Biophysical and physicochemical methods for analyzing plants \textit{in vivo} and \textit{in situ} (II): UV/VIS-Spectroscopy from pigment analysis to quantification of mRNA
(3) UV/VIS fluorescence
→ Principle, example: Chlorophyll

Chlorophyll

S0

S1

S2

intersystem crossing

absorption

intersystem crossing

fluorescence

intersystem crossing

phosphorescence
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.

Atomic Absorption Spectroscopy (AAS)

**Advantages:**
- easy to use,
- fast if only 1 element is needed
- affordable

**Disadvantages:**
- insensitive for some elements (e.g. sulphur)
- slow if many elements are needed
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


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

Important prerequisites 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!

medium inlet

glass window (0.17 mm thick); sample is placed between this window and the layer of cellophane

medium outlet

o-rings (silicon rubber)

layer of cellophane

### Lens Light field diameter

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

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

\[
\text{irr} = (\text{irradiance of 6.3× objective}) \cdot \frac{(\text{radiation throughput of tested objective})}{(\text{irrad. field of tested objective})} \cdot \frac{(\text{radiation throughput of 6.3× objective})}{(\text{irrad. field of 6.3× objective})}
\]
numerical Aperture $NA = n \times \sin \frac{q}{2}$

$n =$ refractive index of the medium
$q =$ half opening angle of the objective
Decisive for measuring LIVING cells:
in order to be able to work with low light:
choose a suitable objective

The Light Gathering Power depends on the NA!

FluoresScience workshops
Carl Zeiss Mikroskopie Dr. Jochen Tham
Decisive for quantification: don’t overexpose!
Decisive for quantification: correct calibration of the detector

Model: $y = ax + b$

Levenberg-Marquardt, statistical weighting

$\chi^2 = 0.10176$

$a = 10.261 \pm 0.157$

$b = -0.039 \pm 0.069$

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

→ The cross talk problem

[Diagram showing the overlap of absorption/emission bands for CFP and GFP with filter sets.]
Preliminary tests with GFP in young leaves of
*Arabidopsis thaliana*

Fluorescence observed through GFP filterset

**NON-transformed plant...**

All the signal was **AUTOFLUORESCENCE**
Purification of *Trichodesmium* phycobiliproteins for deconvoluting spectrally resolved *in vivo* fluorescence kinetics and absorption spectra


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**Absorption**

**Fluorescence**

**Phycourobilin isoforms**

**Phycourobilin (II)**

**Phycoerythrin isoforms**

**Phycocyanin isoforms**

**Allophycocyanin**

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**Diazotrophic cell**

- Measured
- Fitted

**Basic fluorescence yield $F_0$**

**Relative fluorescence quantum yield $F_v$**

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**Observations:**

- Normalised fluorescence (to red maximum)
- Absorption spectra
- Fluorescence spectra
Example for the application of time resolved *in vivo* fluorescence spectra:

**Regulation of Photosynthesis in *Trichodesmium***
Light limitation: acclimation by reversible phycobiliprotein coupling: basic fluorescence yield $F_0$
Use of overlapping Abs/Em-Bands for **Fluorescence**-**Resonance**-**Energy**-**Transfer** (FRET)
Prerequisites for **Fluorescence Resonance Energy Transfer (FRET)**

\[
    Eff = \frac{R_o^6}{(R_o^6 + r^6)}
\]

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![Absorption and Emission Spectra](image)

![FRET Efficiency vs Distance](image)
(3) UV/VIS fluorescence of metal specific fluorescent dyes

Transmitted light:
information about structure and cell type

dye fluorescence:
metal measurement

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

→ Types of dyes

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

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

From: www.Invitrogen.com

fluorescence turn – on
constant absorption
(3) UV/VIS fluorescence of metal specific fluorescent dyes → types of response

From: www.Invitrogen.com

fluorescence constant
ratiometric absorption
(3) UV/VIS fluorescence of metal specific fluorescent dyes

→ types of response


ratiometric fluorescence
constant absorption
(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+}
(3) UV/VIS fluorescence
(a) Metal specific fluorescent dyes → calibration

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

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*!
Analysis of molecule mobility: **Fluorescence Recovery After Photobleaching (FRAP)**
Fluorescence Correlation Spectroscopy (FCS)
Fluorescence Correlation Spectroscopy (FCS) II
Fluorescence Correlation Spectroscopy (FCS) III

--> info about molecular concentration, brightness, diffusion, and chemical kinetics
Quantitative mRNA *in situ* hybridisation (QISH): overview of the method

1. Cut out max. 2x5 mm
2. Vacuum infiltrate with alkaline fixation solution
3. Extract pigments and dehydrate
4. Extract hydrophobic compounds
5. Rehydrate
6. Digest proteins
7. Postfixate
8. Quantify
9. Record images in CLSM
10. Extract and quench background
11. Hybridise with fluorescent oligonucleotides

ZNT1 in young leaves of *Thlaspi caerulescens* Prayon: comparison of different QISH hybridisation probes

- full-length antisense RNA probe
- fragmented (200 b) antisense RNA probe
- 34 b synthetic oligonucleotide

overlays of green autofluorescence and red fluorescence of ZNT1-probes

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

Characteristics of the method: effects of tissue optics.

Regulation of ZNT1 transcription analysed by quantitative mRNA in situ hybridisation (QISH) in a non-hyperaccumulating and a hyperaccumulating *Thlaspi* species

Construct vectors for plant transformation

Transform *Agrobacterium* with the constructs

Transform 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

**Qualitative Observation of Transcription & Translation *in vivo* via Fluorescent Proteins**
35S promoter in young leaves of *Arabidopsis thaliana*:
epidermis

Trichome base, epidermal cells and stoma

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

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

<table>
<thead>
<tr>
<th>In situ hybridisation</th>
<th>Fluorescent proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Easy cellular quantification because whole cells are labelled</td>
<td>- Quantification on a cellular level difficult because only the narrow ring of cytoplasm is labelled</td>
</tr>
<tr>
<td>- No macroscopic (whole plant) quantification possible because of diffusion limits</td>
<td>- Macroscopic (whole plant) observation and quantification easily possible with fluorescence measuring camera (so far only tested with GFP)</td>
</tr>
<tr>
<td>- Low background fluorescence because chlorophyll, carotenoids, flavonoids and many further fluorescent compounds are extracted</td>
<td>- High background fluorescence because all autofluorescent compounds are present in the samples</td>
</tr>
<tr>
<td>- No direct comparison of gene expression with physiology because samples are fixed (dead)</td>
<td>- Direct comparison of gene expression with physiological parameters (photosynthesis, electrophysiology) possible because samples are alive</td>
</tr>
<tr>
<td>- Very fast: Ordering the fluorescently labelled oligonucleotides takes 1-2 weeks, the hybridisation procedure itself takes 3 days</td>
<td>- Very time-consuming because of the cloning, transformation and plant growth/selection steps;</td>
</tr>
<tr>
<td>- All plants can be analysed (→ <em>Thlaspi</em> work)</td>
<td>- The plant has to be transformed (→ <em>Arabidopsis</em>)</td>
</tr>
<tr>
<td>- The gene sequence has to be known</td>
<td>- The promoter has to be cloned</td>
</tr>
</tbody>
</table>