thermolysis: –C–O–O– → *C=O + Chl → *Chl

T_M values are given for data obtained with a 0.5°C/s TL heating rate

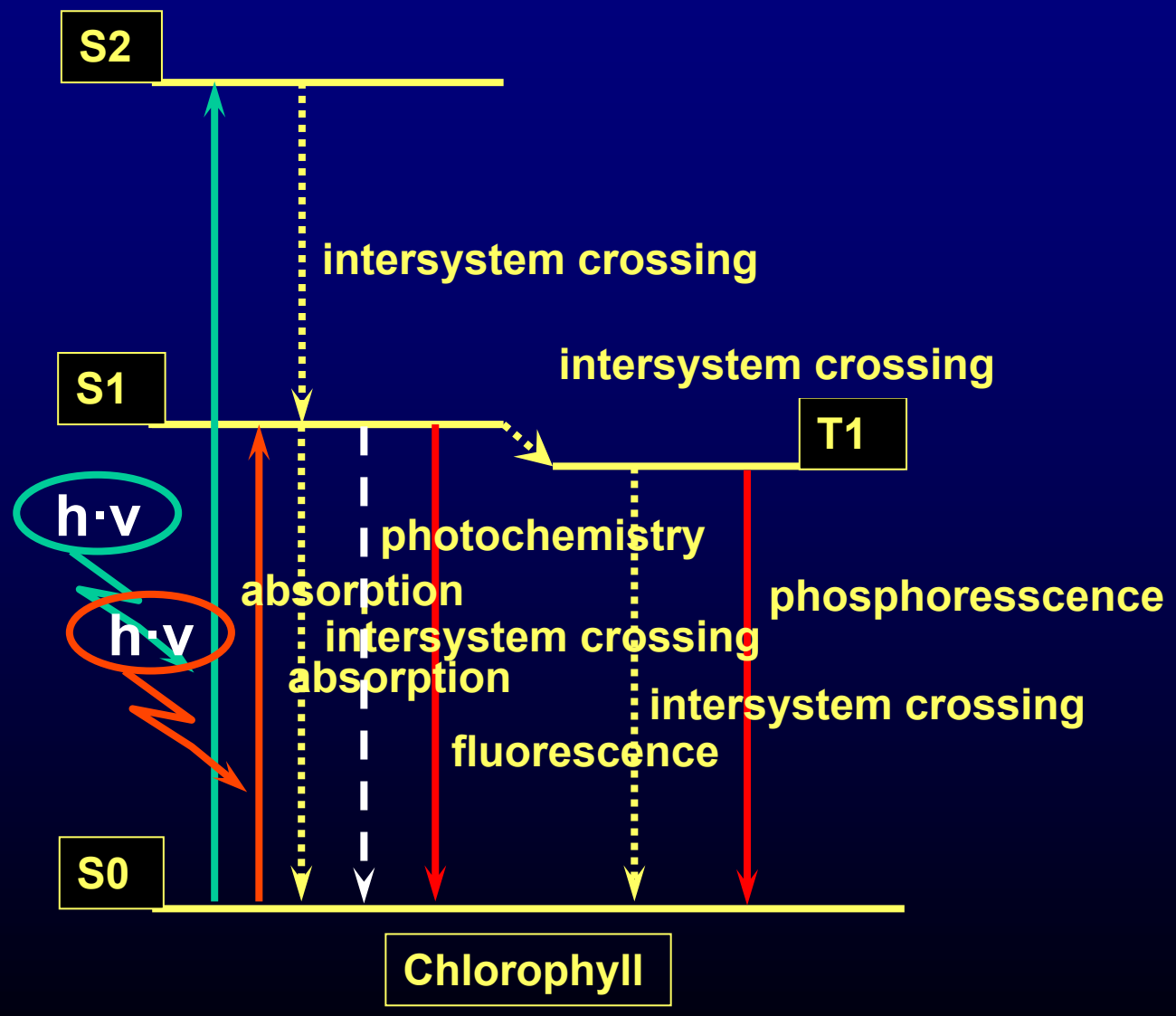
From: Ducruet JM_Vass I_2009_Thermoluminescence experimental_PhotosRes101_195-204

Thermoluminescence measurement: example



From: Ducruet JM_Vass I_2009_Thermoluminescence experimental_PhotosRes101_195-204

The basis for measurement of photosynthesis via fluorescence kinetics: competition for the S1 excited state



Measurement of *in vivo* chlorophyll fluorescence kinetics

Why?

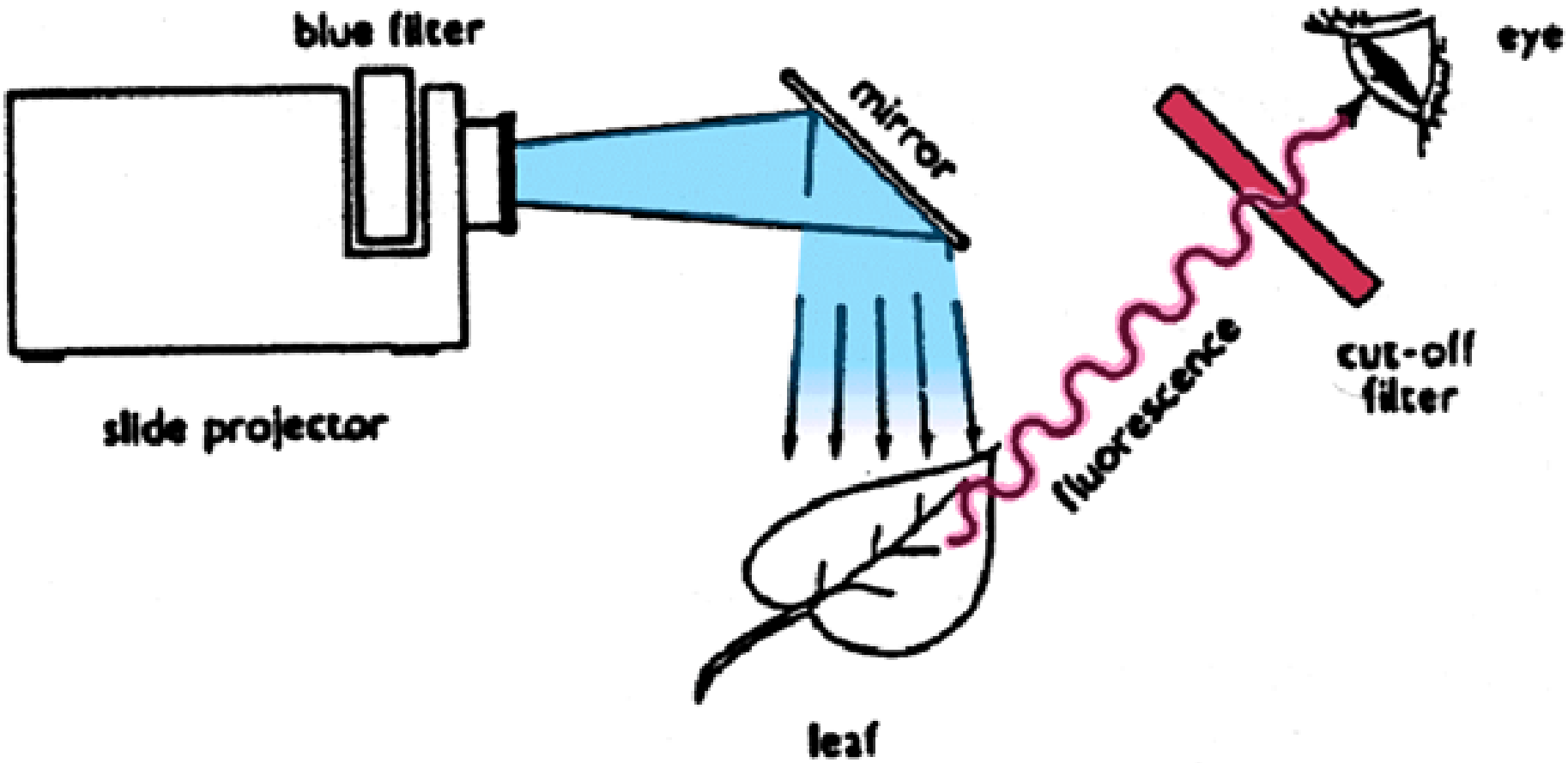
The quantum yield of in vivo chlorophyll fluorescence depends on a competition for excitons between photochemistry (including electron transport after PSII via feedback), thermal relaxation ("nonphotochemical quenching") and fluorescence.

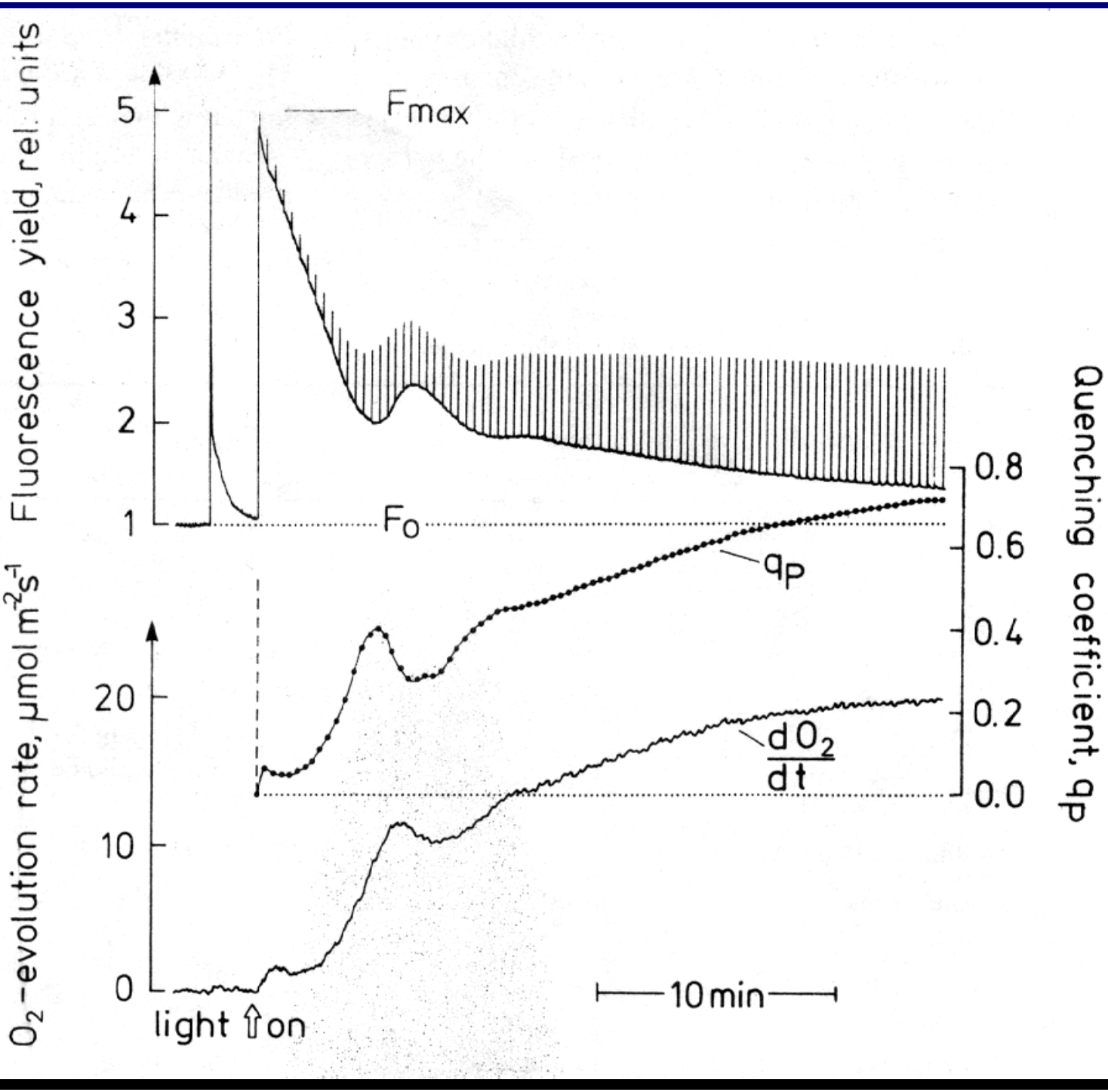
--> The fluorescence quantum yield and especially its change in response to changes in actinic irradiance allows a detailed assessment of photosystem II function und thus the vitality of a cell, tissue, plant or even ecosystem.

Examples of Applications

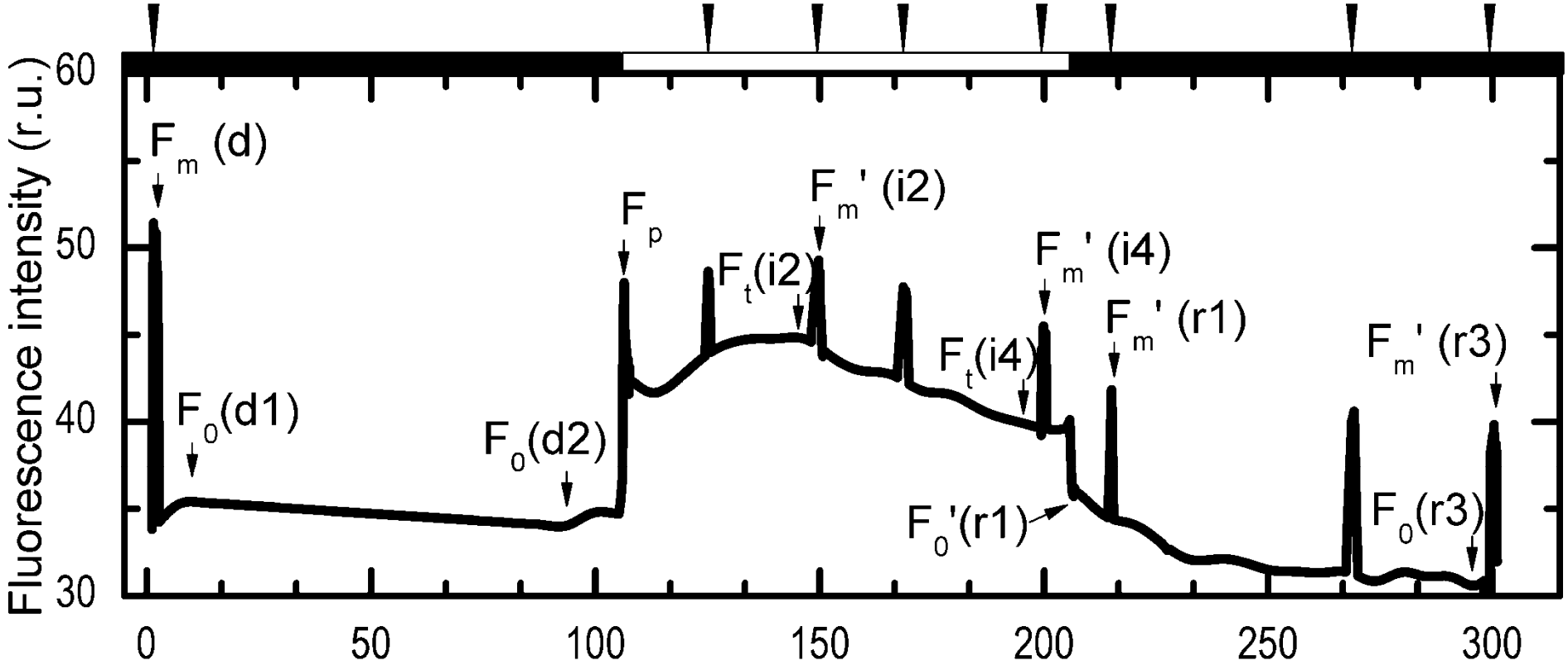
- biophysical investigations of mechanisms of photosynthesis
- studies of the effects of abiotic and biotic stress on plants
- ecophysiological studies
- fruit quality assessment

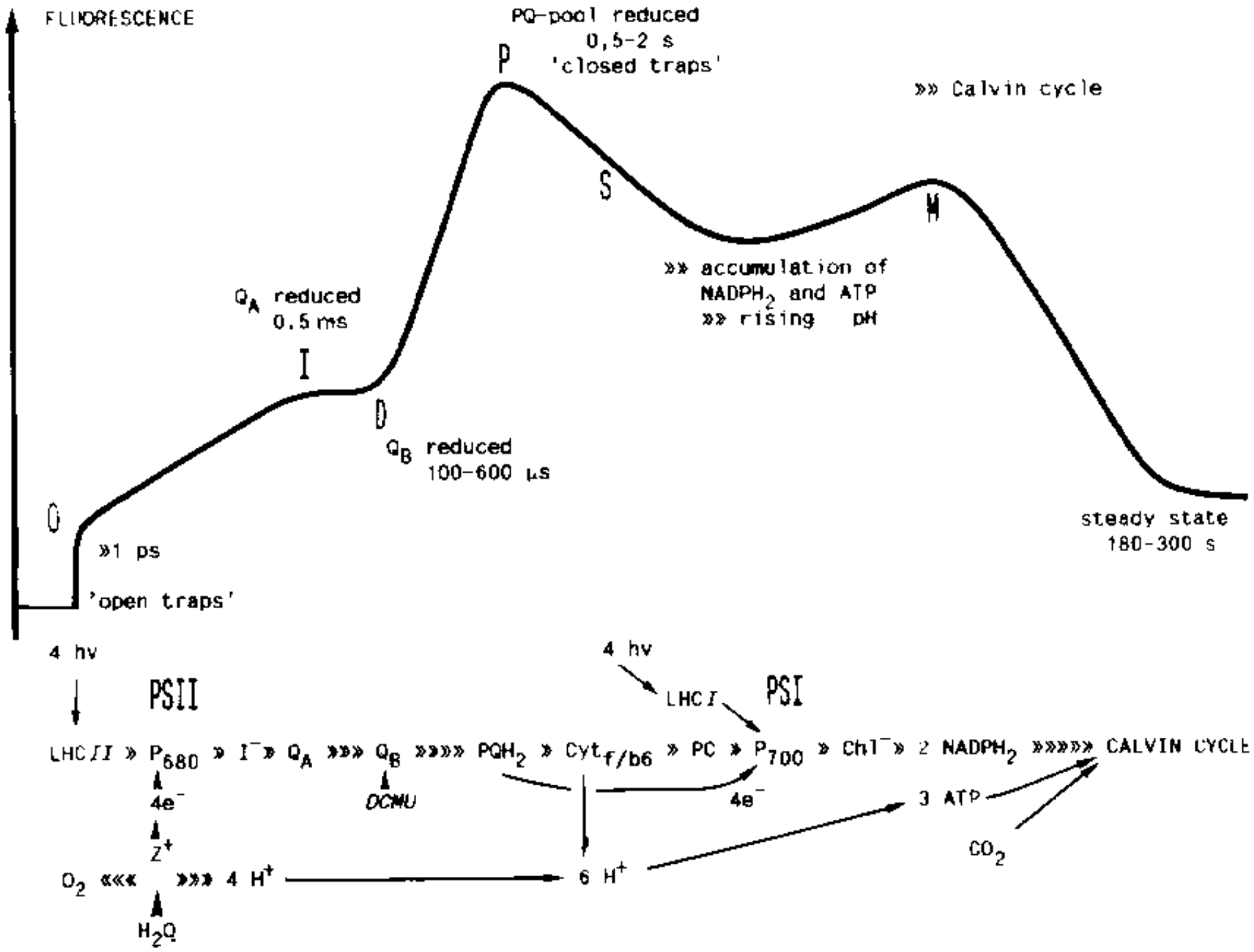
Chlorophyll-Fluoreszenz

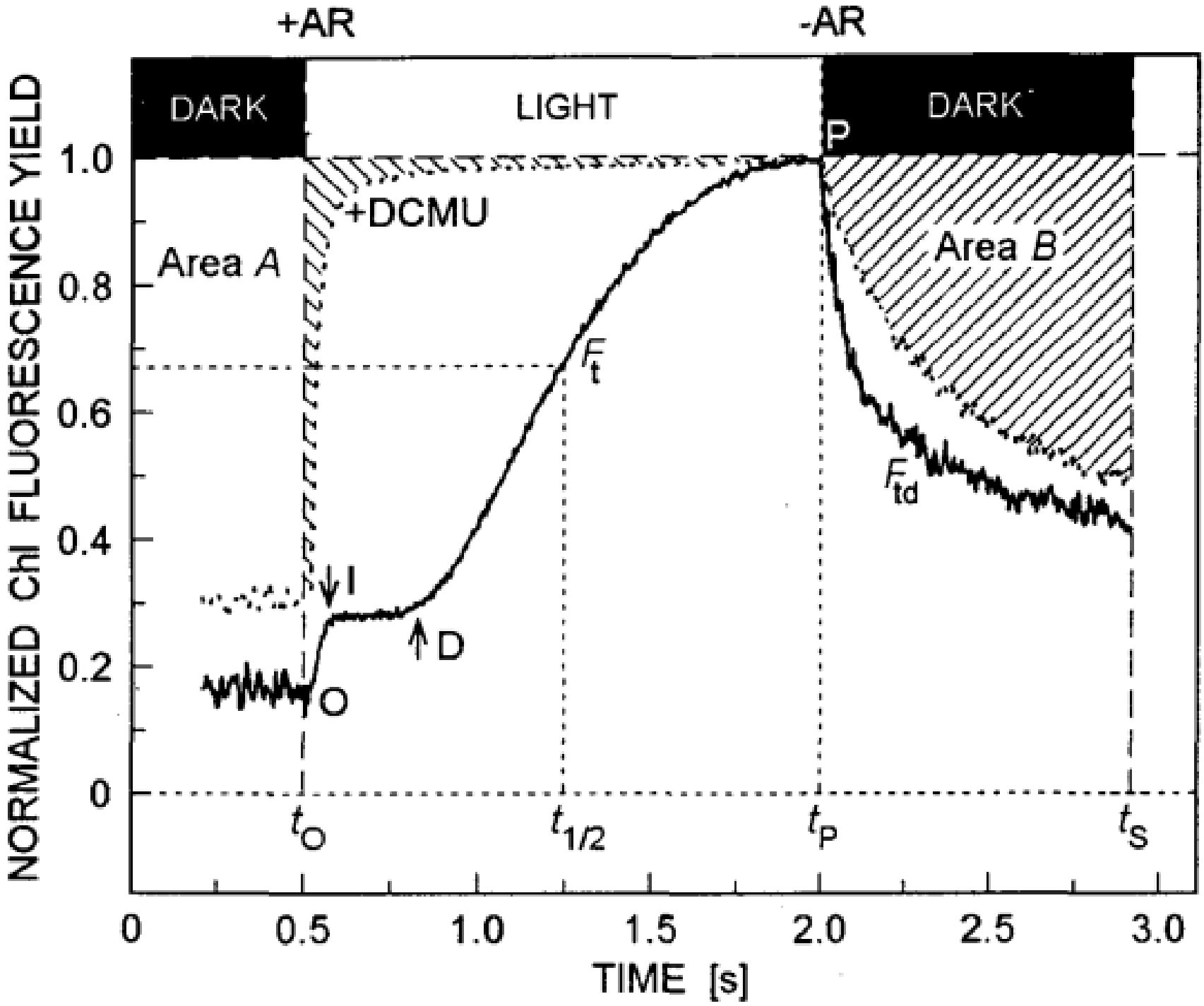


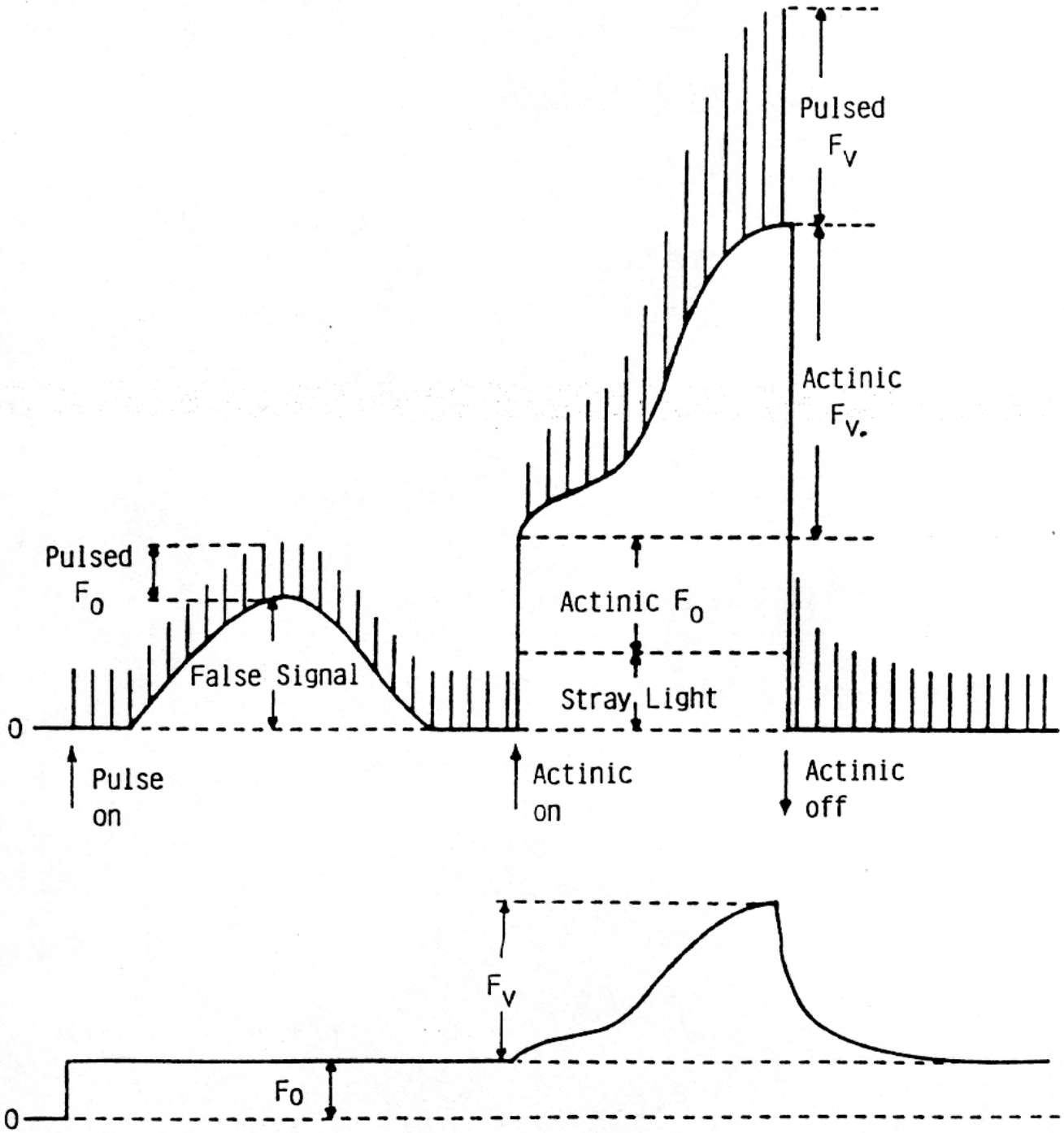

Fluorescence yield, rel. units
5
4
3
2
1
Fmax
Fo
qp
Quenching coefficient, qp
0.8
0.6
0.4
0.2
0.0
O2-evolution rate, µmol m-2s-1
20
10
0
dO2/dt
light ⇧on
10 min

Wichtigste Symbole in der Chlorophyll-Fluoreszenzmessung

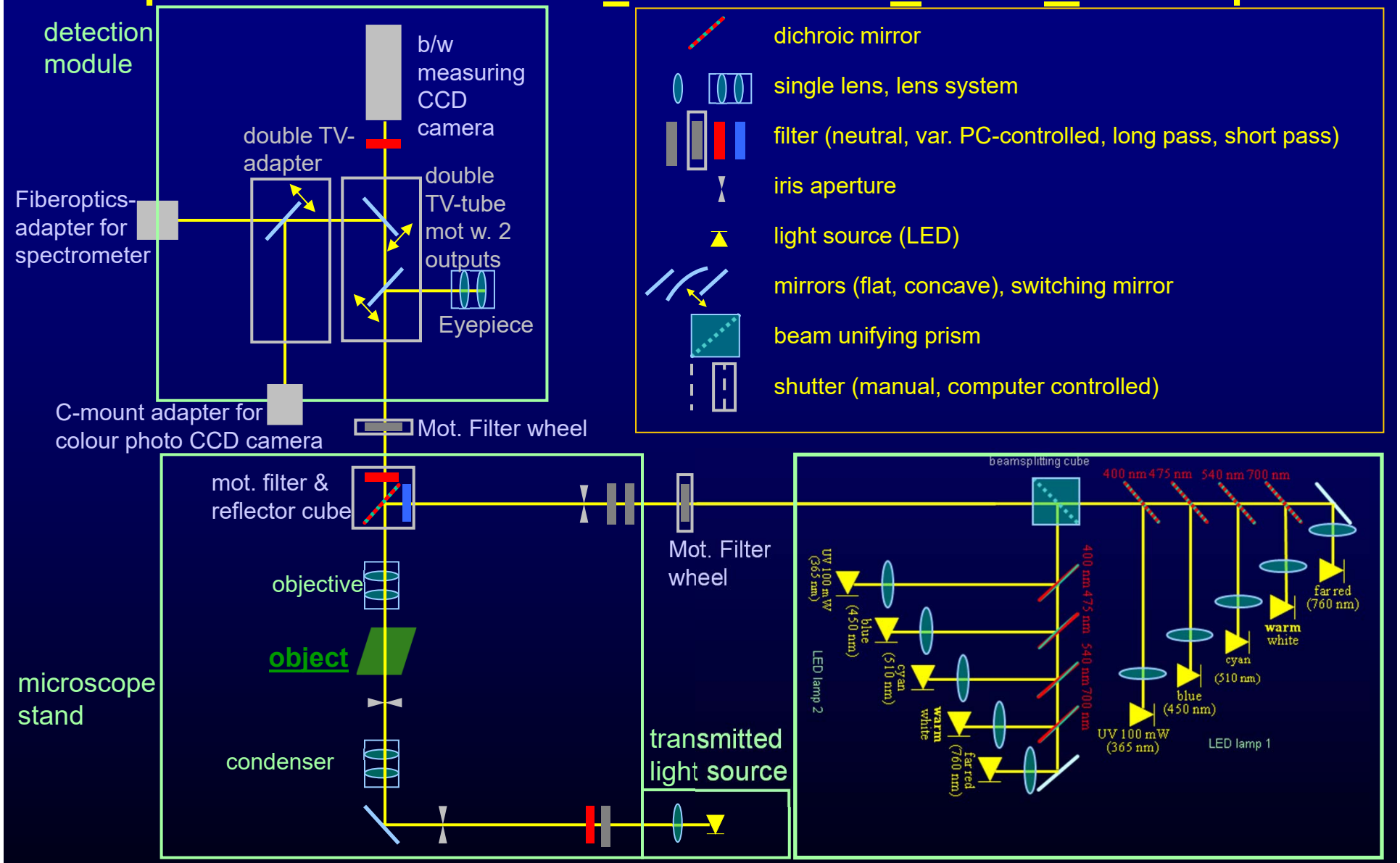



FLUORESCENCE
PQ-pool reduced
0,5-2 s
'closed traps'
P
»» Calvin cycle
S
M
»» accumulation of
NADPH2 and ATP
»» rising pH
QA reduced
0.5 ms
I
D
QB reduced
100-600 µs
O
»1 ps
'open traps'
steady state
180-300 s
4 hv
PSII
4 hv
LHC I
PSI
LHC II » P680 » I⁻» QA »»» QB »»»» PQH2 » Cytf/b6 » PC » P700 » Chl⁻» 2 NADPH2 »»»»» CALVIN CYCLE
4e⁻
DCMU
4e⁻
3 ATP
Z+
CO2
O2 ««« »»» 4 H+
6 H+
H2O


+AR
-AR
DARK
LIGHT
DARK
P
+DCMU
Area A
Area B
F_t
F_{td}
I
D
O
NORMALIZED Chl FLUORESCENCE YIELD
1.0
0.8
0.6
0.4
0.2
0
t_O
$t_{1/2}$
t_P
t_S
0
0.5
1.0
1.5
2.0
2.5
3.0
TIME [s]


Pulsed
F_v
Actinic
F_v.
Pulsed
F_o
Actinic F_o
False Signal
Stray Light
0
Pulse
on
Actinic
on
Actinic
off
F_v
F_o
0

Biophysical measurements *in vivo* with temporal, spatial and spectral resolution: the Fluorescence *Kinetic* Microscope



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

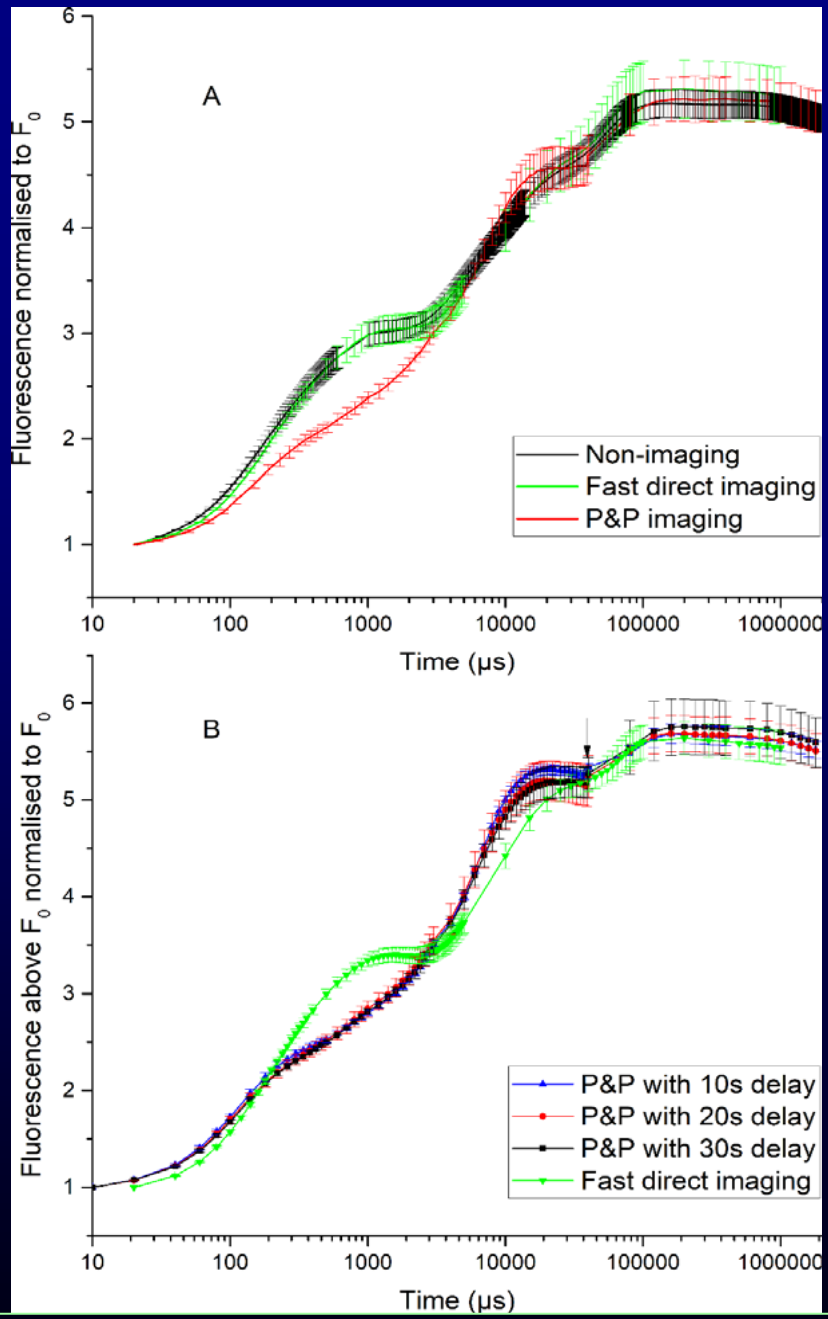
Küpper H, Šetlík I, Trtilek M, Nedbal L (2000) Photosynthetica 38(4), 553-570

Analysis of OJIP chlorophyll fluorescence kinetics & QA re-oxidation kinetics by direct fast imaging

Comparison of different OJIP measuring methods using *Arabidopsis thaliana* leaves

A) Comparison of non-imaging direct measurement, imaging measurement with pump-and-probe (P&P), and fast direct imaging. The values represent the average ±SD of five independent measurements (leaves of similar age on different plants).

B) Comparison of different delay times for the P&P sequences with fast direct imaging.
The values represent the average ±SD of four independent measurements (leaves of similar age on different plants).

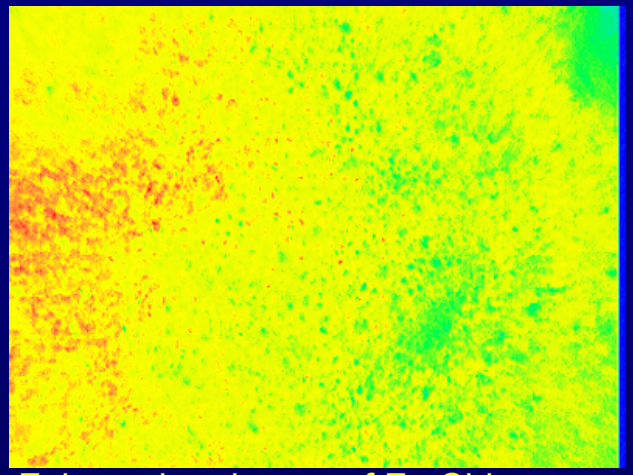


Küpper H, Benedikty Z, Morina F, Andresen E, Mishra A, Trtílek M (2019) Plant Physiology 179, 369-381, DOI: https://doi.org/10.1104/pp.18.00953

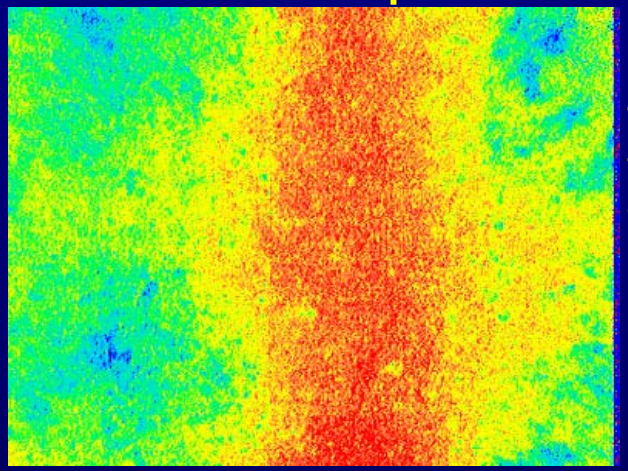
Fluorescence *kinetic* microscopy

Methods of data processing

Method 1: images of fluorescence parameters



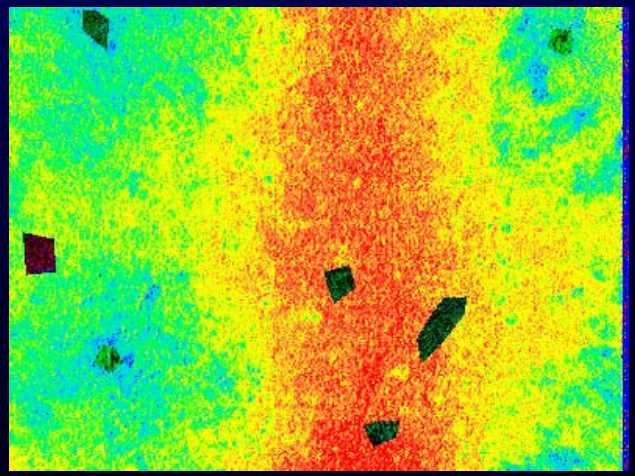
False colour image of F_m Chl fluorescence calculated from fluorescence kinetic film



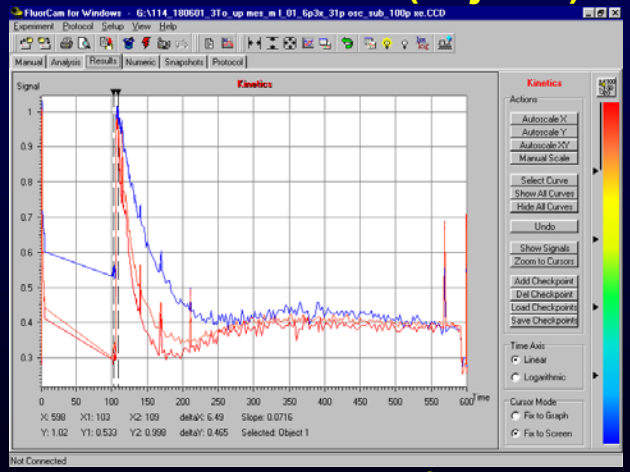
False colour map of F_v/F_m, showing the **differences in this parameter over the entire image**.

To obtain images of fluorescence parameters, frames within the relevant time periods are selected and the necessary mathematical operations are performed on every pixel.

Method 2: kinetics of selected areas (objects)



Manual selection of objects for kinetic analysis.

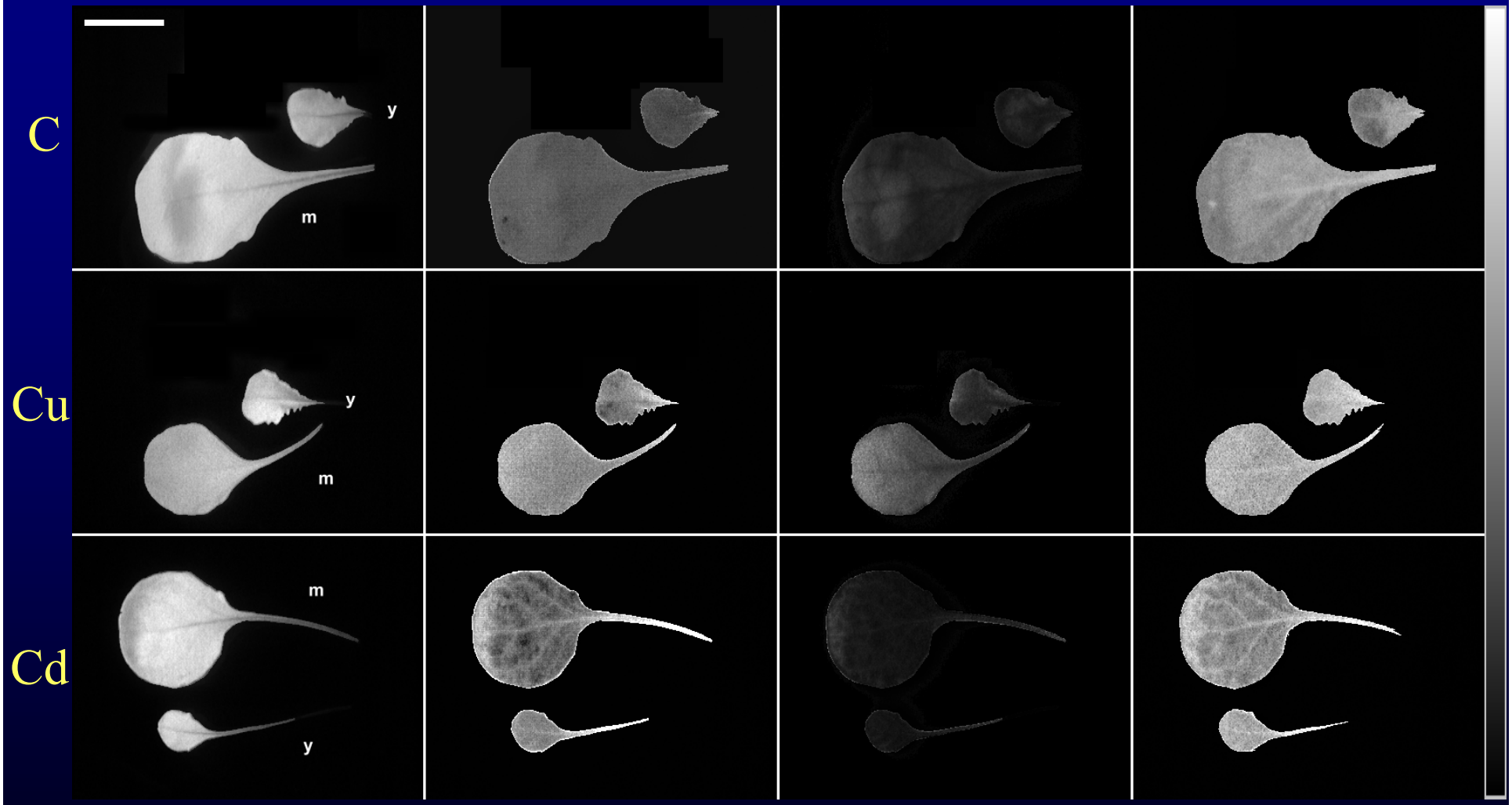


Fluorescence induction of selected objects, showing **all differences in kinetics for representative cells**.

To obtain kinetic traces, the relevant regions are selected on a captured frame or parameter image. The kinetics of all pixels within the selected areas are averaged.

Cd-stressed *Thlaspi caerulescens*

Images of PS II activity parameters



Fluorescence yield during F_m

Efficiency of PS II F_v/F_m

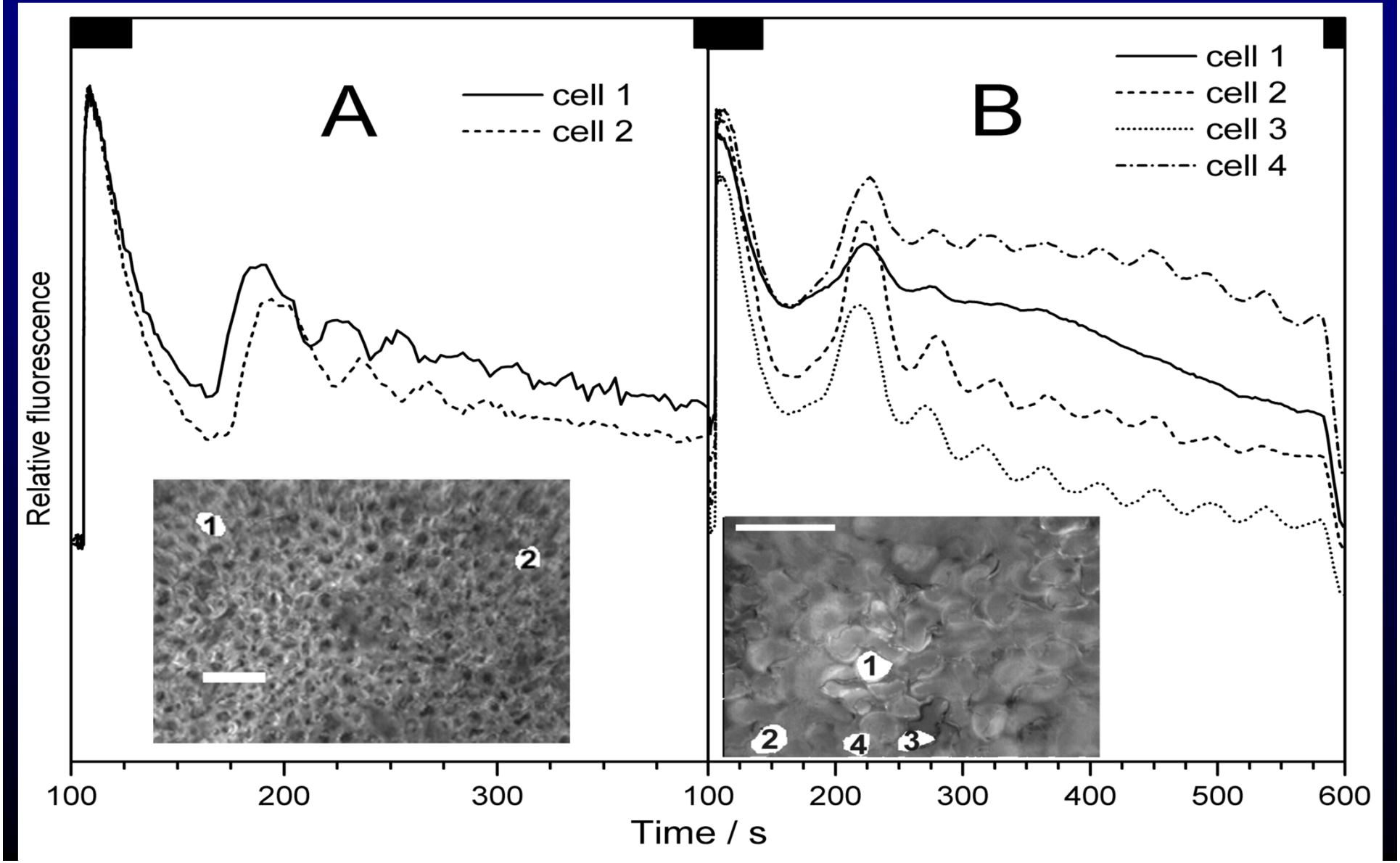
Light saturation F_m/F_p

Electron flow through PS II during actinic irradiance $(F_m'-F_t)/F_m'$

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

Spatial heterogeneity of photosynthetic oscillations over the leaf surface

Insets: fluorescence emission images (F_p); the white bar represents 100 µm



Cd-stress in the Zn-/Cd-hyperaccumulator *T. caerulescens:* images of PSII activity parameters

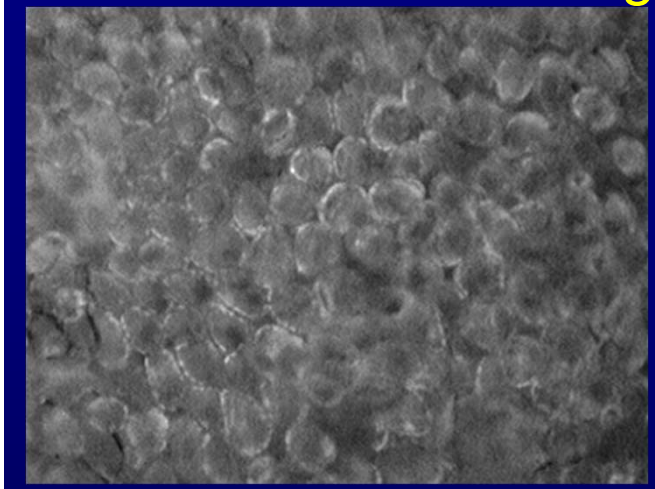


Image of F_m of an unstressed mature leaf

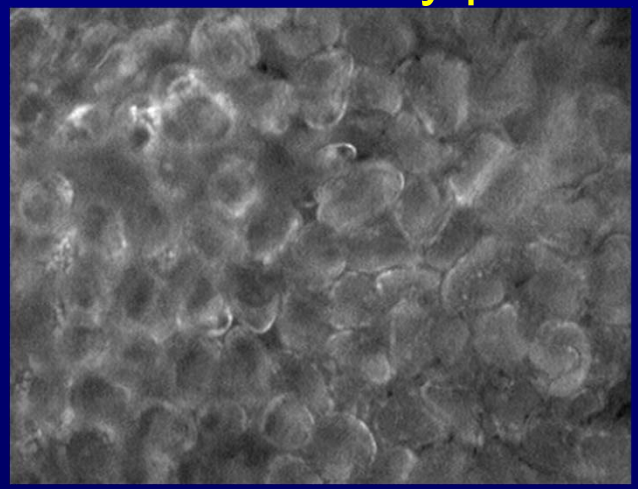


Image of F_m of a leaf stressed with 50µM Cd^2, showing bright cells

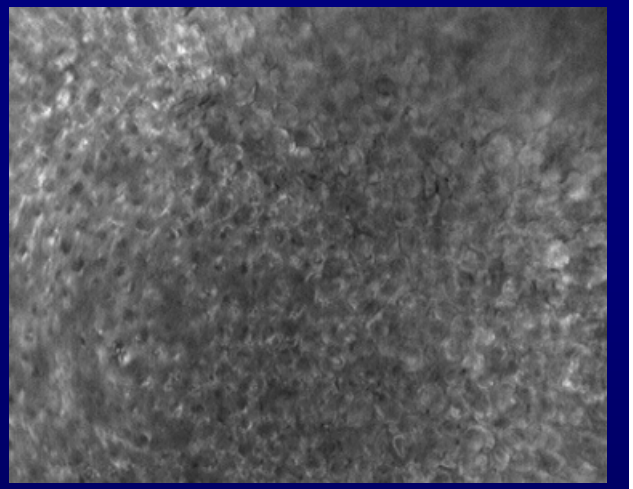


Image of F_m of the same sample as on the left, lower magnification

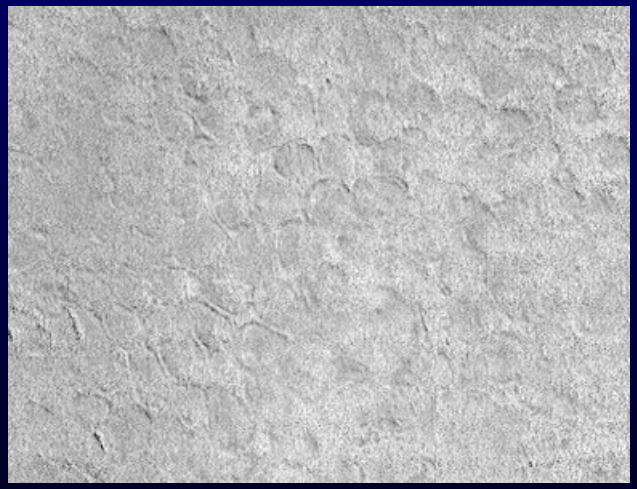


Image of F_v/F_m of the same sample as above, showing the homogeneously high photosynthtic activity of a healthy leaf of this plant

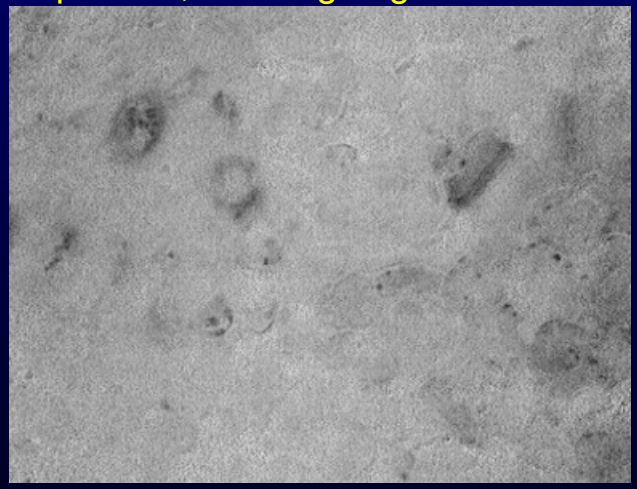


Image of F_v/F_m of the same sample as above, showing the low photosynthtic activity of the bright cells

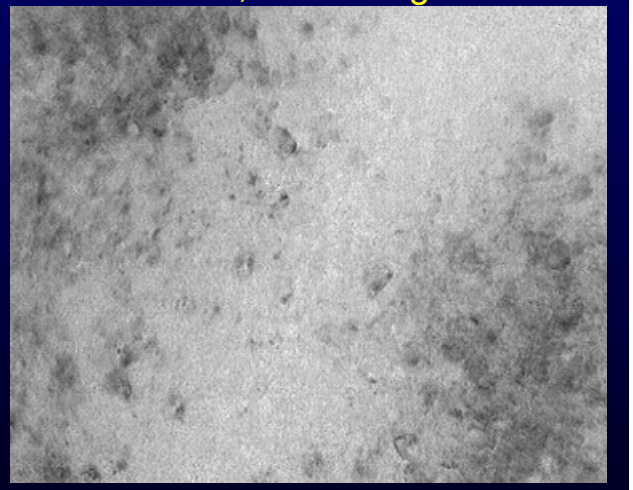
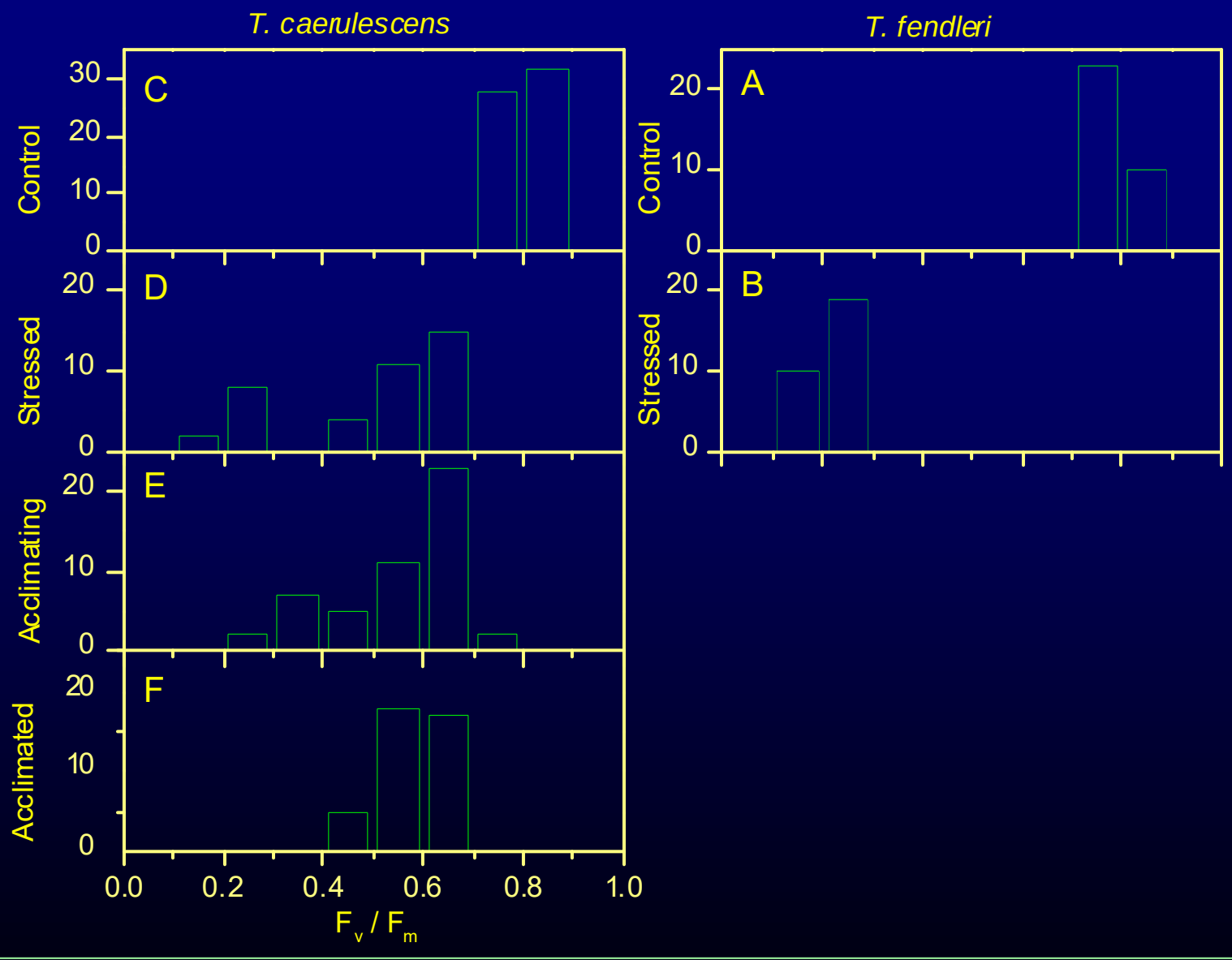


Image of F_v/F_m of the same sample as on the left, but with lower magnification

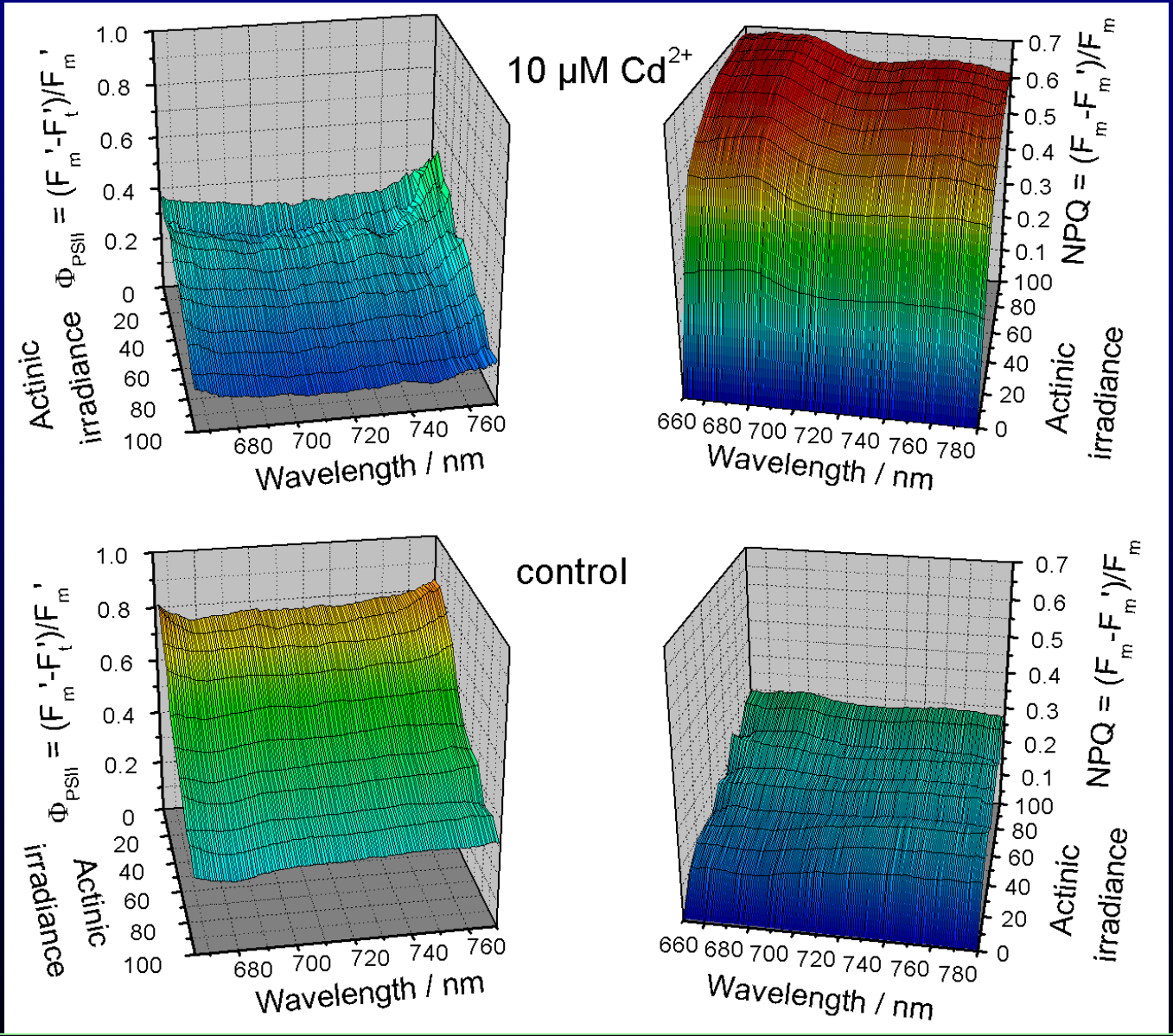
Küpper H, Aravind P, Leitenmaier B, Trtilek M, Šetlík I (2007) New Phytol175, 655-74

Cd-stress and acclimation in *T. caerulescens & T. fendleri:* histograms of F_v/F_m



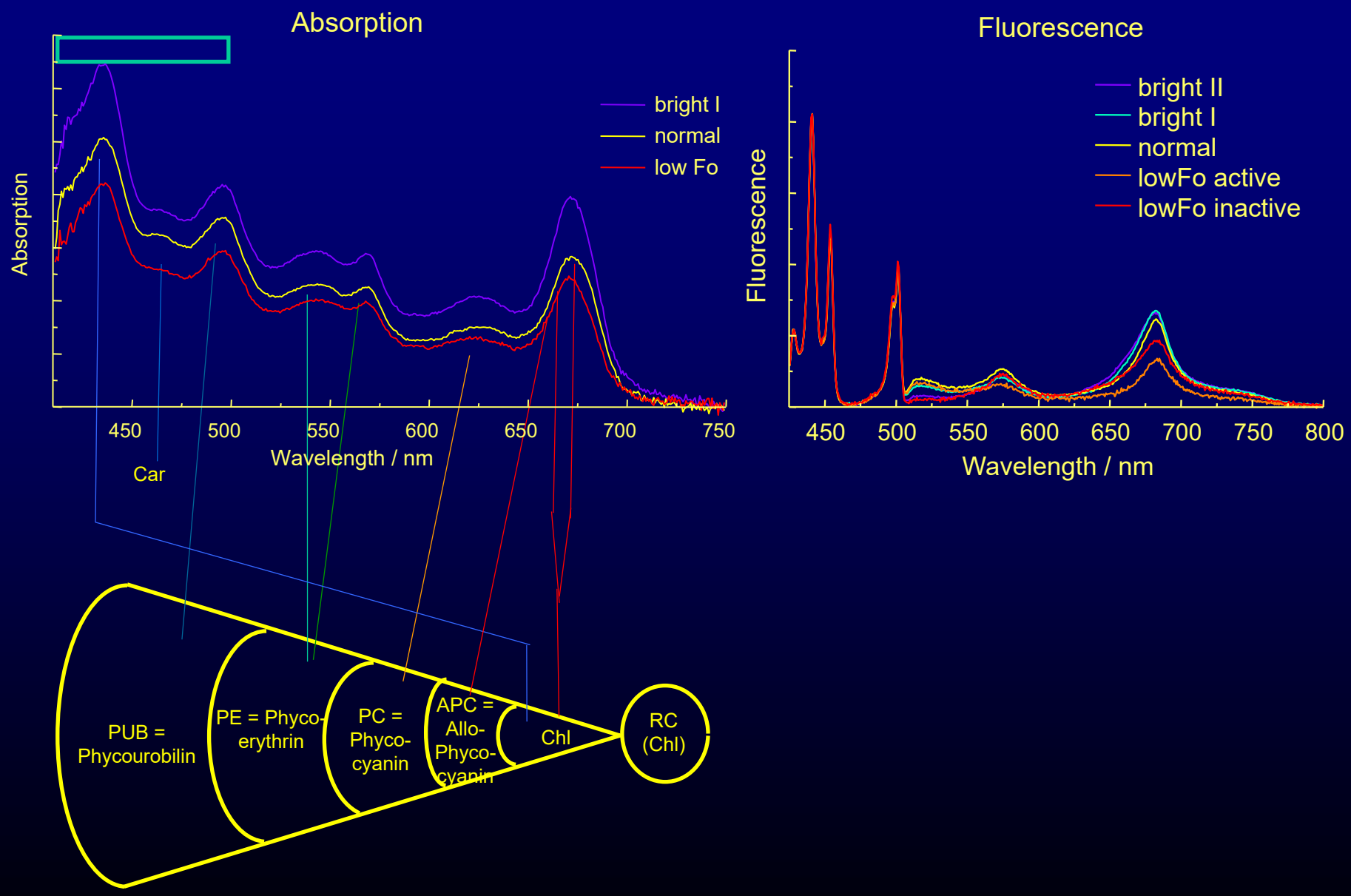
Küpper H, Aravind P, Leitenmaier B, Trtilek M, Šetlík I (2007) New Phytol175, 655-74

Spectrally resolved fluorescence kinetic parameters (II)



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

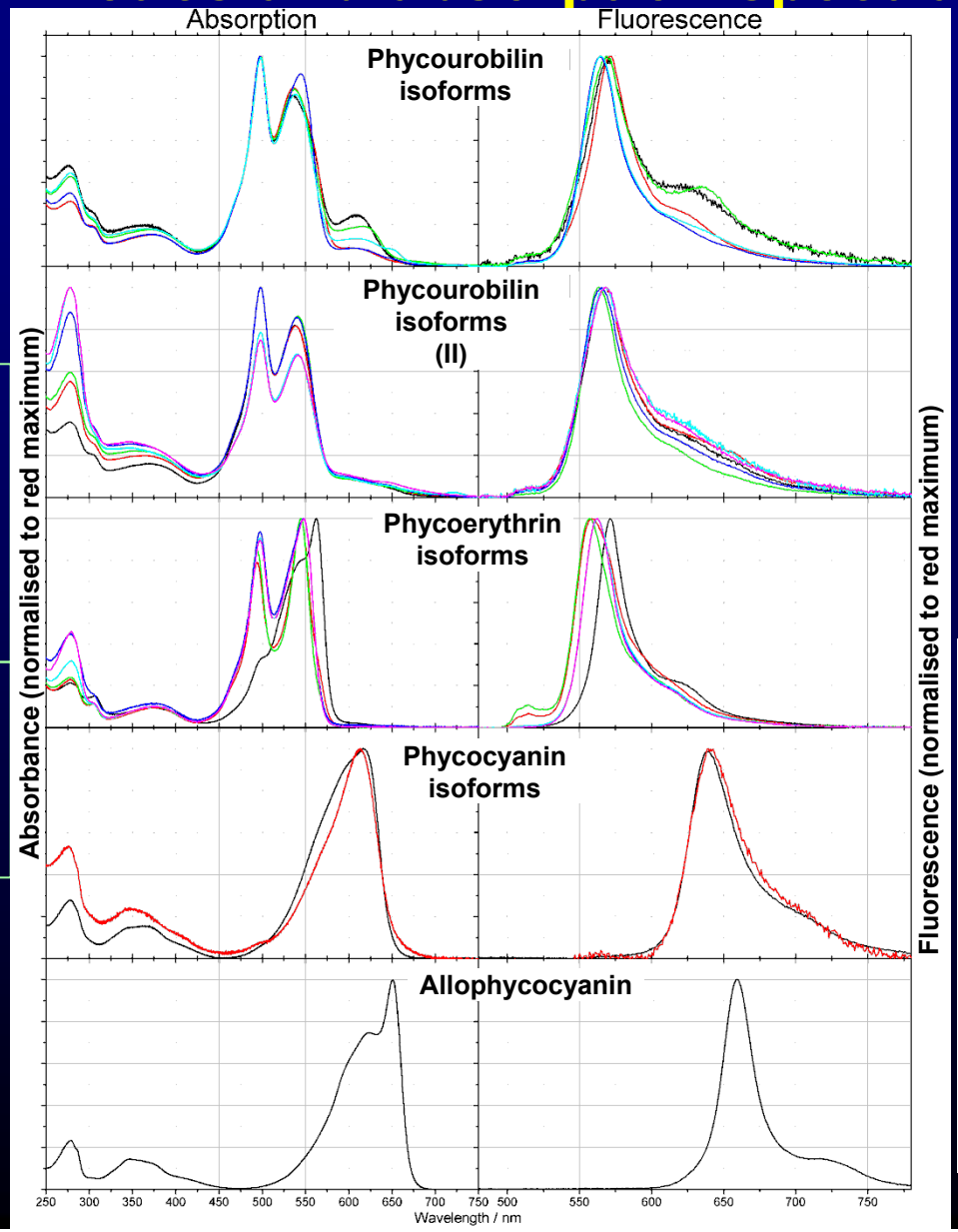
Spectrally resolved fluorescence kinetic parameters (I)

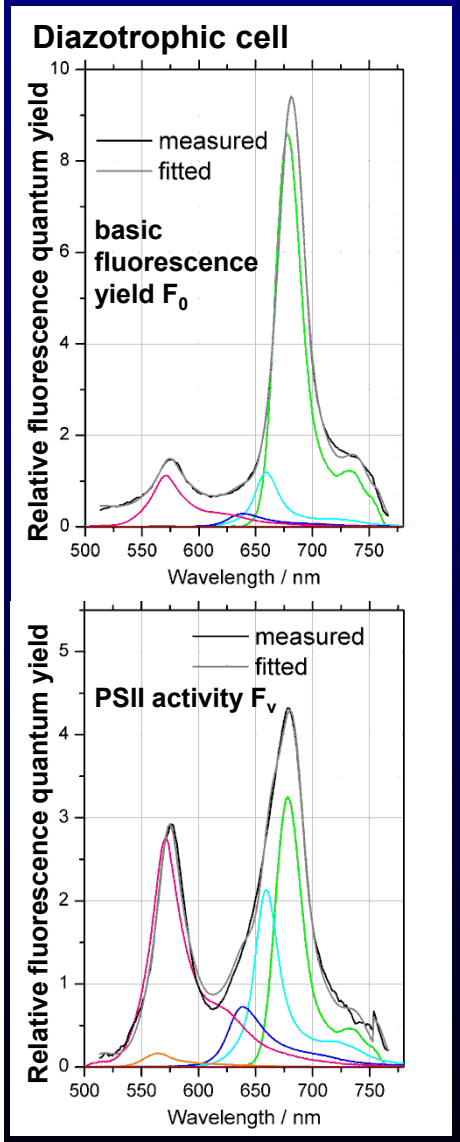


Purification of *Trichodesmium* phycobiliproteins for deconvoluting spectrally resolved *in vivo* fluorescence kinetics and absorption spectra

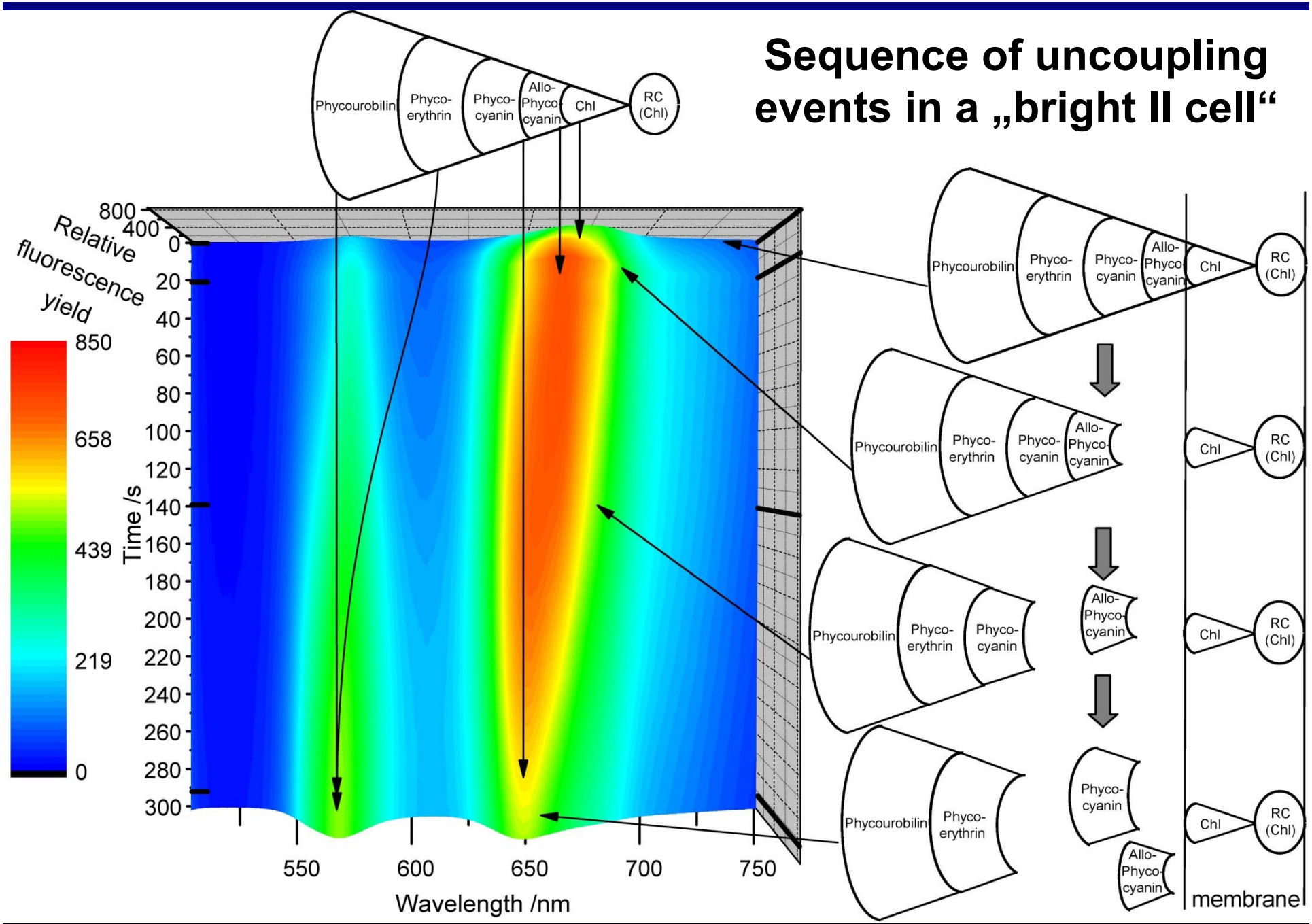
Phycobiliprotein purification + characterisation: Küpper H, Andresen E, Wiegert S, Šimek M, Leitenmaier B, Šetlík I (2009) Biochim. Biophys. Acta (Bioenergetics) 1787, 155-167

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



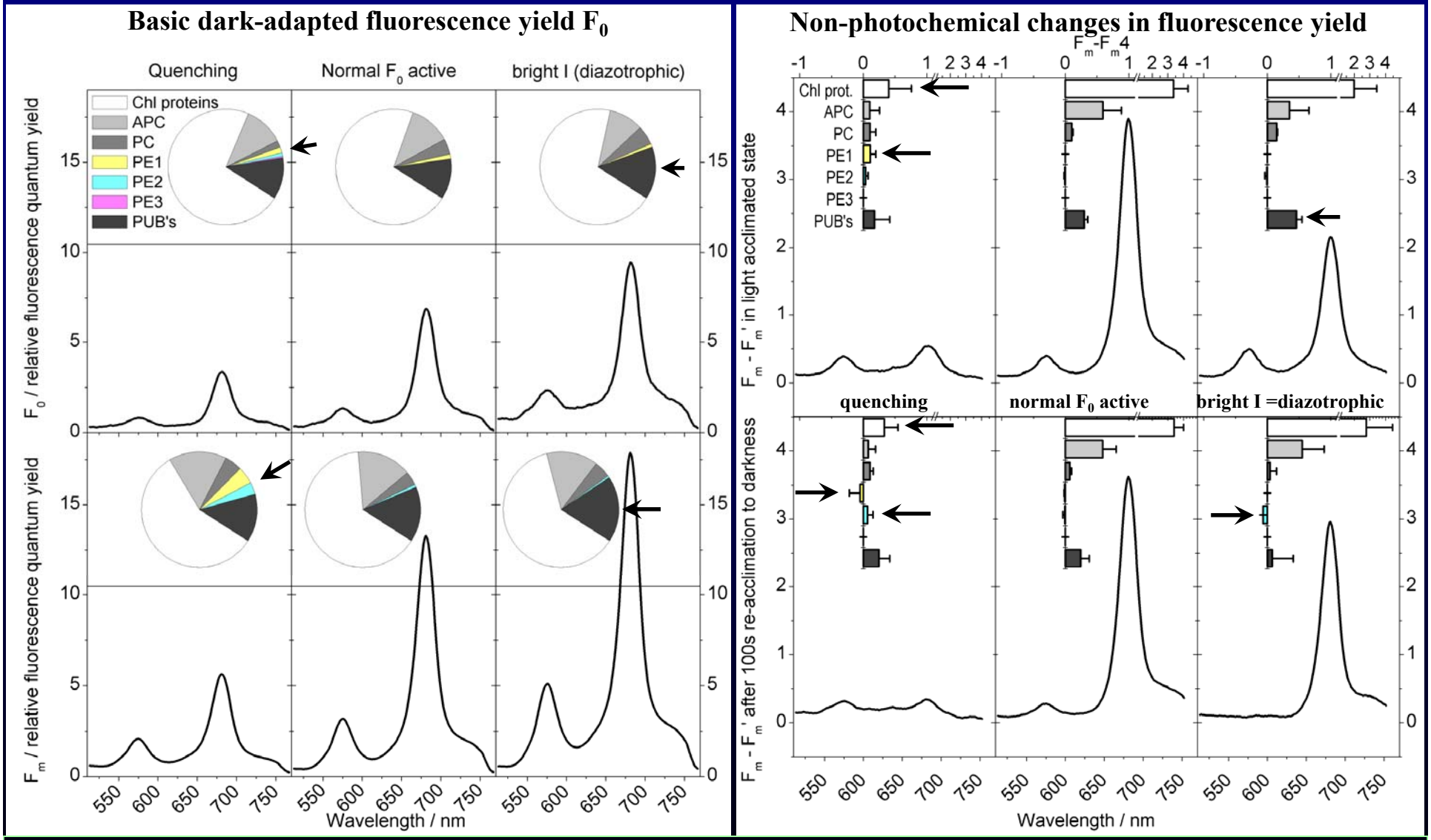


Sequence of uncoupling events in a „bright II cell“

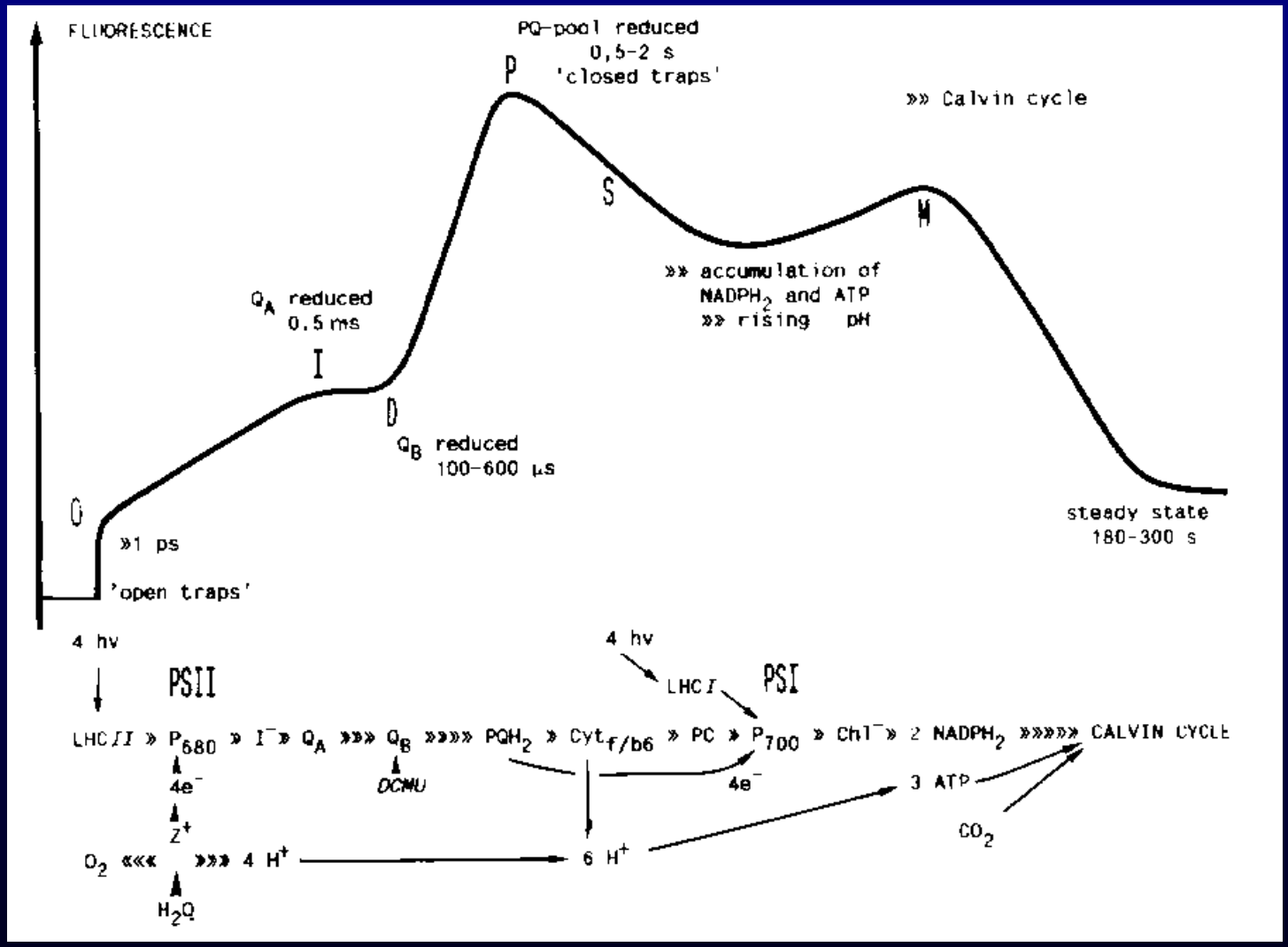


Küpper H, Andresen E, Wiegert S, Šimek M, Leitenmaier B, Šetlík I (2009) Biochim. Biophys. Acta (Bioenergetics) 1787, 155-167

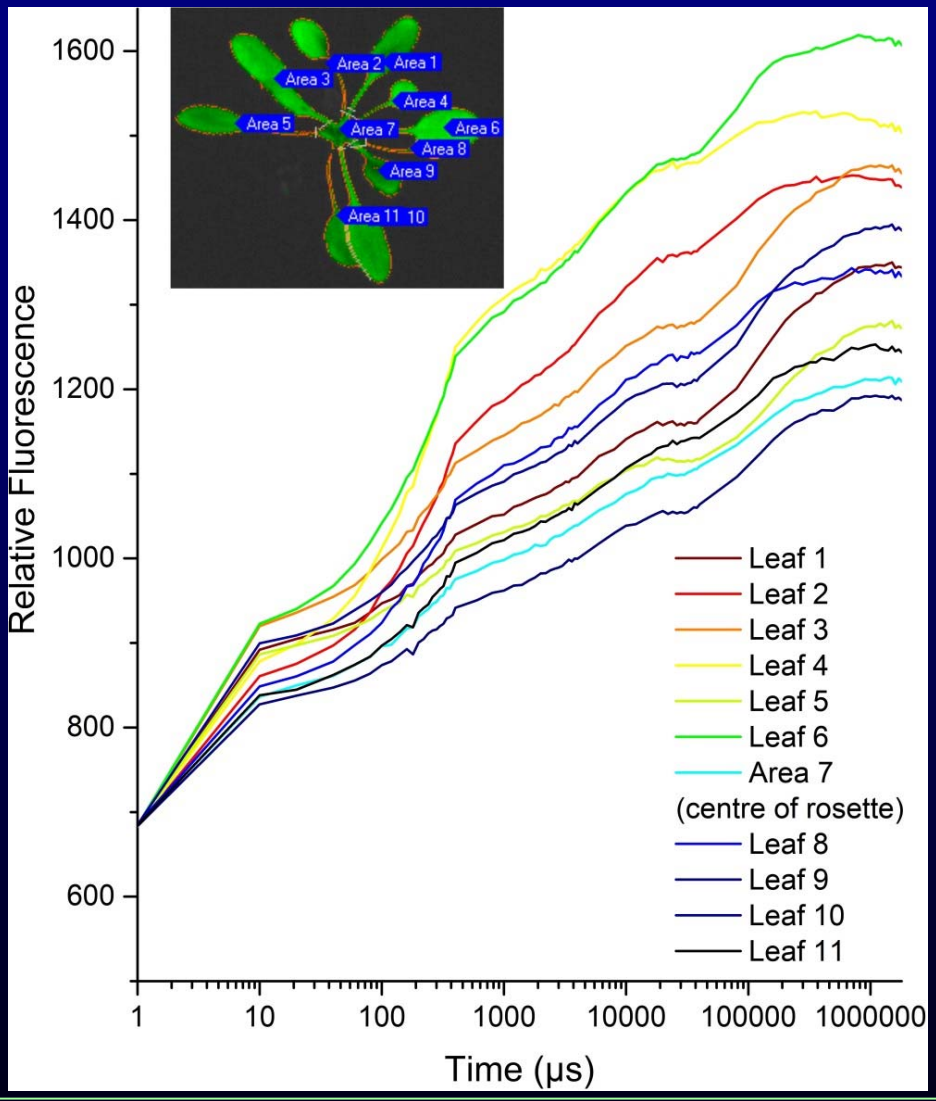
Deconvolution of spectrally resolved *in vivo* fluorescence kinetics shows reversible coupling of individual phycobiliproteins

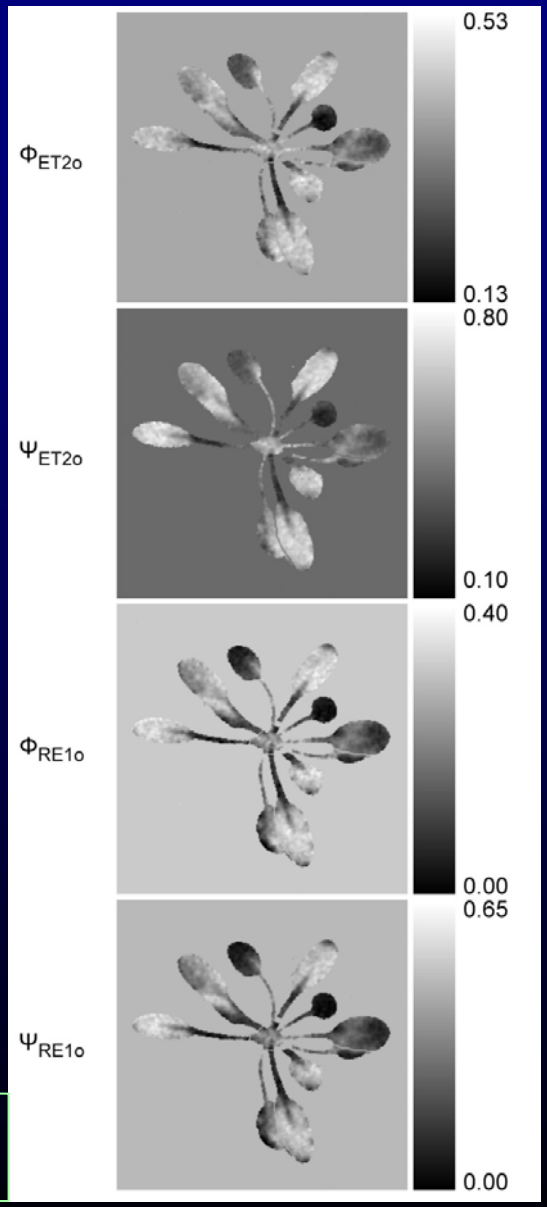


Küpper H, Andresen E, Wiegert S, Šimek M, Leitenmaier B, Šetlík I (2009) Biochim. Biophys. Acta (Bioenergetics) 1787, 155-167


FLUORESCENCE
PQ-pool reduced
0,5-2 s
'closed traps'
P
»» Calvin cycle
S
M
»» accumulation of
NADPH2 and ATP
»» rising pH
QA reduced
0.5 ms
I
D
QB reduced
100-600 µs
O
»1 ps
'open traps'
steady state
180-300 s
4 hv
PSII
4 hv
LHCI
PSI
LHCII » P680 » I⁻» QA »»» QB »»»» PQH2 » Cytf/b6 » PC » P700 » Chl⁻» 2 NADPH2 »»»»» CALVIN CYCLE
4e⁻
DCMU
4e⁻
3 ATP
Z+
CO2
O2 «««
»»» 4 H+
6 H+
H2O

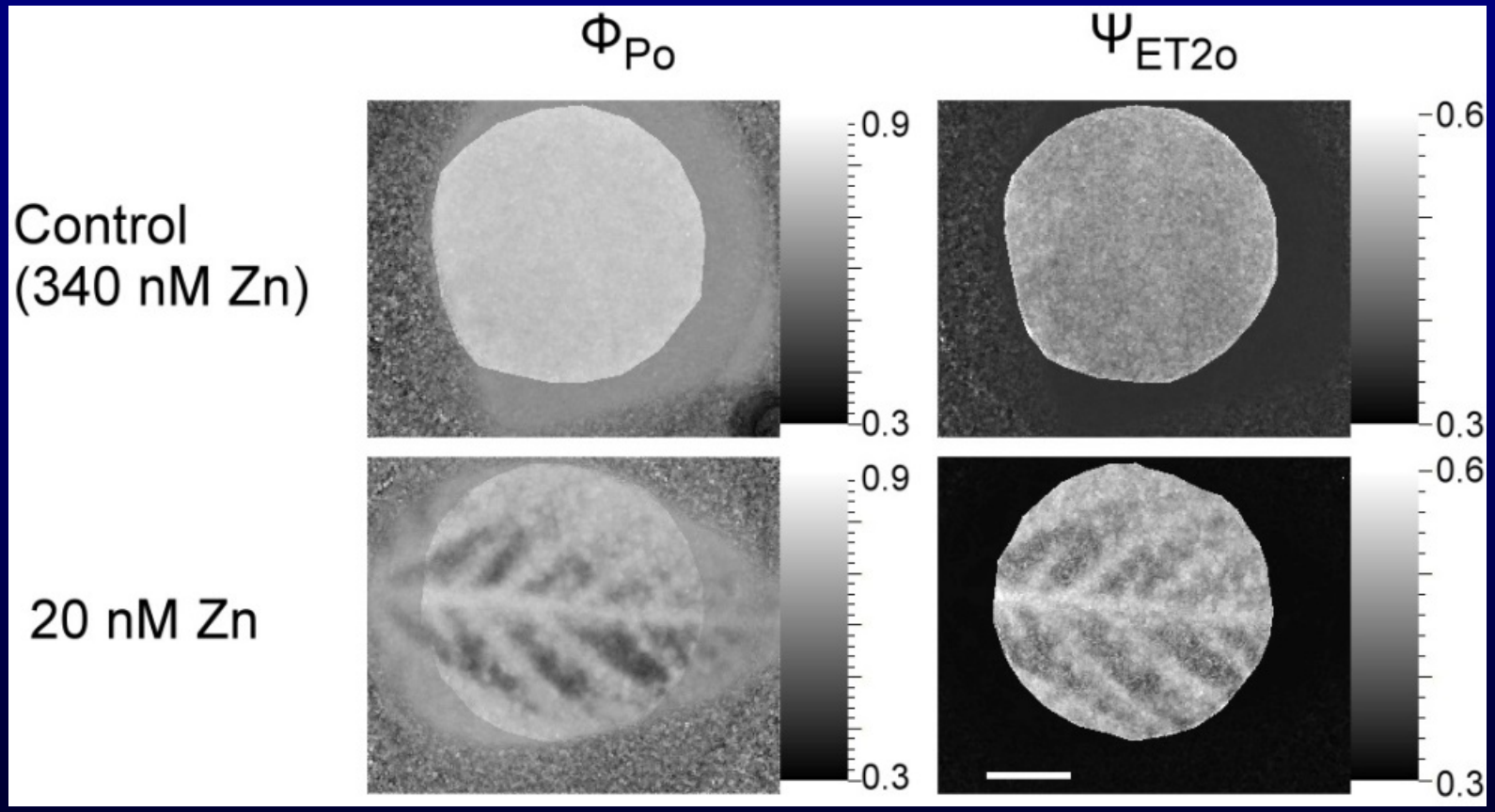
Differences in OJIP kinetics between leaves of an *A. thaliana* plant measured by direct fast imaging





Küpper H, Benedikty Z, Morina F, Andresen E, Mishra A, Trtílek M (2019) Plant Physiology 179, 369-381, DOI: https://doi.org/10.1104/pp.18.00953

Changes of Ψ_{ET2o} and Φ_{Po} in response to zinc deficiency stress



Soybeans treated with "0" Zn addition (20 nM residue from chemicals and water) compared to "control" (340 nM Zn) for 6 weeks. Scale bar is 1 cm.

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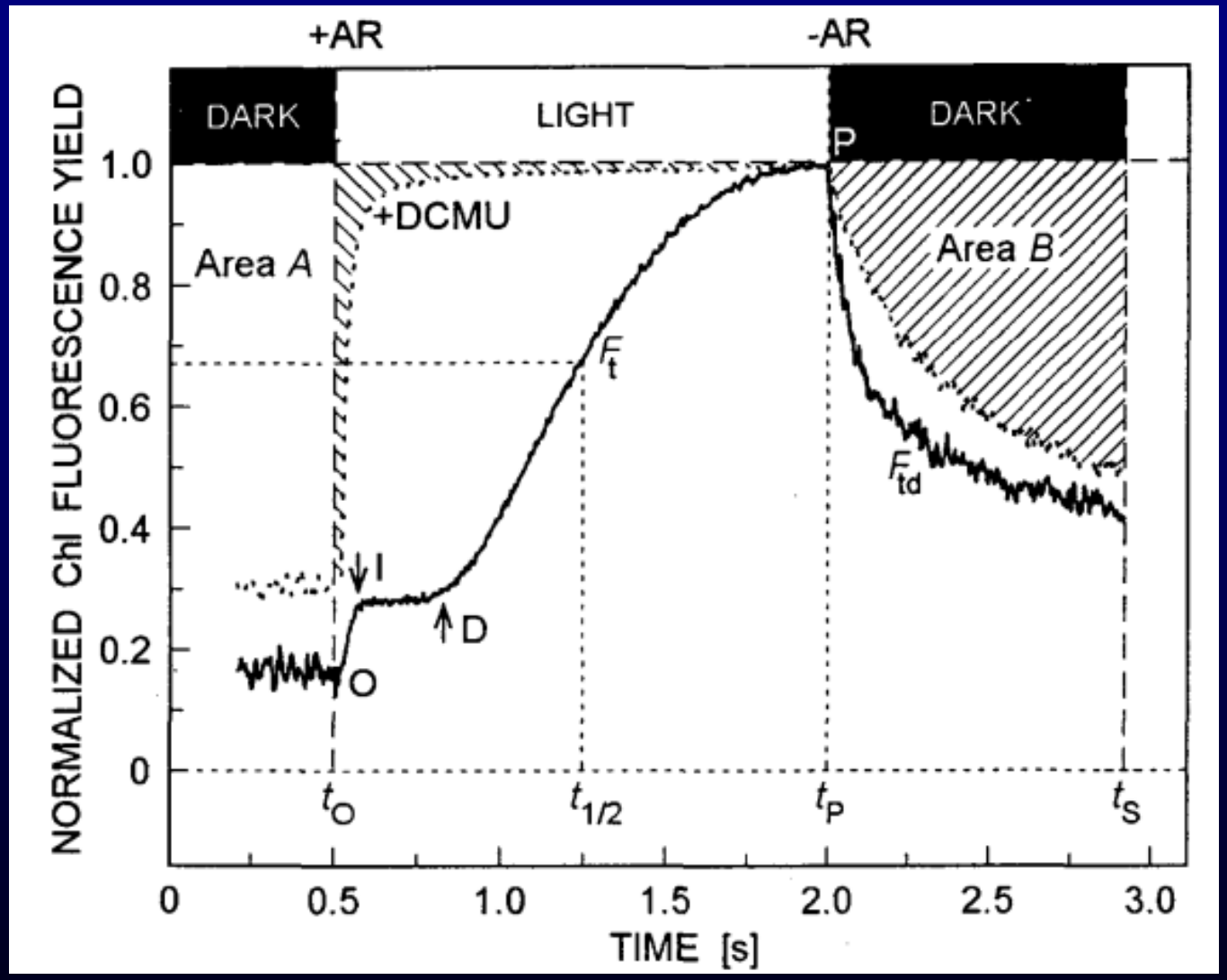
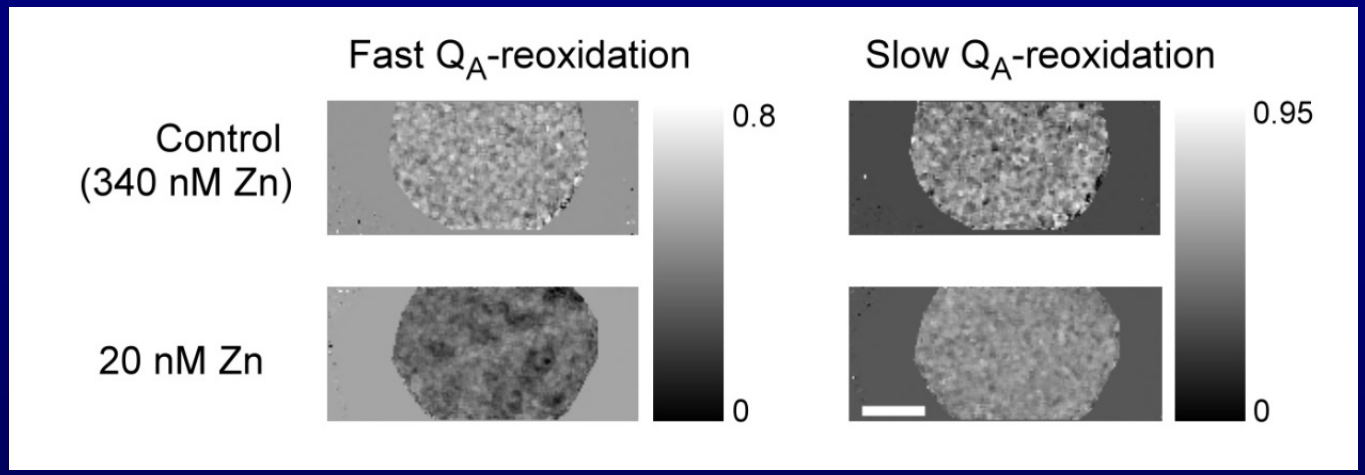
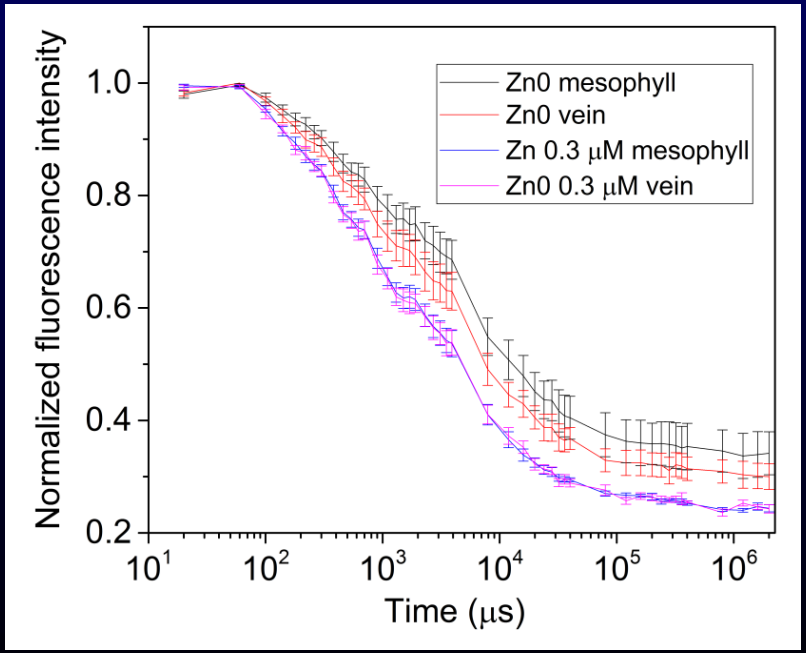

+AR
-AR
DARK
LIGHT
DARK
P
+DCMU
Area A
Area B
F_t
F_{td}
I
D
O
NORMALIZED Chl FLUORESCENCE YIELD
1.0
0.8
0.6
0.4
0.2
0
t_O
$t_{1/2}$
t_P
t_S
0
0.5
1.0
1.5
2.0
2.5
3.0
TIME [s]

Differences in Q_A re-oxidation kinetics of veins (bundle sheath cells) and regular mesophyll cells in response to zinc deficiency stress



Soybeans treated with "0" Zn addition (20 nM residue from chemicals and water) compared to "control" (340 nM Zn) for 6 weeks.
Scale bar is 1 cm.



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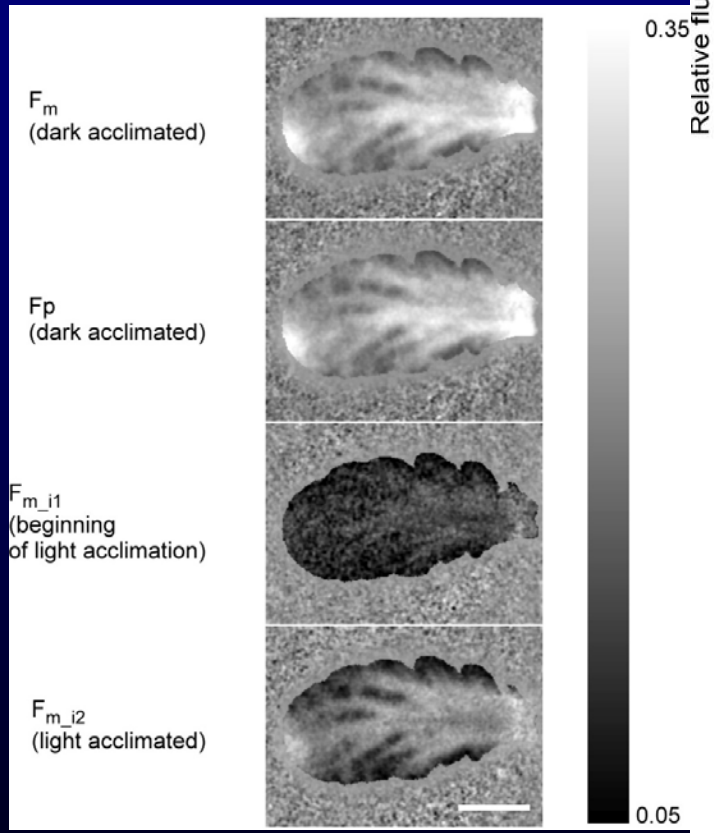
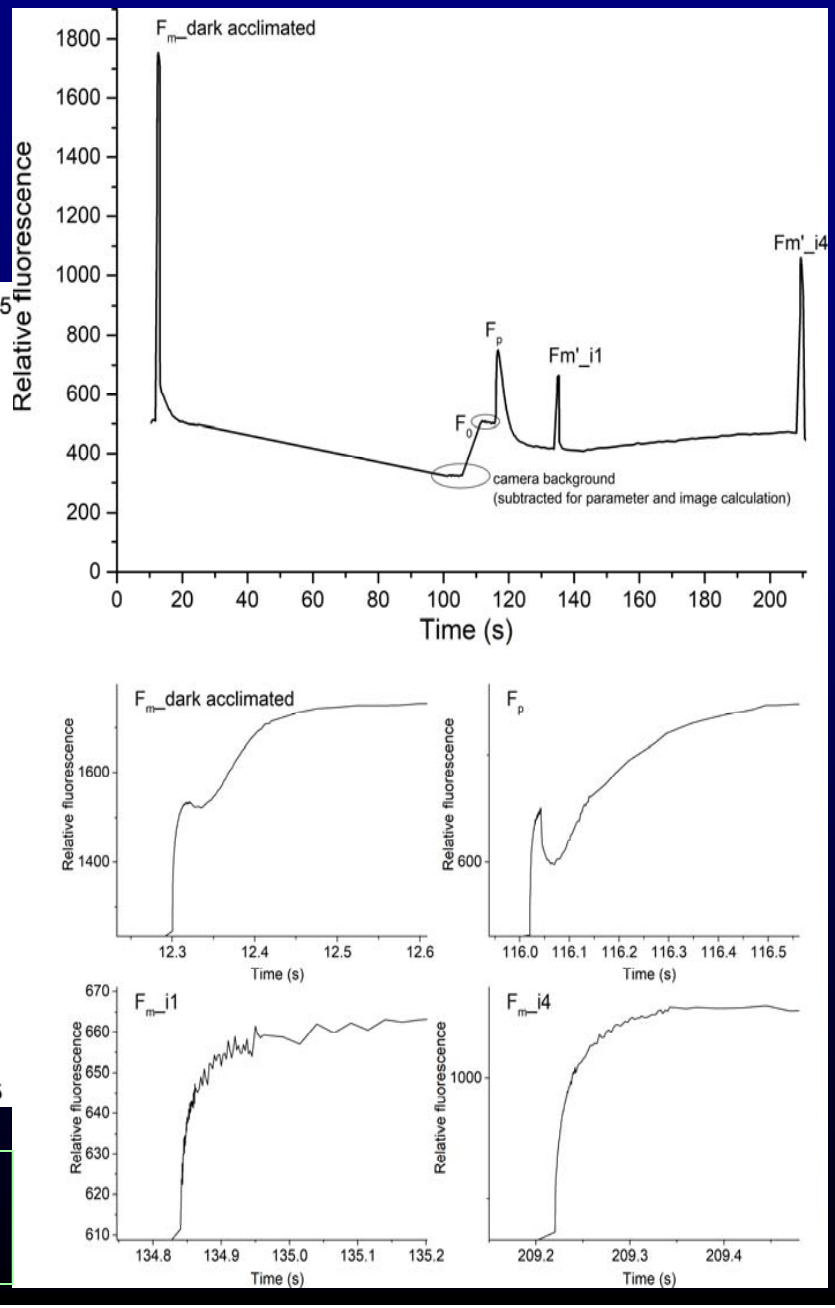
Multi-OJIP protocol for analysis of adaptation to actinic light

Right: The shape of the fluorescence rises of each peak are shown below the main graph. The measured sample was a young-mature leaf of the Cd/Zn hyperaccumulator Noccaea caerulescens grown with replete (non-toxic) 100 µM Zn^{2+} for three months.

Left: Φ_{ET2o} parameters derived from the multi-OJIP protocol of Noccaea caerulescens leaves grown with replete (non-toxic) 100 µM Zn^{2+} for three months. Scale bar is 1 cm.





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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