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An Introduction to Photosynthetic Spectroscopy

Jeffrey Cruz, PhotosynQ Inc.
Hosted by: Carmela Rosaria Guadagno, University of Wyoming

Beginners Guide to Nausea and Confusion

by Some Lesser-Known Evil Entity

Please feel free to interrupt and ask questions

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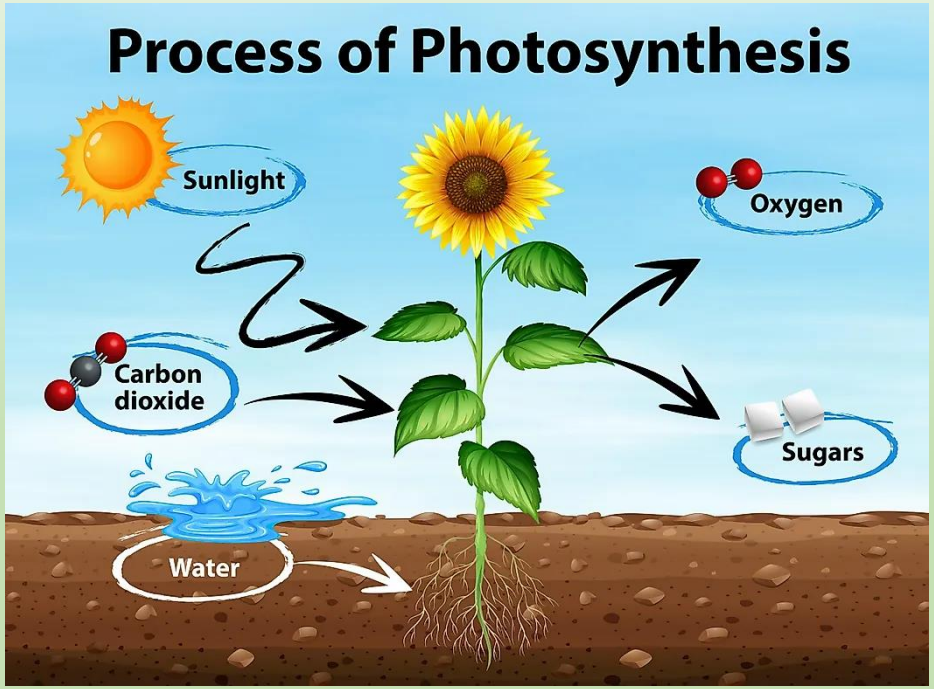
A Simplified View



Photosynthesis Explained - WorldAtlas

Plants convert water and carbon dioxide to oxygen and carbohydrate using sunlight as the energy source.

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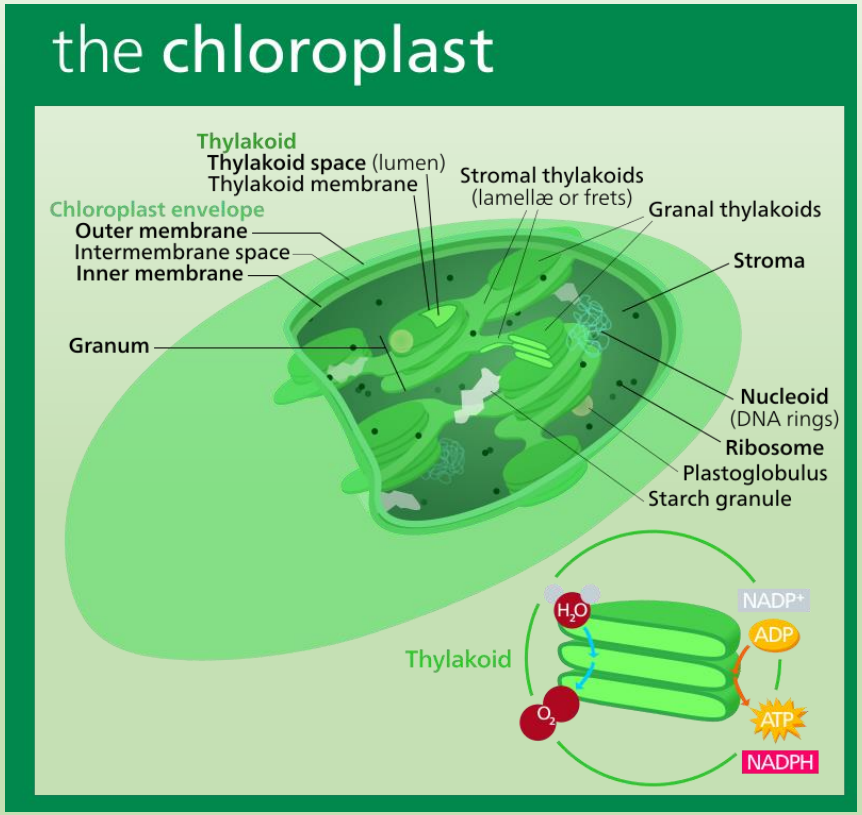
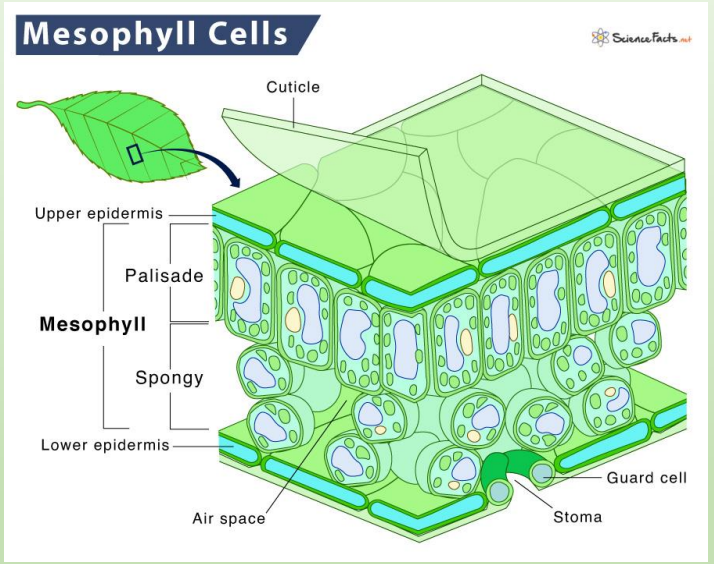
Photosynthetic reactions are contained within the chloroplast.



Chloroplast II - Chloroplast - Wikipedia



Mesophyll.jpg (900×718) (sciencefacts.net)

Light reactions are localized to the thylakoid membranes.

The Calvin-Benson-Bassham Cycle (carbon fixation) is localized to the stroma.

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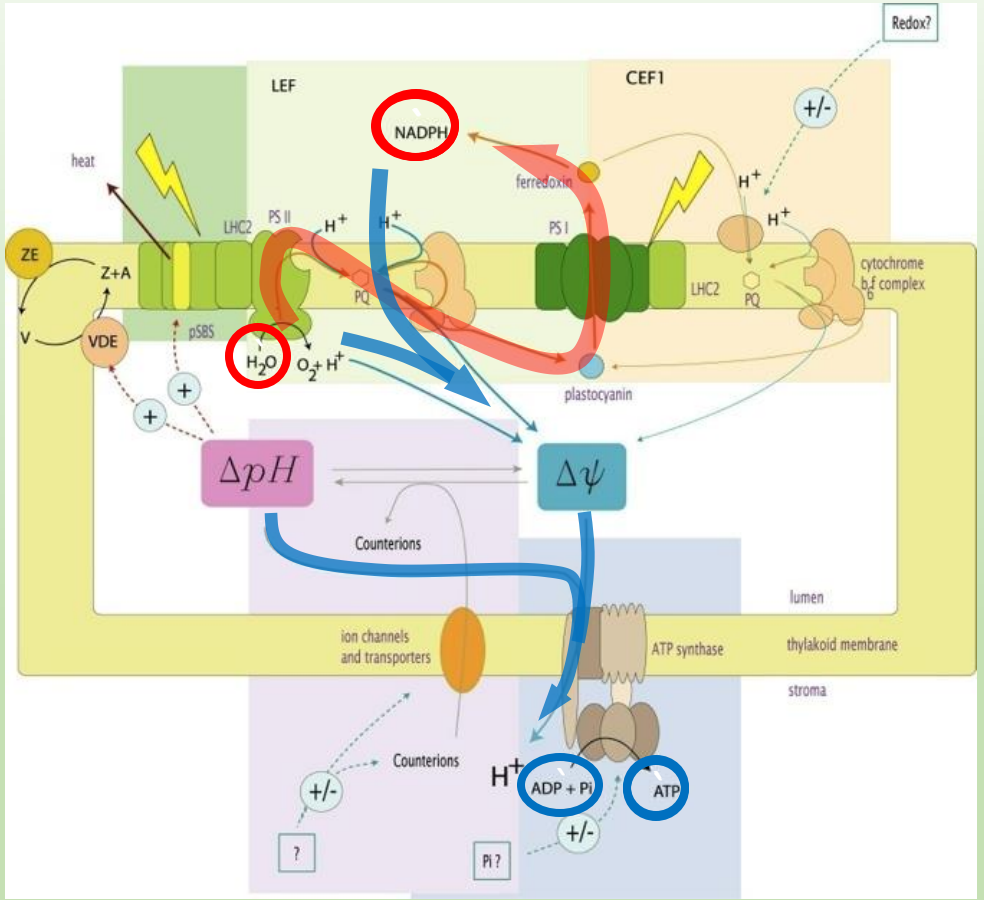
Primary Reactions of Photosynthesis

- The light reactions convert solar energy into chemical energy.
- Powered by light energy electrons are driven through a series of redox carriers from water to NADPH.
- Electron transfer is coupled to proton accumulation in the lumen forming a proton motive force (*pmf*). Dissipation of *pmf* is coupled to ATP synthesis via the chloroplast ATP Synthase.
- NADPH and ATP provide the reducing power and chemical bond energy required to fix CO_2 to carbon skeletons.



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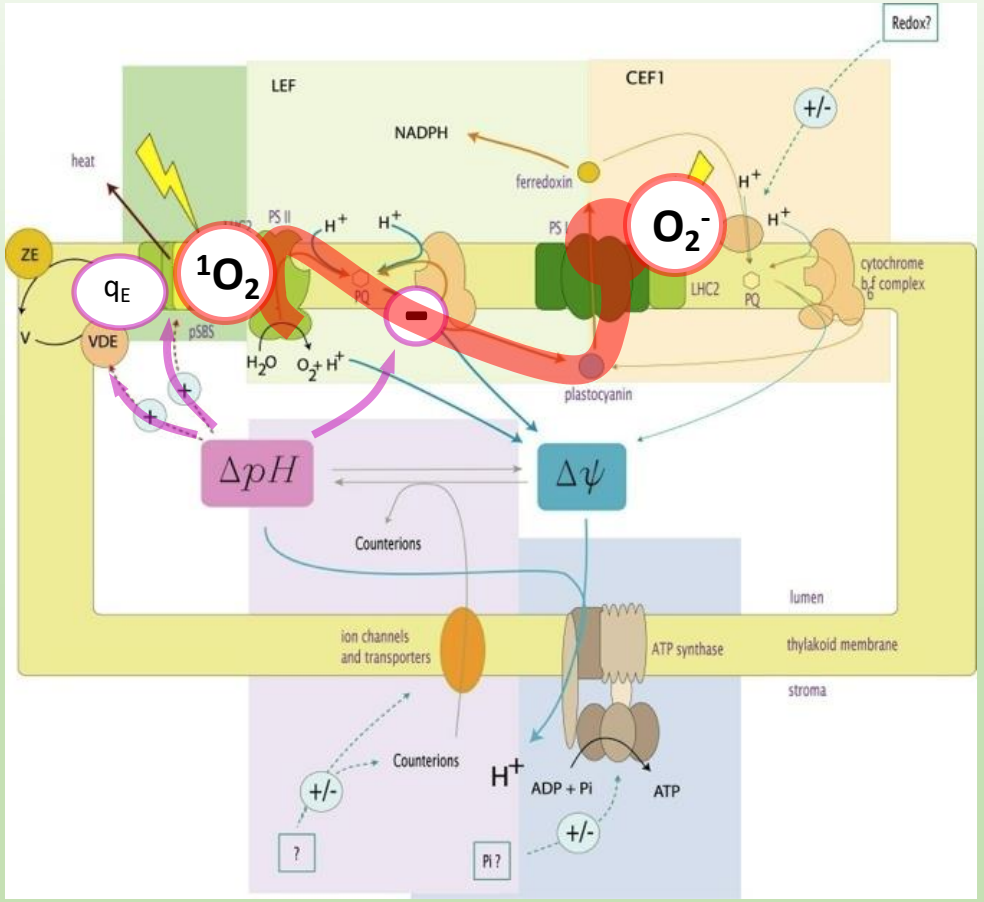
The Need for Photoprotection

- The potential for damage is high, especially if the plant absorbs more energy than it needs.
- Plants have evolved mechanisms to slow electron transfer and shed excess energy.
  - For example, the ΔpH component of *pmf* will slow pH sensitive electron transfer at the *cyt* b_6f complex
  - It will also induce, energy dependent quenching of PSII antennae mediated by Violaxanthin De-epoxidase and the PSBS protein
- Too much down regulation leads to a loss of efficiency. Supply cannot satisfy demand.



Are there ways we can optimize the balance between photochemistry and the need for photoprotection in a natural or field environment?

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Why measure photosynthesis?

- Increasing/maintaining productivity
  - Climate change
  - Precision agriculture
  - Biofuels
  - Indoor farming
- Breeding & Genetic Engineering
  - Stress tolerance
  - Disease resistance

Productivity relies on 'efficient' regulation of photosynthesis, and effects on photosynthesis may be early indicators of stress.

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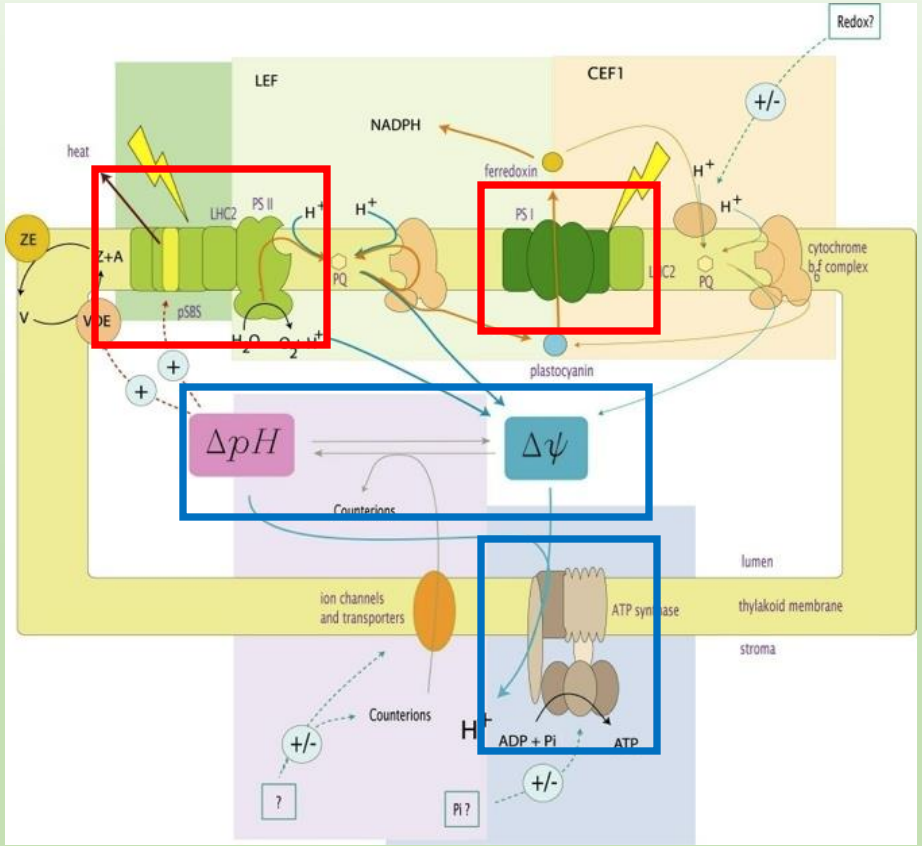
Measuring photosynthesis in the field

- Chlorophyll fluorescence
  - Photosystem II (P_{680})
- Electrochromic Shift
  - *pmf*
  - ATP synthase
- 820 nm Absorbance
  - Photosystem I (P_{700})
- CO_2
  - CBB Cycle
  - Stomatal function



We will focus on the measurements used by MultispeQ in the RIDES 2.0 protocol.

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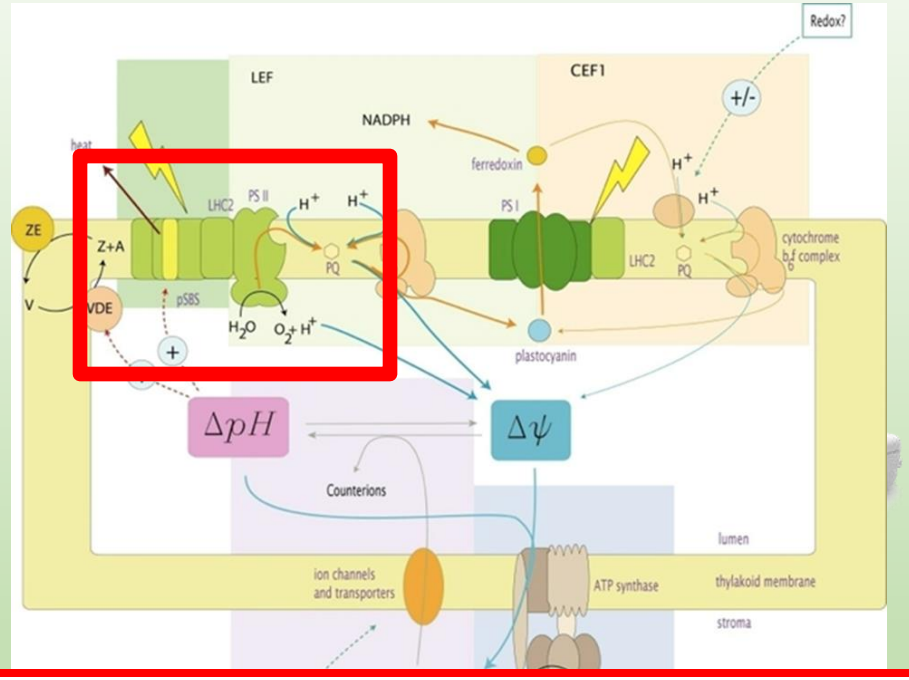
Methods using chlorophyll a fluorescence have become a standard approach for tracking photosynthetic performance.

- Robust signal
- Measure the variable fluorescence associated with Photosystem II (PSII)
- Easy to implement.
  - Laboratory/Benchtop
  - Field measurements
  - Imaging

https://commons.wikimedia.org/wiki/File:Chlorophyll_fluorescence_of_Elodea_canadensis_under_405_nm_light.jpg



Common feature:
All use relatively weak, uniform pulses of light to measure a change in fluorescence that is proportional to the fluorescence yield.

The most common method for probing photosynthesis is Pulse Amplitude Modulated (PAM) fluorescence.

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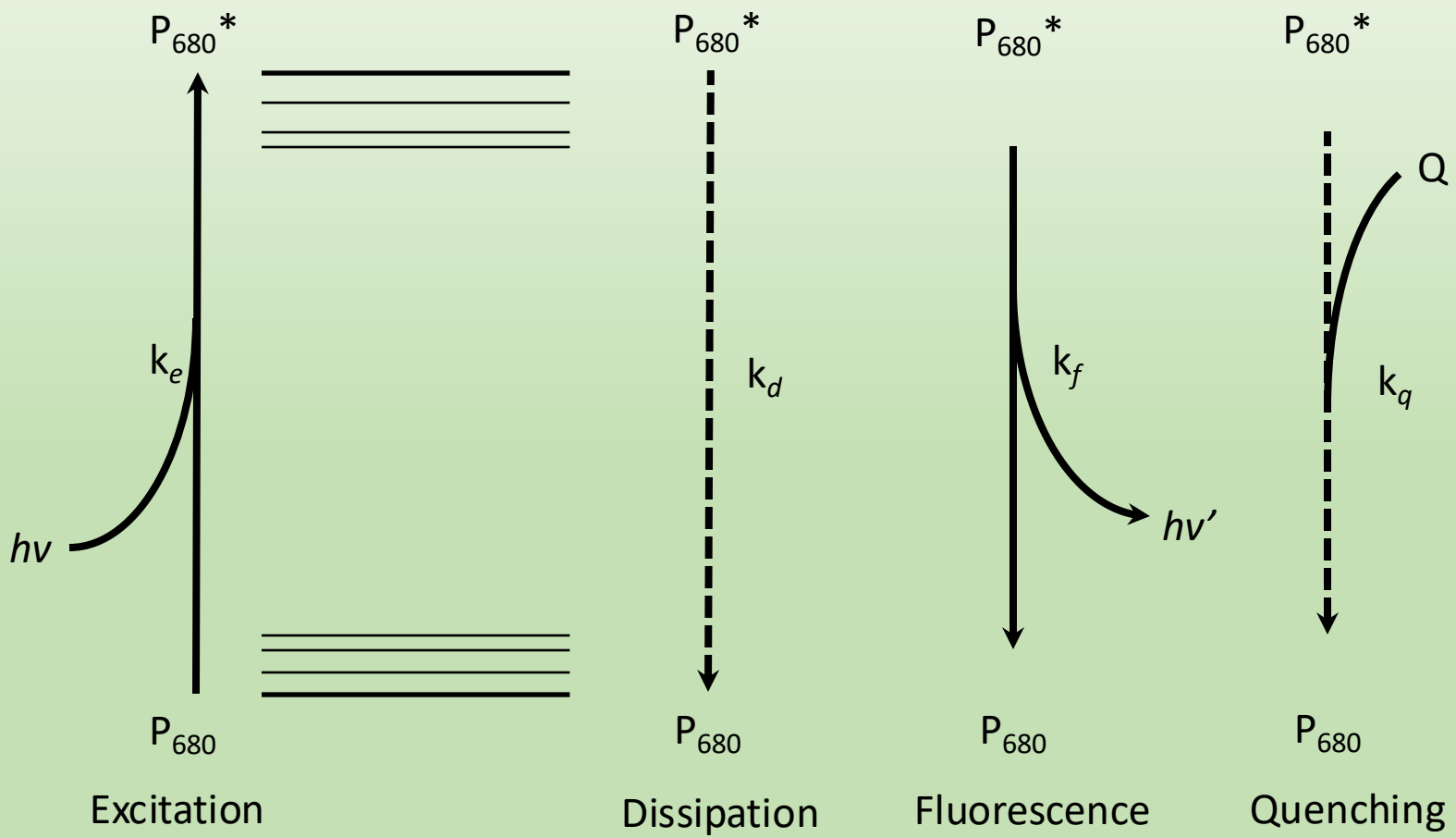
PAM Fluorescence of Photosystem II is based on Stern-Volmer model



Given a fixed level of excitation, the fluorescence signal will depend on rates of dissipation and 'quenching' processes that compete with it for the absorbed light energy.

This is an imperfect model. Most of the quenching processes do not directly quench the excited state of P_{680}.

Mathematically, it works.

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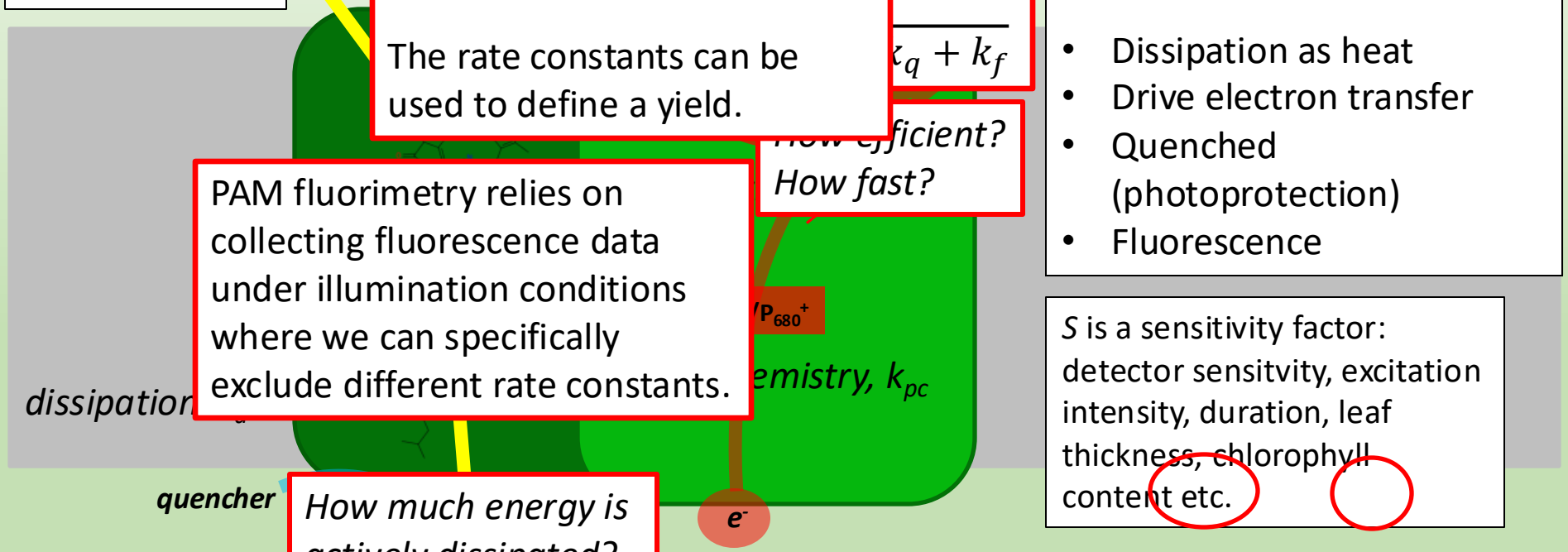
PAM Fluorescence as interpreted by the Stern-Volmer model

The fluorescence produced from a weak pulse will depend on the 'openness' of other competing pathways

Processes that compete for absorbed light energy can be described with a rate constant.

The rate constants can be used to define a yield.



Light energy absorbed by PSII antenna has a number of potential fates:

- Dissipation as heat
- Drive electron transfer
- Quenched (photoprotection)
- Fluorescence

PAM fluorimetry relies on collecting fluorescence data under illumination conditions where we can specifically exclude different rate constants.

S is a sensitivity factor: detector sensitvity, excitation intensity, duration, leaf thickness, chlorophyll content etc.

How much energy is actively dissipated?

$$NPQ = \frac{k_q}{k_d + k_f}$$

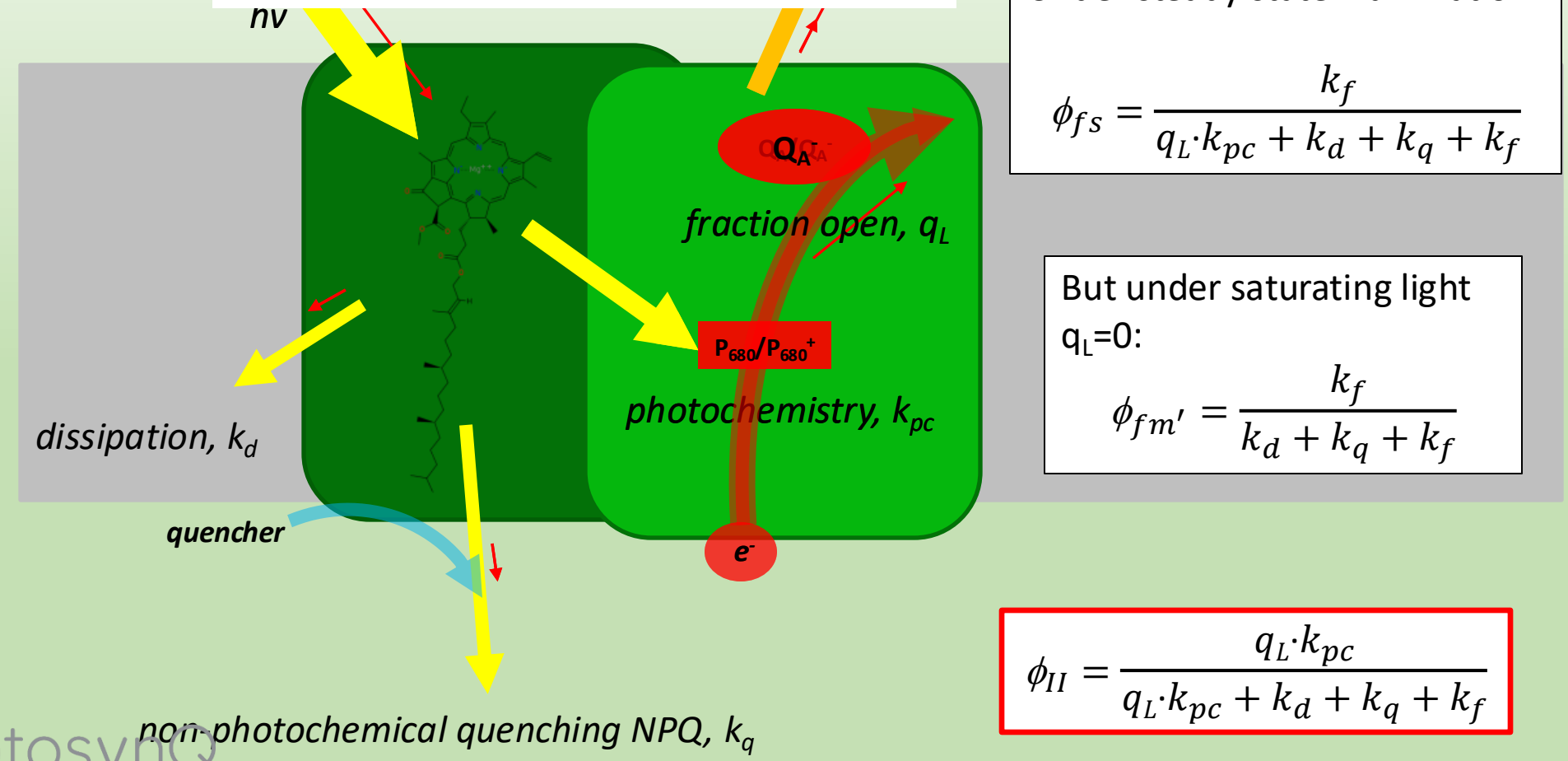
non-photoch… NPQ, k_q → $k_q = k_{qE} + k_{qI}$

→ *energy dependent quenching (q_E), k_{qE}*

→ *inhibitory quenching (q_I), k_{qI}*

One sample derivation: Quantum Yield of PSII (ϕ_{II})

-Efficiency of light consumption at PSII
-The fraction of light absorbed by PSII that is used for photosynthesis



Under steady state illumination:

$$\phi_{fs} = \frac{k_f}{q_L \cdot k_{pc} + k_d + k_q + k_f}$$

But under saturating light q_L=0:

$$\phi_{fm'} = \frac{k_f}{k_d + k_q + k_f}$$

$$\phi_{II} = \frac{q_L \cdot k_{pc}}{q_L \cdot k_{pc} + k_d + k_q + k_f}$$

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A little bit of math

$$\phi_{fm'} - \phi_{fs} = \frac{k_f \times \left(q_L \cdot k_{pc} + k_d + k_q + k_f\right) - k_f \times \left(k_d + k_q + k_f\right)}{k_d + k_q + k_f \times \left(q_L \cdot k_{pc} + k_d + k_q + k_f\right)}$$

$$\phi_{fm'} - \phi_{fs} = \frac{k_f \times q_L \cdot k_{pc}}{\left(k_d + k_q + k_f\right) \times \left(q_L \cdot k_{pc} + k_d + k_q + k_f\right)}$$

$\phi_{fm'}$ ϕ_{II}

$$\frac{\phi_{fm'} - \phi_{fs}}{\phi_{fm'}} = \frac{q_L \cdot k_{pc}}{\left(q_L \cdot k_{pc} + k_d + k_q + k_f\right)} = \phi_{II}$$

$$\frac{S \cdot F_M{}' - S \cdot F_S}{S \cdot F_M{}'} = \frac{F_M{}' - F_S}{F_M{}'} = \frac{\phi_{fm'} - \phi_{fs}}{\phi_{fm'}} = \phi_{II}$$

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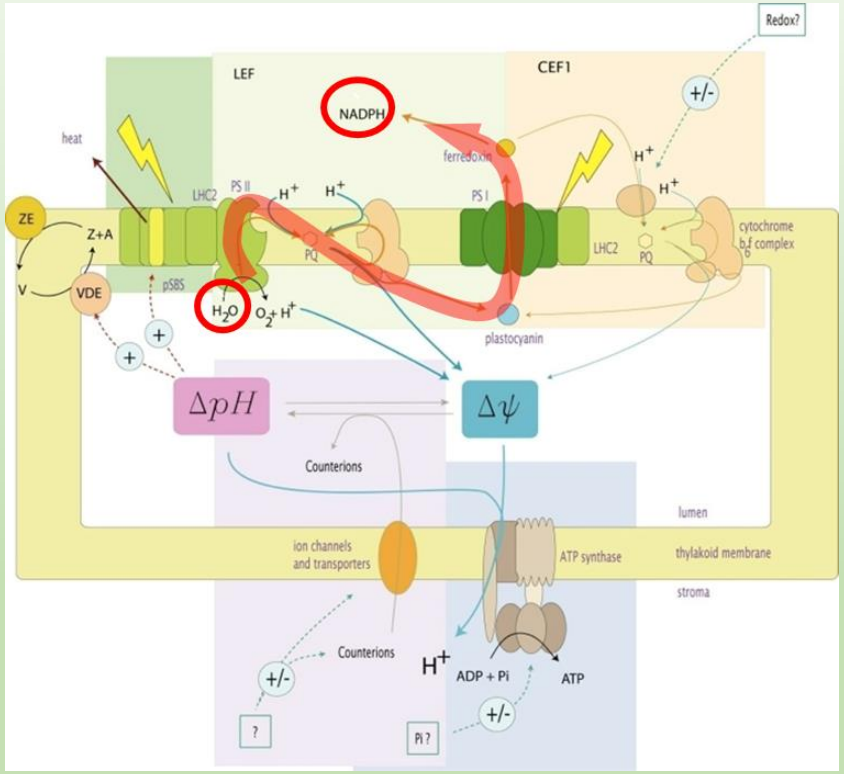
Photosystem II electron flux rate

- The quantum yield of PSII, ϕ_{II}, is the fraction of absorbed light energy funneled to PSII, that is used for electron transfer.
- It can be used to calculate electron transfer rate through PSII.
- I is the incident light intensity (PAR).
- 0.41 assumes 82% percent of incident light is absorbed by the chlorophyll antenna system and that the energy is equally distributed between PSII and PSI.



$$LEF = I \cdot 0.41 \cdot \phi_{II}$$

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Classic Lab Fluorescence Measurements

Dark Adapted State

-Quenching Inactive (k_q = 0)

-Photosystem II is fully "open" (q_L=1)

-**F_0**, Baseline Fluorescence in Dark

-**F_M**, Maximum Fluorescence

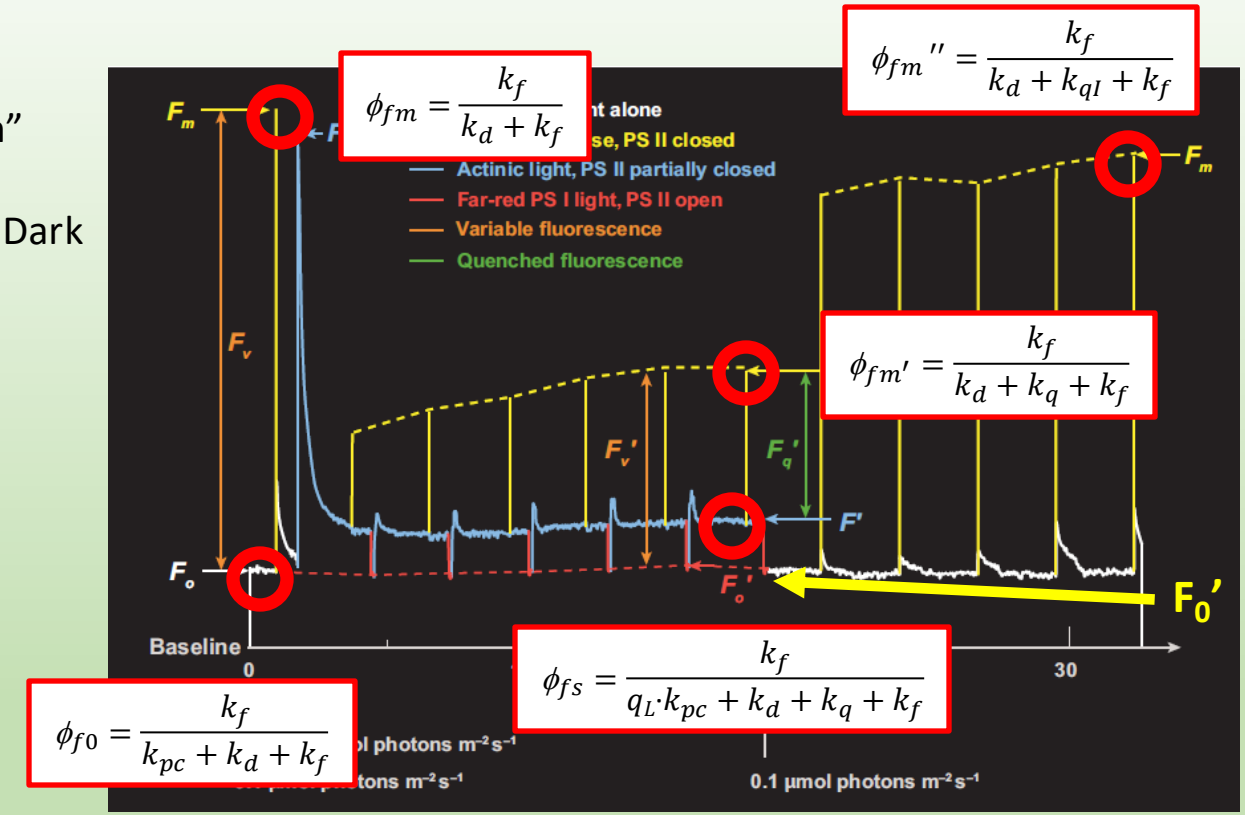
Steady State

-**F_S**, Fluorescence at steady state under constant 'actinic' illumination

-**F_M'**, Fluorescence maximum at steady state.

Dark Relaxation

-**F_M"**, Fast response quenching mechanisms (q_E) relax, slowly reversible quenching (q_I) remains (k_{qE} = 0)



Baker NR (2008) Chlorophyll Fluorescence: A Probe of Photosynthesis In Vivo. Annual Review of Plant Biology **59:** 89-113

For a field instrument collecting most of these values would be challenging.

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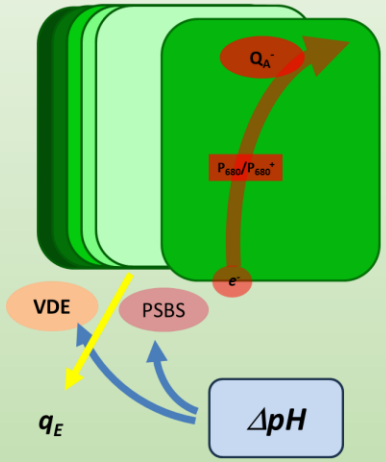
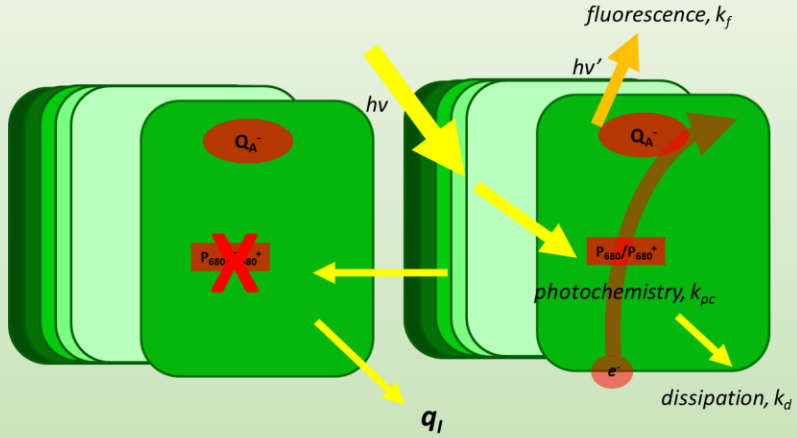
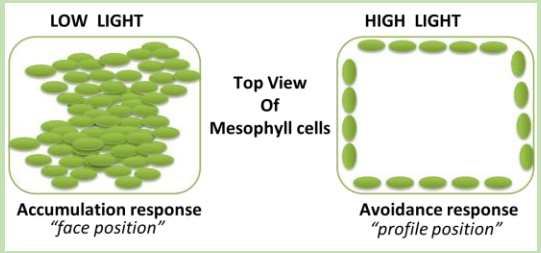
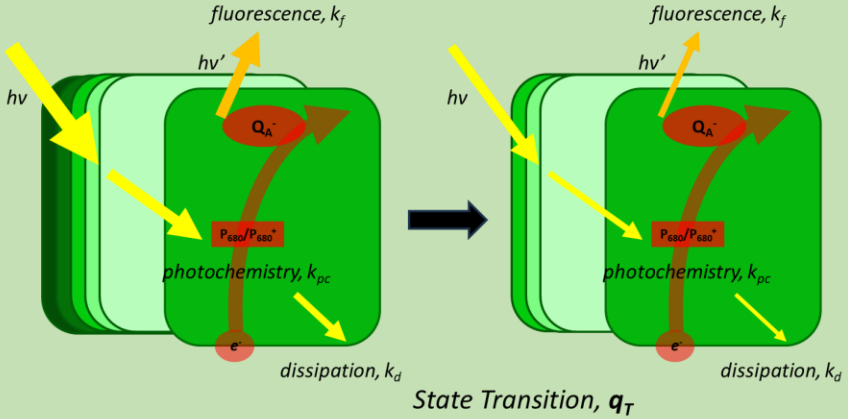
Non-Photochemical quenching (NPQ)

- Sum of all processes that quench or appear to quench absorbed light energy to reduce (redox) pressure on PSII
- Accumulates when excitation pressure is high and/or downstream electron transfer is restricted
- Antenna quenching
  - Energy dependent quenching, q_E
  - Inhibitory quenching, q_I
- Energy avoidance
  - State transitions, q_T
  - Photorelocation of chloroplast, q_Z(?)









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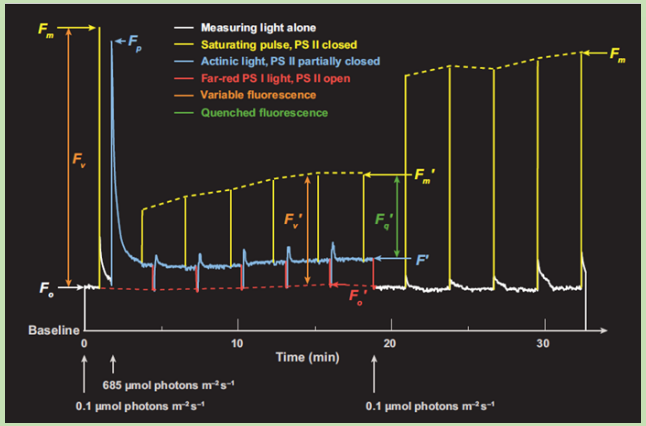
Specific challenges to measurement of quenching

All the quenching parameters require measurement of F_M:

$$NPQ = \frac{F_M - F_M'}{F_M'}$$

- Requires a long dark adaptation time
- Difficult to implement in field studies without significant perturbations to the plant or inconvenience to the researcher
- Incomplete relaxation of quenching (e.g. residual damage to PSII, or insufficient dark time) can lead to artifacts (underestimation of F_M)
- Significant delay (minutes to hours) between measurement of FM and FM' or FM" limits the capacity to measure in high throughput



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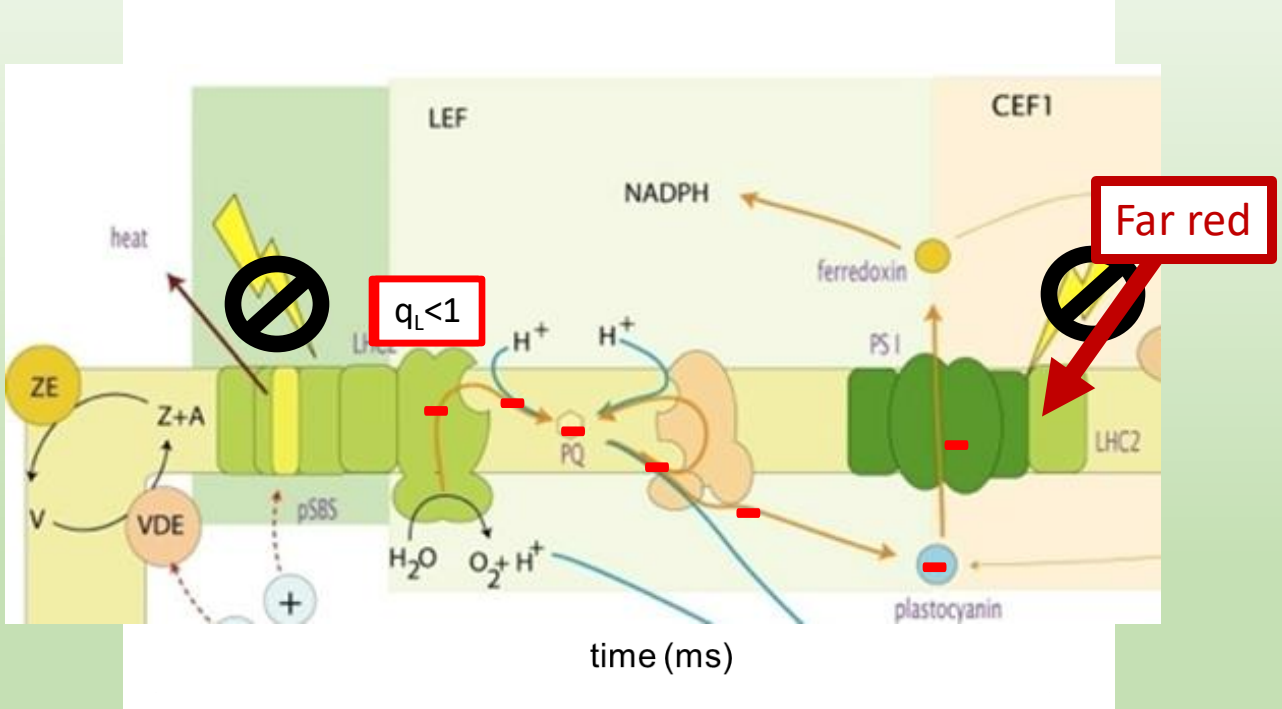
Introducing F_0'



$$\phi_{f0'} = \frac{k_f}{k_{pc} + k_d + k_q + k_f} \qquad \phi_{fm'} = \frac{k_f}{k_d + k_q + k_f}$$

Collected immediately after the light/dark transition.

Far red illumination is used to preferentially excite PSI, leading to near complete oxidation of Q_A ($q_L \approx 1$).

Since this occurs within seconds after the light dark transition, quenching has not completely relaxed.

This measurement can be used with F_M' to calculate total quenching.

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Derivation of NPQ_T

$$\frac{\phi_{fm\prime}}{\phi_{f0\prime}} = \frac{k_f+k_d+k_q+k_p}{k_f+k_d+k_q}$$

$$\frac{S \cdot F_M{}'}{S \cdot F_0{}'} = 1 + \frac{k_p}{k_f + k_d + k_q}$$

$$\frac{F_M{}'}{F_0{}'} - 1 = \frac{k_p}{k_f + k_d + k_q}$$

$$\frac{F_M}{F_0} = \frac{k_f + k_d + k_p}{k_f + k_d}$$

$$\frac{F_M}{F_0} = 1 + \frac{k_p}{k_f + k_d}$$

$$\frac{F_M}{F_0} - 1 = \frac{k_p}{(k_f + k_d)}$$

$$\mathrm{NPQ} = \frac{k_q}{k_f + k_d}$$

$$\phi_{f0'} = \frac{k_f}{k_{pc} + k_d + k_q + k_f}$$

$$\phi_{fm'} = \frac{k_f}{k_d + k_q + k_f}$$

$$\phi_{f0} = \frac{k_f}{k_f + k_d + k_p}$$

$$\phi_{fm} = \frac{k_f}{k_f + k_d}$$

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Derivation Continued

$$\frac{\left(\frac{F_M}{F_0}-1\right)}{\left(\frac{F_M'}{F_0'}-1\right)}=\frac{(k_f+k_d+k_q)}{(k_f+k_d)}$$

$$\frac{\left(\frac{F_M}{F_0}-1\right)}{\left(\frac{F_M'}{F_0'}-1\right)}=1+\frac{(k_q)}{(k_f+k_d)}$$

$$\frac{\left(\frac{F_M}{F_0}-1\right)}{\left(\frac{F_M'}{F_0'}-1\right)}=1+\mathrm{NPQ}$$

$$\mathrm{NPQ}=\frac{\left(\frac{F_M}{F_0}-1\right)}{\left(\frac{F_M'}{F_0'}-1\right)}-1$$

$$\mathrm{NPQ}=\frac{k_q}{k_f+k_d}$$

The literature average for F_V/F_M is ~0.83

$$\frac{F_V}{F_M}=1-\frac{F_0}{F_M}=0.83$$

$$\frac{F_M}{F_0}=5.88$$

$$\mathrm{NPQ}_T=\frac{4.88}{\left(\frac{F_M'}{F_0'}\right)-1}-1$$

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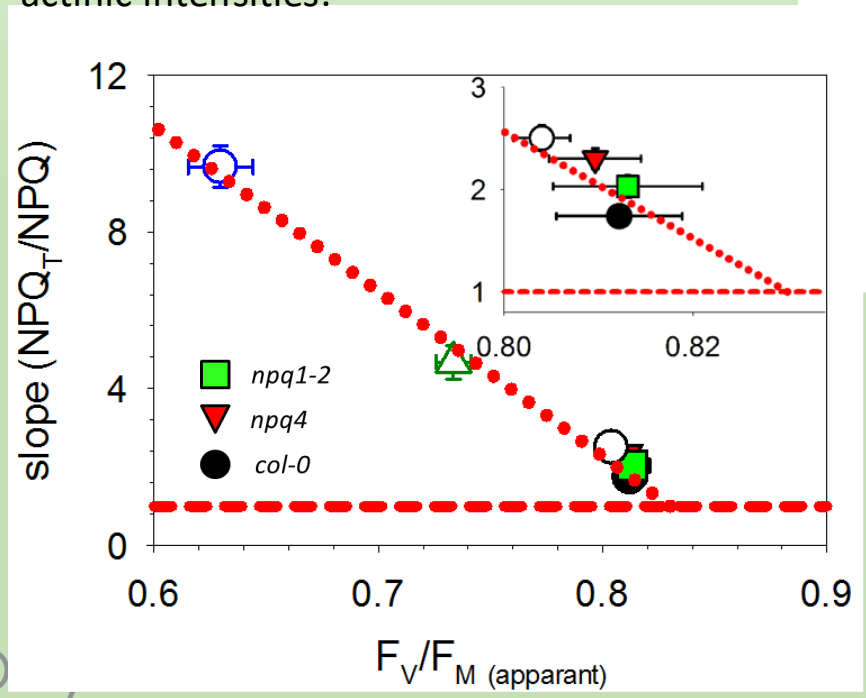
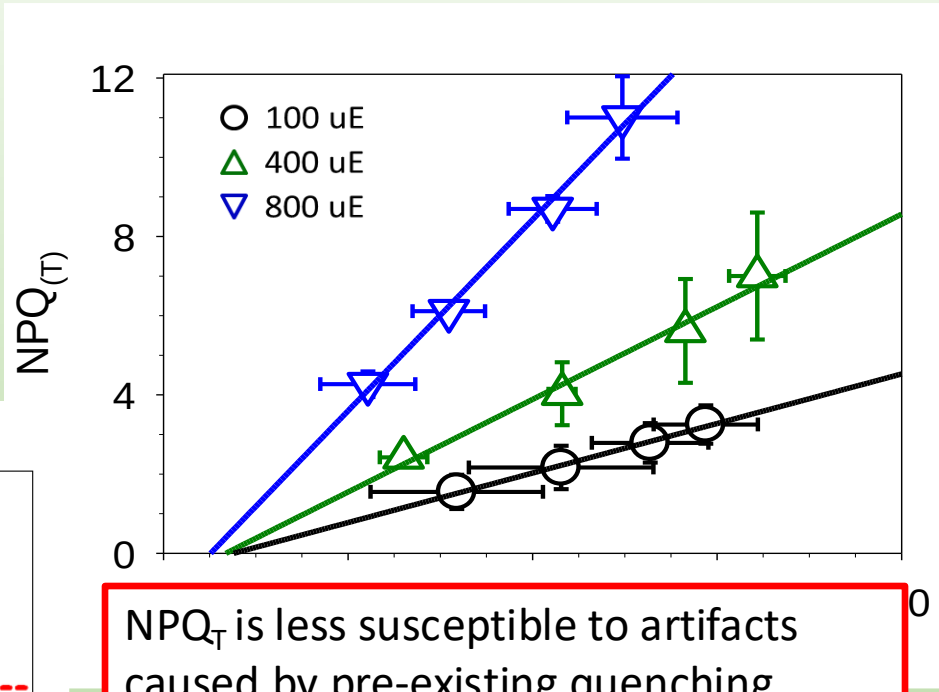
NPQ_T is less susceptible to artifacts caused by pre-existing quenching

Pre-illuminated 30 minutes at different intensities to induce different levels of q_I (i.e. pre-existing quenching).

10 minutes dark adaptation.

Measure NPQ and NPQ_T at different actinic intensities.





NPQ_T is less susceptible to artifacts caused by pre-existing quenching

Small differences between measured and ideal F_V/F_M values can cause relatively large deviations in slope.

Ideal values of F_V/F_M may vary between species/ecotypes?

Other PSII quantum yields

- ϕ_{NO} is fraction of energy lost through non-regulated paths (fluorescence and dissipation)
  - Changes to ϕ_{NO} likely reflect changes to k_d.
  - Increase in ϕ_{NO} -> insufficient NPQ
  - Decrease in ϕ_{NO} -> too much NPQ

- ϕ_{NPQ} is the fraction of dissipated by photoprotective mechanisms
  - Should increase with light intensity
  - As ϕ_{NPQ} increase ϕ_{II} should decrease (in proportion).

$$\phi_{NO} = \frac{k_d + k_f}{q_L \cdot k_{pc} + k_d + k_q + k_f}$$

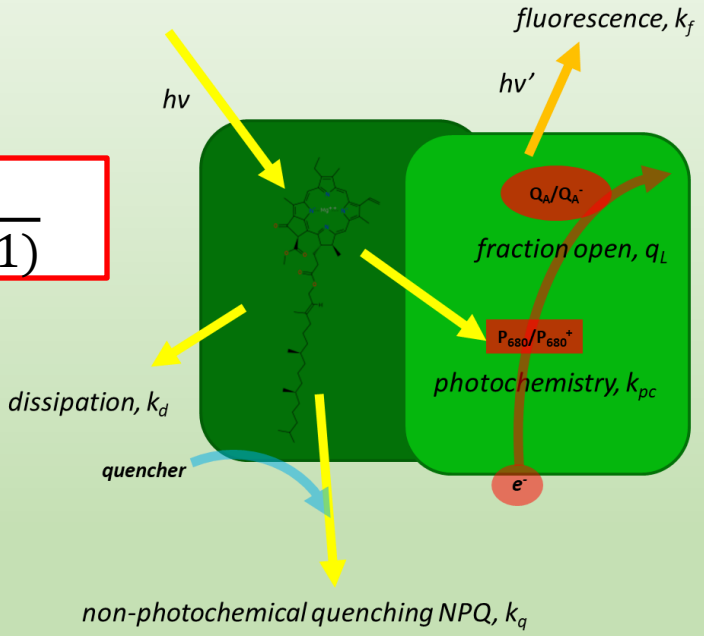
$$\phi_{NO} = \frac{1}{NPQ + 1 + q_L \cdot (F_M/F_0 - 1)}$$

$$\phi_{NO} = \frac{F_S}{F_M} \quad (4.88)$$

$$\phi_{NPQ} = \frac{k_q}{q_L \cdot k_{pc} + k_d + k_q + k_f}$$

$$1 = \phi_{II} + \phi_{NO} + \phi_{NPQ}$$

$$\phi_{NPQ} = 1 - \phi_{II} - \phi_{NO}$$



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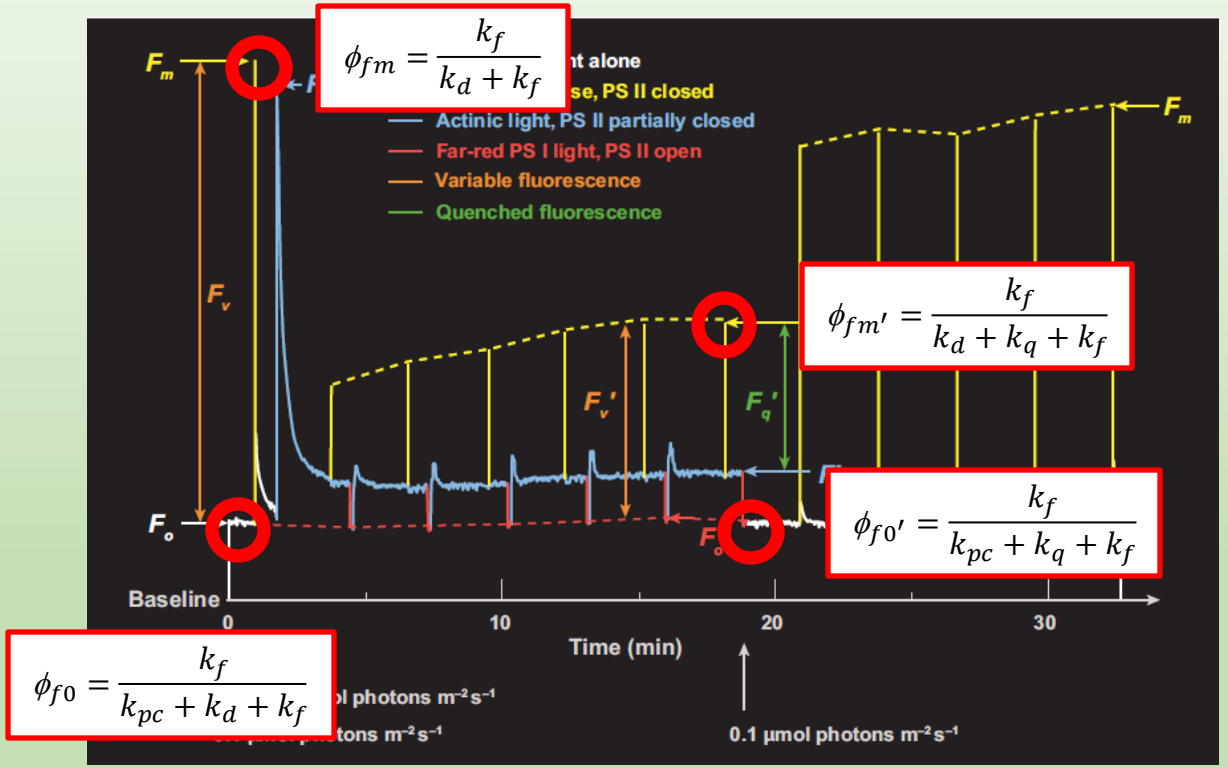
F_V'/F_M'

- Similar to F_V/F_M, the maximum quantum yield.
- F_V/F_M is calculated using F_M and F_0, which are measured in the dark-adapted state.
- F_V'/F_M' uses F_M' and F_0'.
- It is the maximum quantum yield of the steady state.
- Relative decreases may indicate increased rates of dissipation (susceptibility to photodamage) or quenching (downregulation).



$$F_V/F_M = \frac{k_{pc}}{k_{pc}+k_d+k_f}$$

$$F_V'/F_M' = \frac{k_{pc}}{k_{pc}+k_d+k_q+k_f}$$

$$F_V/F_M = \frac{F_M - F_0}{F_M}$$

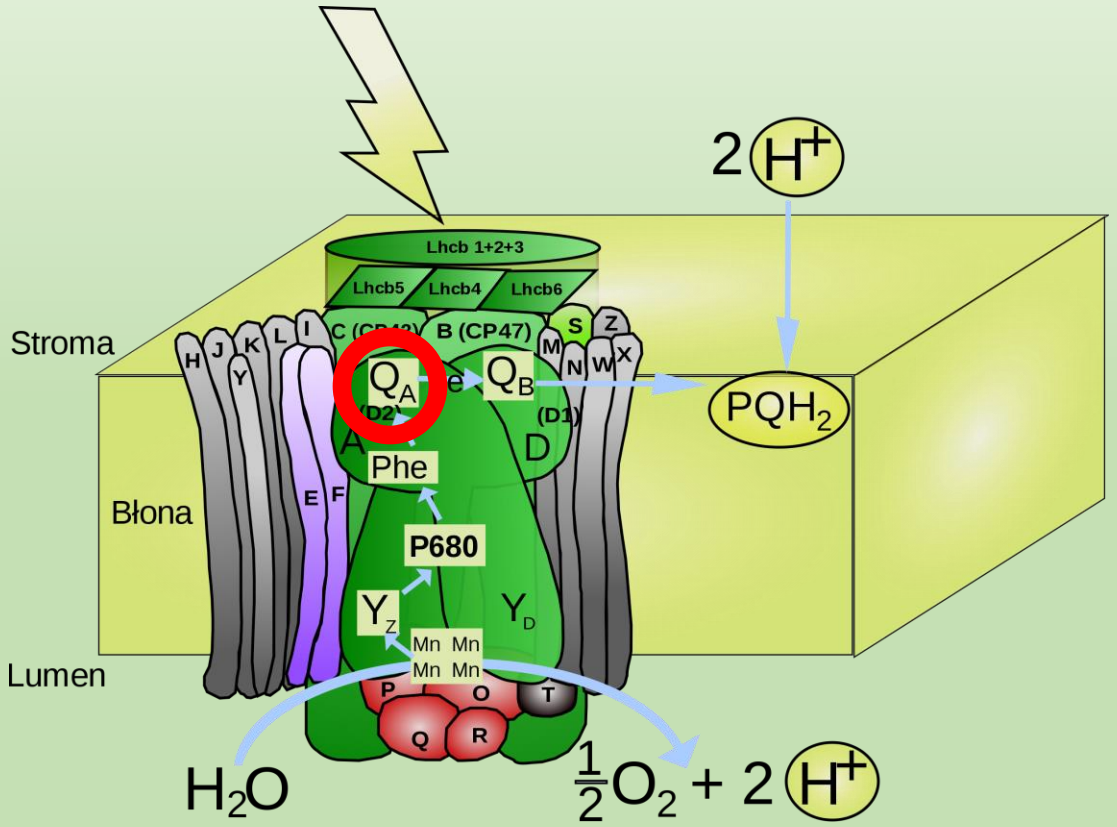
$$F_V'/F_M' = \frac{F_M' - F_0'}{F_M'}$$

q_L is a measure of how open PSII is to electron transfer.

- It is the fraction of PSII centers that have a downstream electron acceptor, Q_A, in the oxidized state and open (or active) to electron transfer from P_{680}.
- It is an indicator of the redox state of the bound quinone at PSII, Q_A. (The Q_B site binds PQ and releases it after it is sequentially reduced to PQH_2.)
- Major factor in calculation of quantum yield and PSII rate.
- May be an indicator of over-reduction (redox stress), when LEF is relatively slow

$$q_L = \frac{F_M\prime - F_S}{F_M\prime - F_0\prime}\left(\frac{F_0\prime}{F_S}\right)$$

$$\phi_{II} = \frac{q_L \cdot k_{pc}}{q_L \cdot k_{pc} + k_d + k_q + k_f}$$



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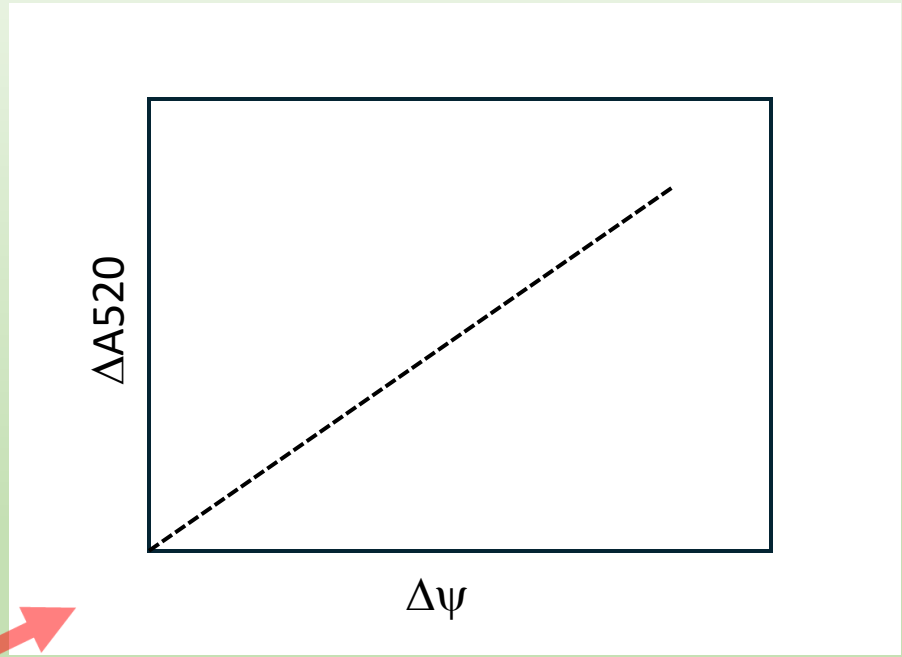
End of chlorophyll fluorescence

Electrochromic shift (ECS)

- Shift in the absorbance spectrum of pigments embedded in thylakoid membranes in response to changes in ‘electric field’ across the membrane
- Peak change at 520 nm
- The change in absorbance is proportional to the change in ‘electric field’.
- Since the formation of pmf leads to a net positive electric field inside the thylakoid lumen, the ECS can be used to probe proton flux through ATP synthase and *pmf*.



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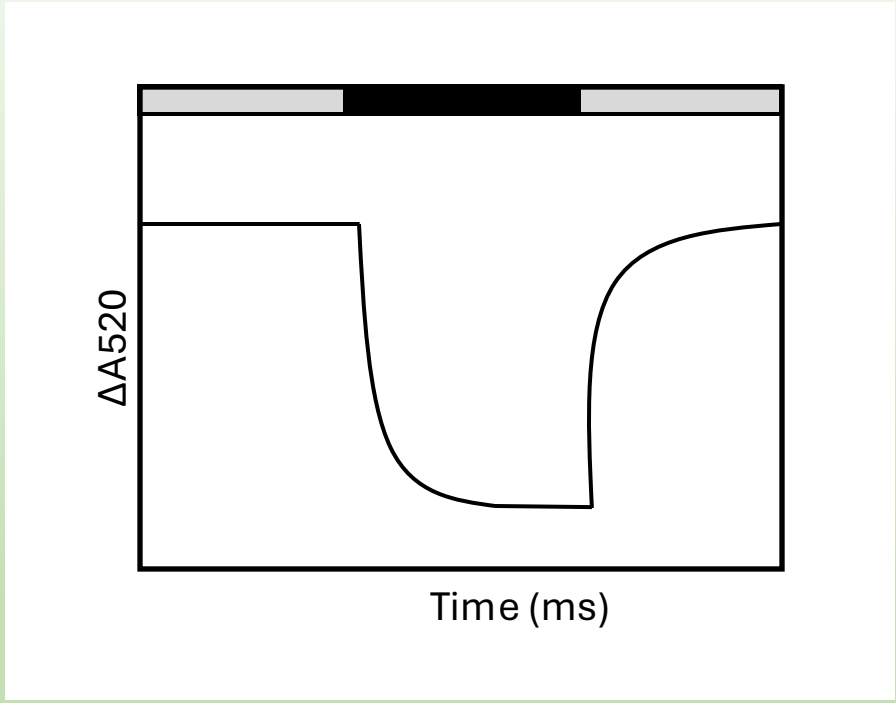
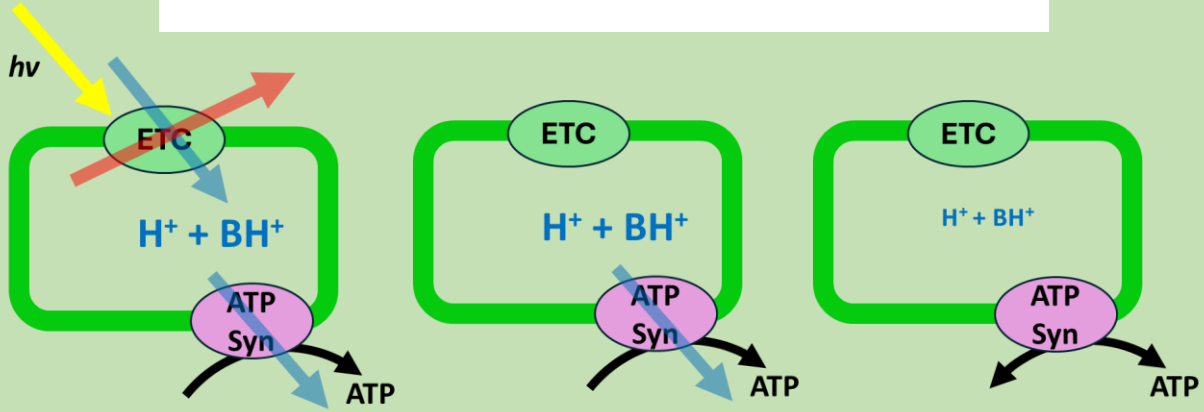
Dark Interval Relaxation Kinetics (DIRK)

- At steady state, the rate of proton accumulation equals the rate of dissipation.
- Shuttering of the light stops electron transfer and coupled proton influx, but *pmf* remains.
- *Pmf* drives proton flux through the ATP synthase until it equilibrates with DG_{ATP}.
- Resume actinic illumination to re-establish steady state.





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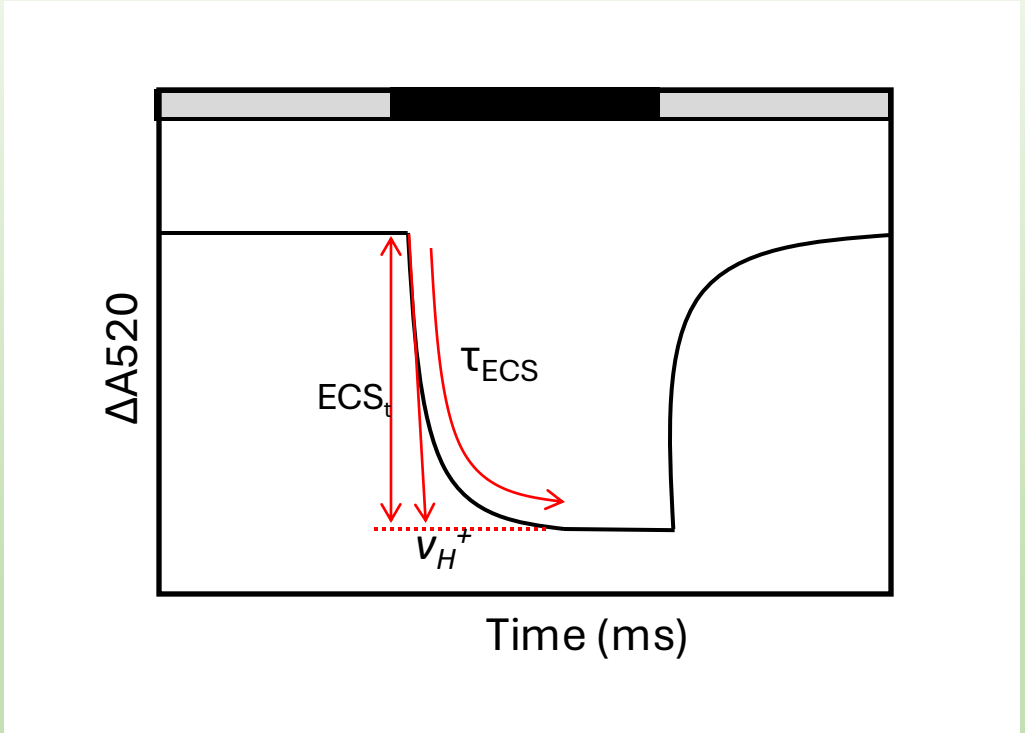
ECS Parameters

- The extent of the ECS decay ECS_t is proportional to the light induced *pmf* (pmf_L).
- ECS decays with first order kinetics and can be fit to a single exponential with decay time of ECS_tau (t_{ECS}).
- The inverse of the exponential decay time is the proton conductivity (g_{H^+}).
- Multiplying conductivity by ECS_t yields a relative measure of steady state proton flux (v_{H^+}).
- Alternatively, v_{H^+} could be extracted from the initial slope of the decay.



$$g_{H^+} = 1/\tau_{ECS}$$

$$v_{H^+} = ECS_t \cdot g_{H^+}$$

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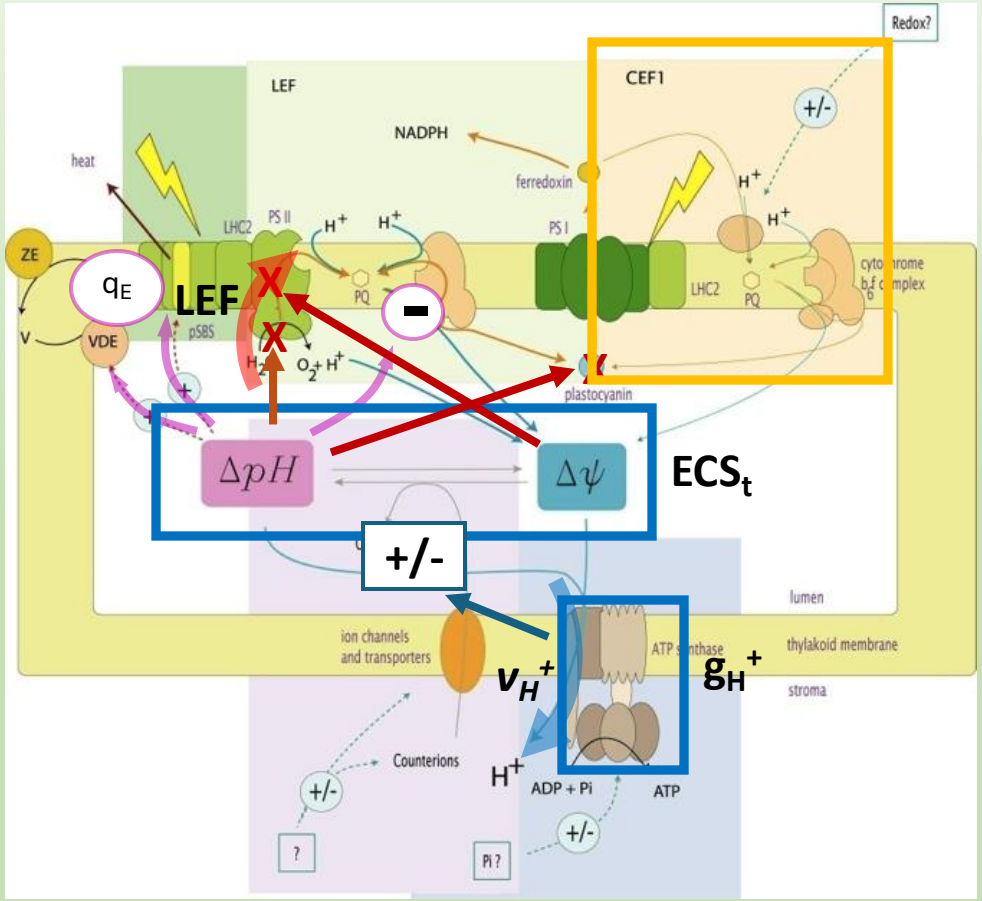
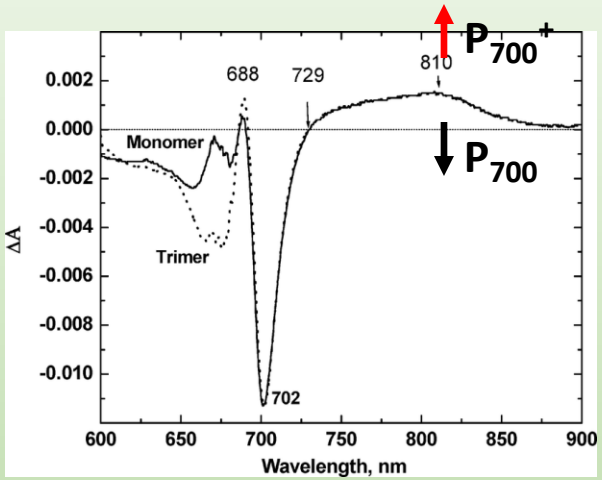
Modulation of *pmf* is crucial to photosynthetic regulation

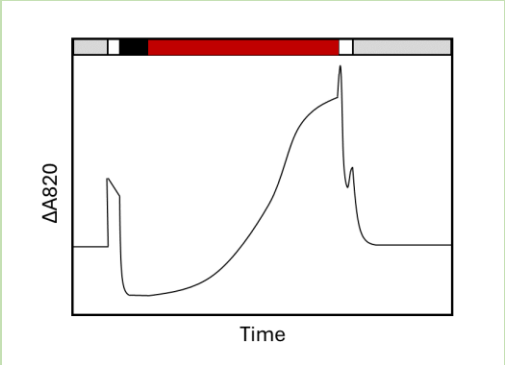
- Extent to which *pmf* forms (ΔpH) determines the extent to which major downregulatory mechanisms occur.
- Excessive *pmf* can damage the ETC or increase susceptibility to photodamage.
- Proton conductivity is a relative measure of ATP synthase activation state.
- Regulating g_{H^+} is a mechanism for modulating *pmf*.
- Comparison of v_{H^+} with LEF allows detection of alternative electron flux pathways that have been implicated in downregulation (cyclic electron flux).

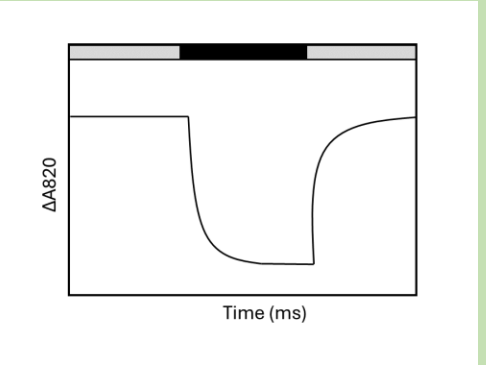


P_{700} absorbance spectroscopy

- The oxidized state of P_{700} (P_{700}^{+}) has an absorbance peak at ~810-820 nm.
- ΔA820 is linearly proportional to the content of P_{700}^{+}.
- Saturation pulse spectroscopy is state method (redox state).
- DIRK is a kinetic method (rates and rate constants).

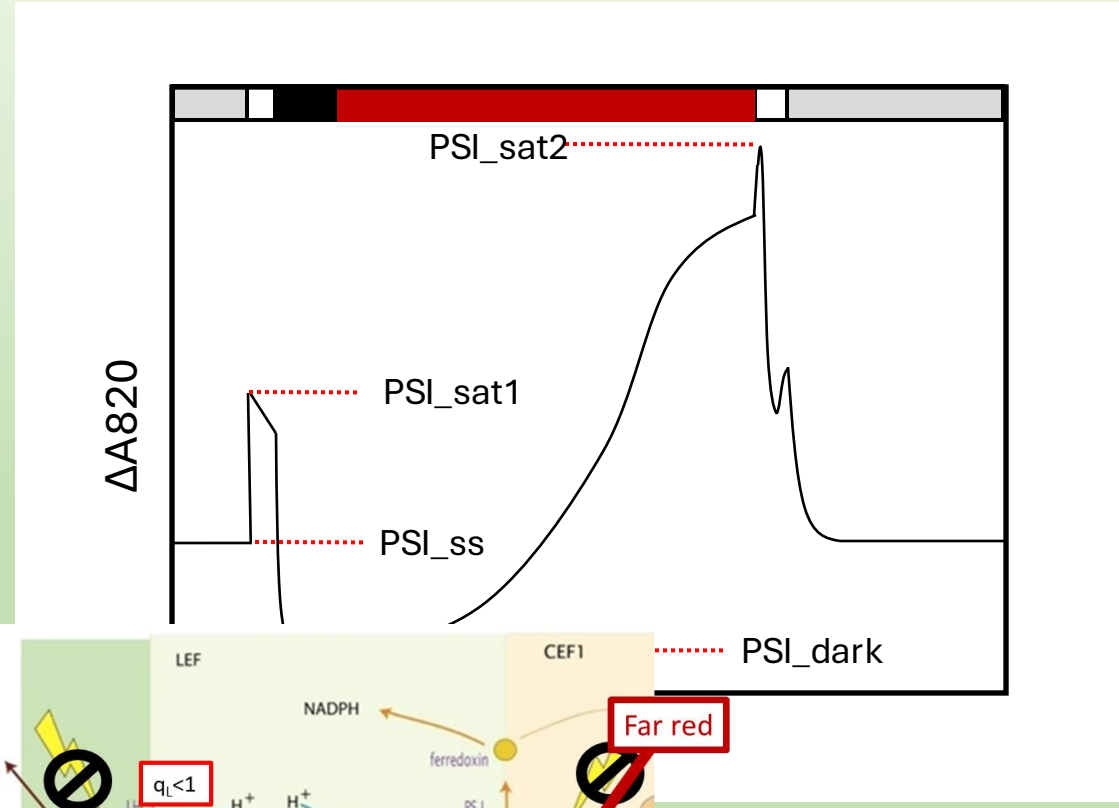






P700 sat pulse spectroscopy (aka ϕ_I)

- Absorbance at 820 is measured under 4 different conditions.
- Under steady state illumination the pool of PSI is partially oxidized (PSI_ss).
- Under saturating light centers that are not ‘over-reduced’ (PSI_sat1) are fully oxidize.
- In the dark the pool oxidized PSI relaxes to the open, or reduced state (PSI_dark).
- Using far-red pre-illumination to preferentially excite PSI, allows full oxidation of the total active pool of PSI under a second saturation pulse (PSI_sat2) .



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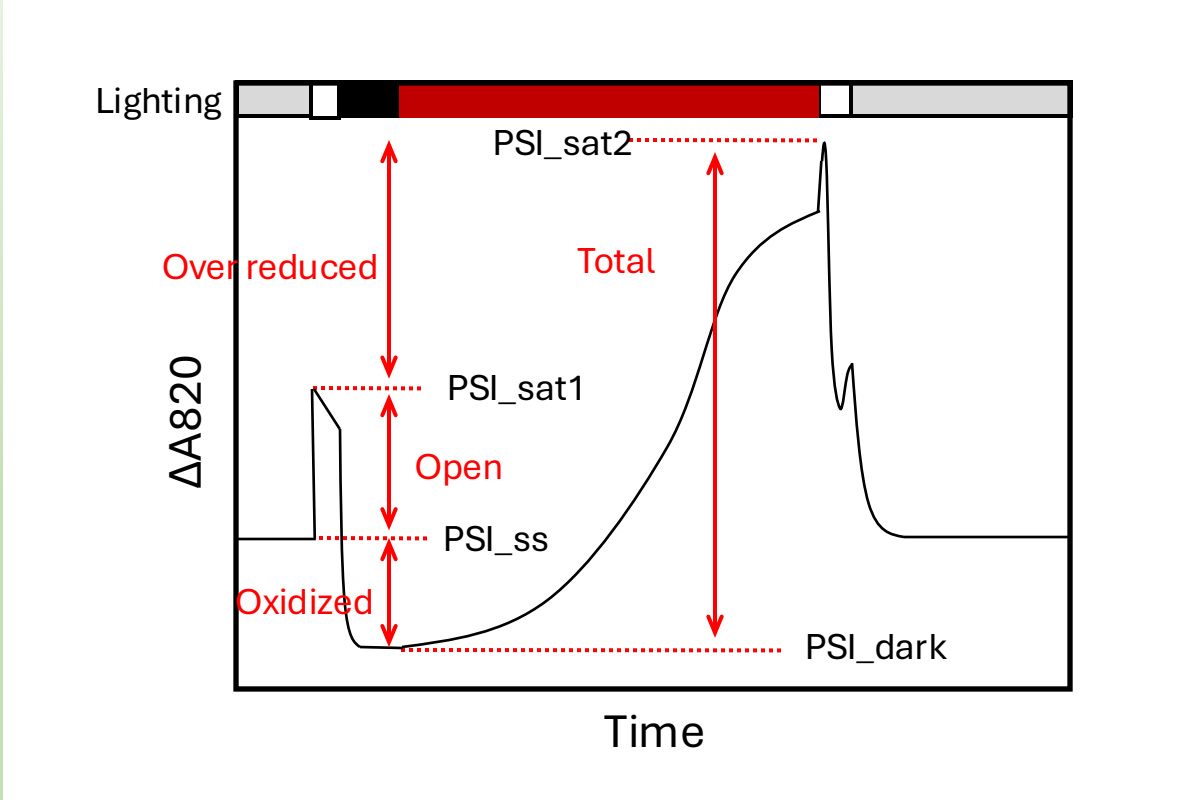
Calculated Parameters (as reported by RIDES).

- PSI Active Centers- Total pool of redox active PSI.
- PSI Open Centers (Also known as ϕ_I)- Fraction of PSI open to charge separation.
- PSI Oxidized Centers - Fraction of centers in the oxidized state.
- PSI Over-reduced Centers- Fraction of PSI in which the electrons are fully saturated and electron transfer is effectively blocked.



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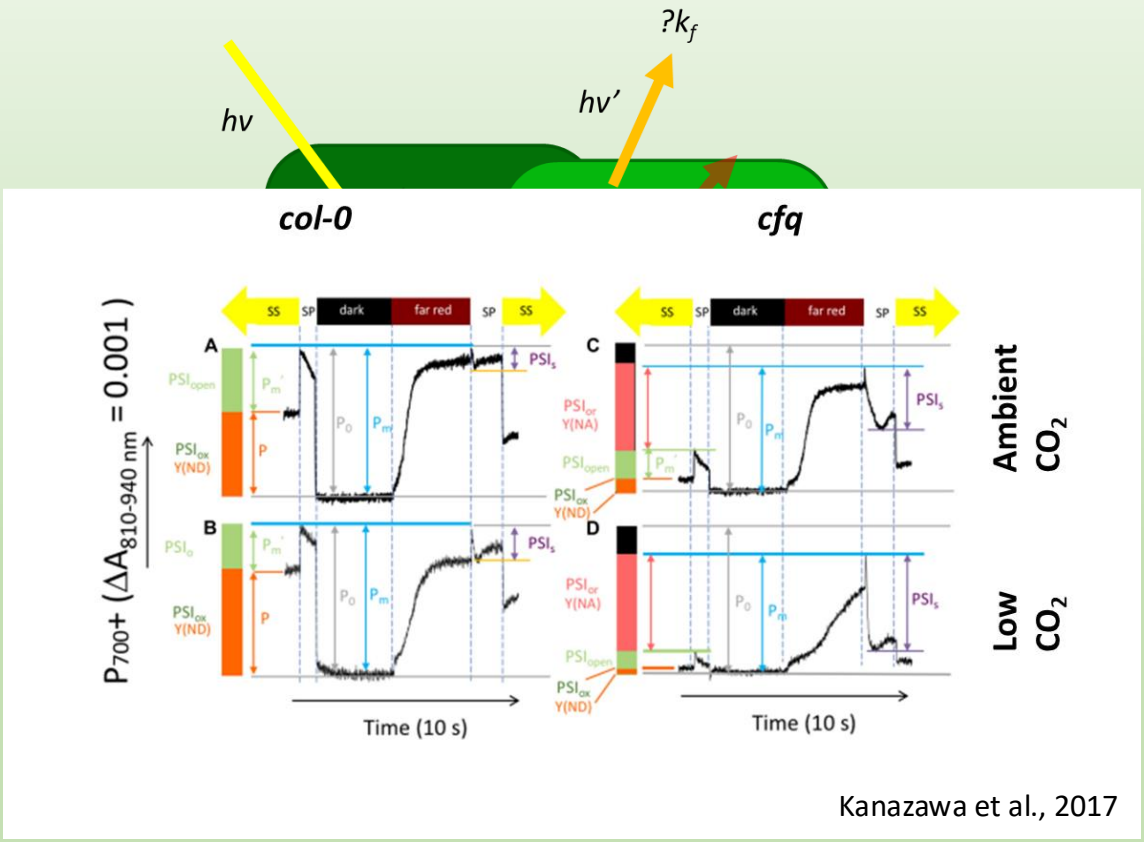
Can ϕ_I be used to calculate PSI rate?

- ϕ_I is not a true quantum yield since it does not factor in losses to competing pathways.
- Representing the fraction of open PSI, it is more comparable to q_L.
- Indicator of PSI redox status/stress, e.g. over-reduction of electron transfer.
- DIRK would be a more theoretically sound approach for determining rate



Kanazawa et al., 2017

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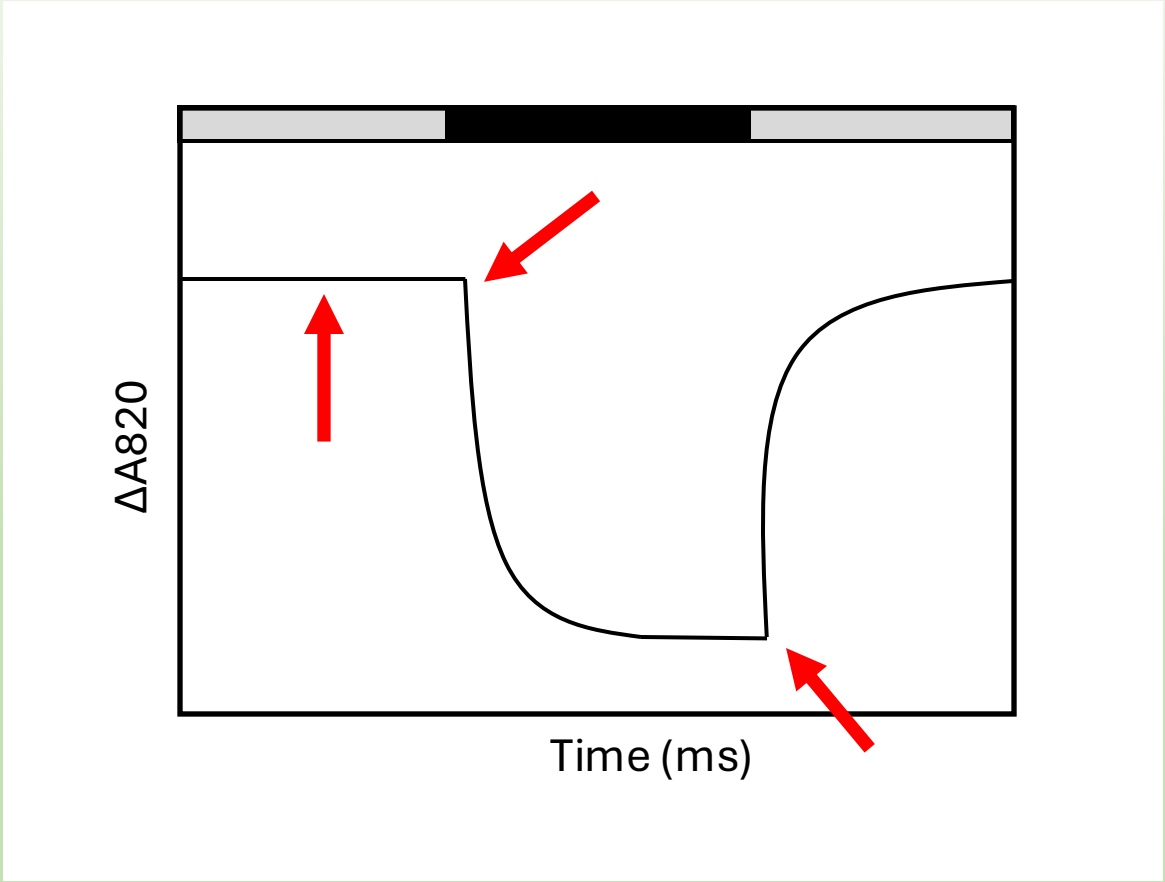
P_{700} DIRK

- Similar to ECS DIRK.
- Under steady-state illumination the P_{700} pool is partially oxidized.
- When the light is shuttered, oxidation of P_{700} stops and the oxidized pool of P_{700}^{+} is rapidly reduced, leading to a decrease in absorbance.
- Actinic illumination is restored to re-establish the steady-state.



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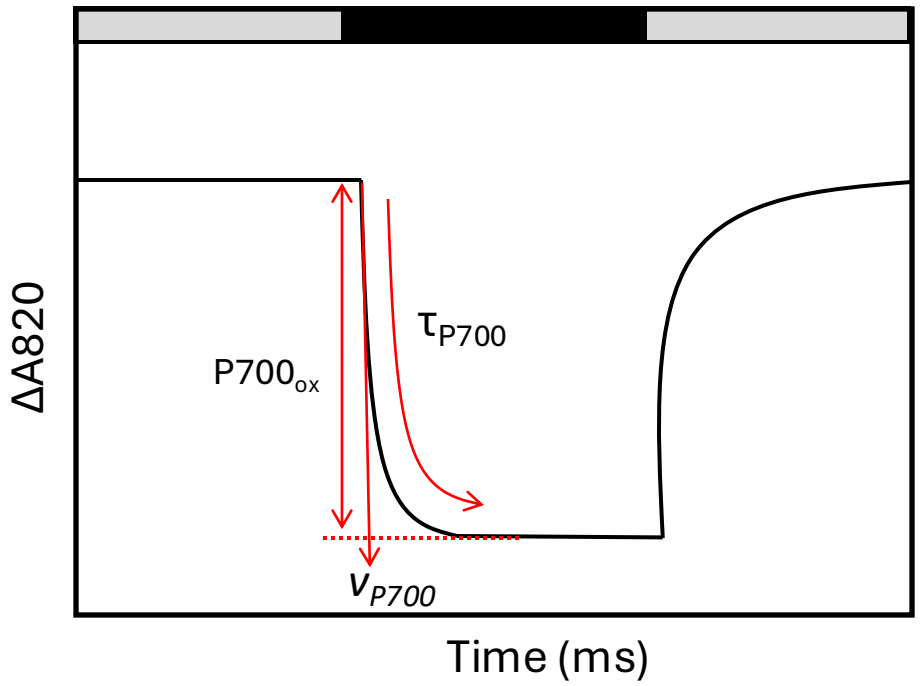
P700 DIRK parameters

- The extent of the decay, P700_DIRK_AMPL, is proportional to the amount of oxidized PSI at steady state.
- In most cases the decay can be fit to a single exponential with decay time, tP700 (τ_{P700}).
- The inverse of the decay time is equal to the rate constant for the decay kP700 (k_{P700}).
- The steady state rate of electron flux through PSI, V_initial_P700 (or v_{P700}) can be obtained by multiplying the amplitude of the decay by the rate constant or from the initial slope of the decay.

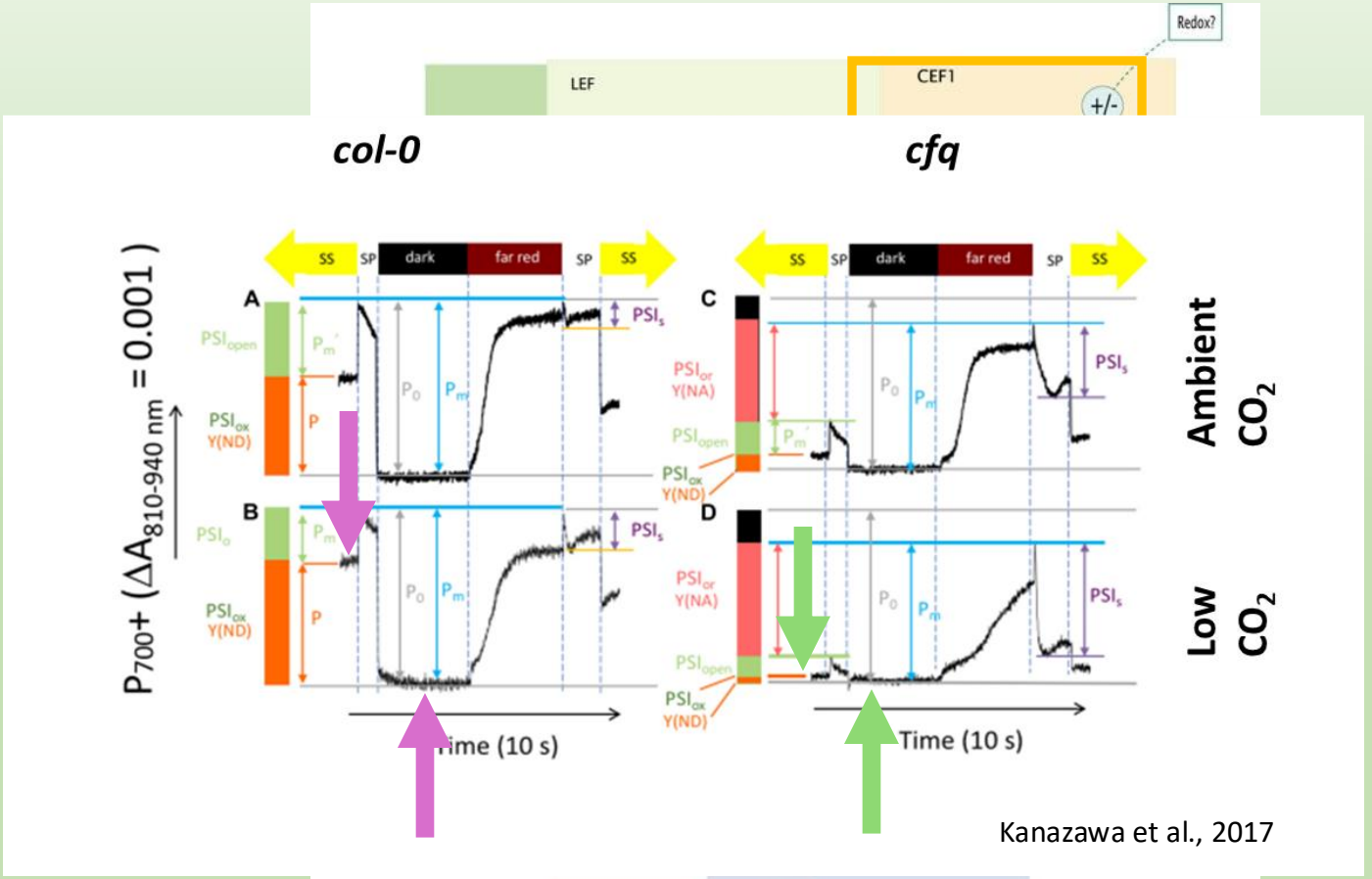


$$k_{P700} = 1/\tau_{P700}$$

$$v_{P700} = P700_{ox} \cdot k_{P700}$$

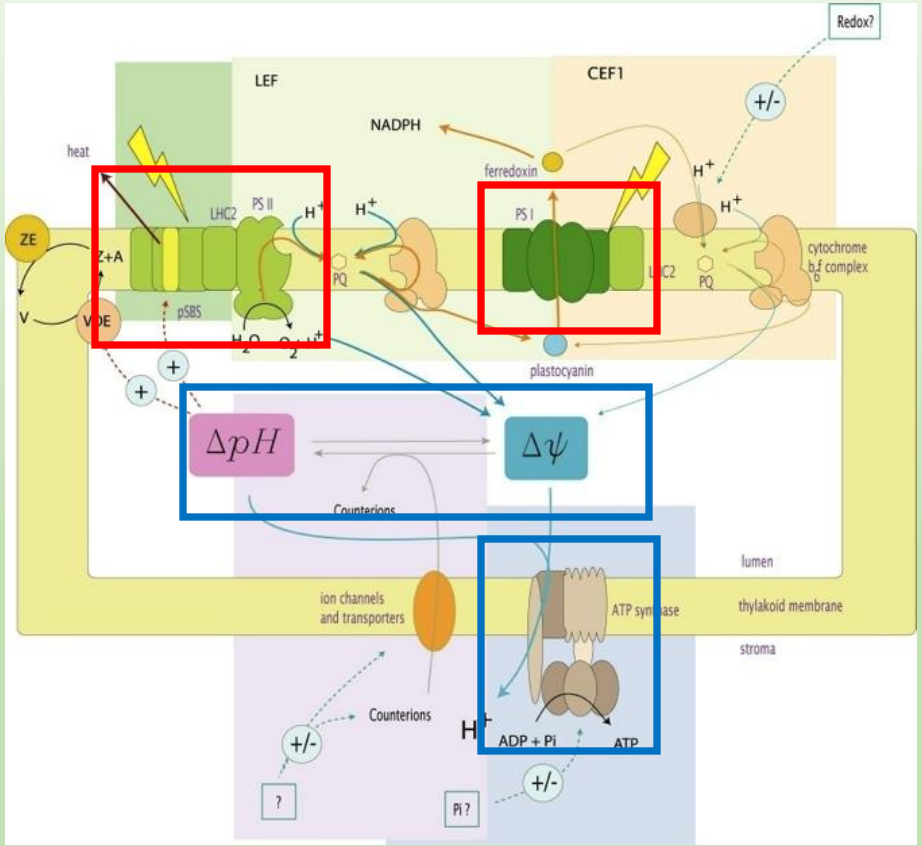
P700 DIRK

- An increase in the v_{P700} relative to LEF could indicate an increase in CEF.
- Some potential issues:
  - Signal to noise is tends to be lower.
  - Biphasic decay at high light intensity if upstream electron transfer (cyt b_6f to PSI) is limiting
  - Over-reduction can flatten the decay. The macro will fit to noise.



Parameters of interest (summary)

- Chlorophyll fluorescence (PSII)
  - Quantum yields: ϕ_{II}, ϕ_{NO}, ϕ_{NPQ}
  - Photosynthetic rate, LEF
  - Downregulation, NPQ_T
  - Redox status, q_L
- Electrochromic Shift (*pmf*, H^+ flux)
  - Light-induced *pmf,* ECS_t
  - Proton conductivity, g_{H^+}
  - Rate of flux, v_{H^+}
- 820 nm Absorbance (PSI)
  - PSI redox status: Fractions Oxidized, Reduced and Over-reduced
  - P_{700} initial rate, v_{P700}



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