Functional dynamics of proteins revealed by ultrafast fluorescence and transient absorption spectroscopy

LAMELIS 2016

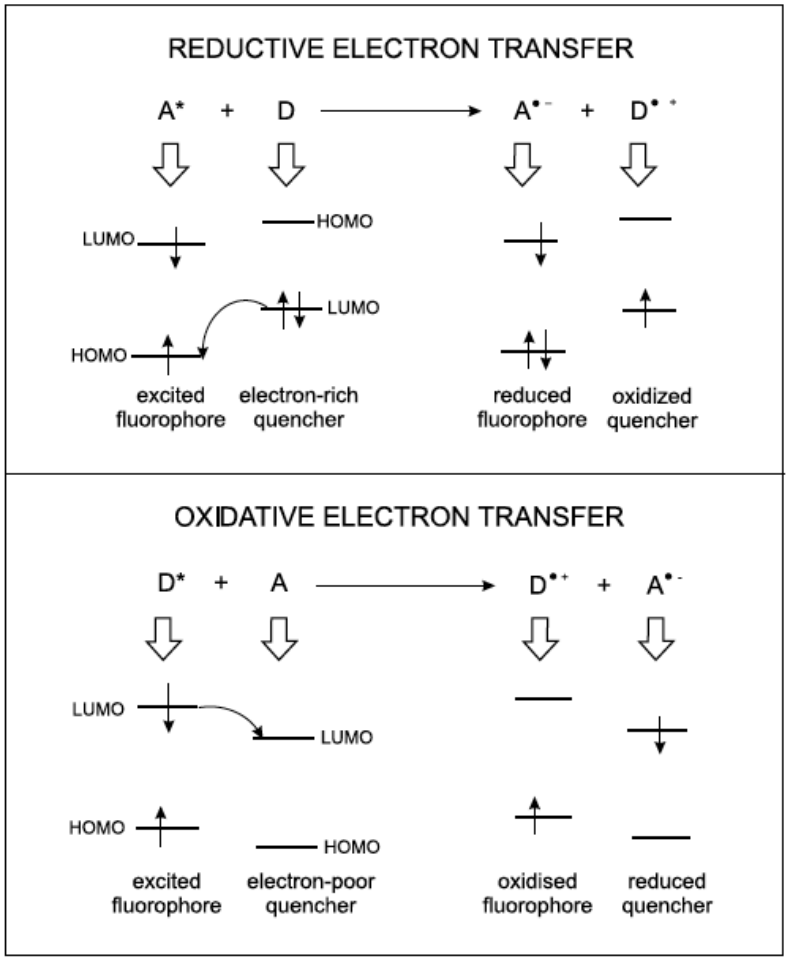
Outline

- Why do we need ultrafast laser spectroscopy?
- What do we need for ultrafast laser spectroscopy?
- Fluorescence lifetime measurement using upconversion
- Fluorescence lifetime measurement using Kerr-gate method
- Visible transient absorption
- Infrared transient absorption

Why do we need ultrafast spectroscopy?

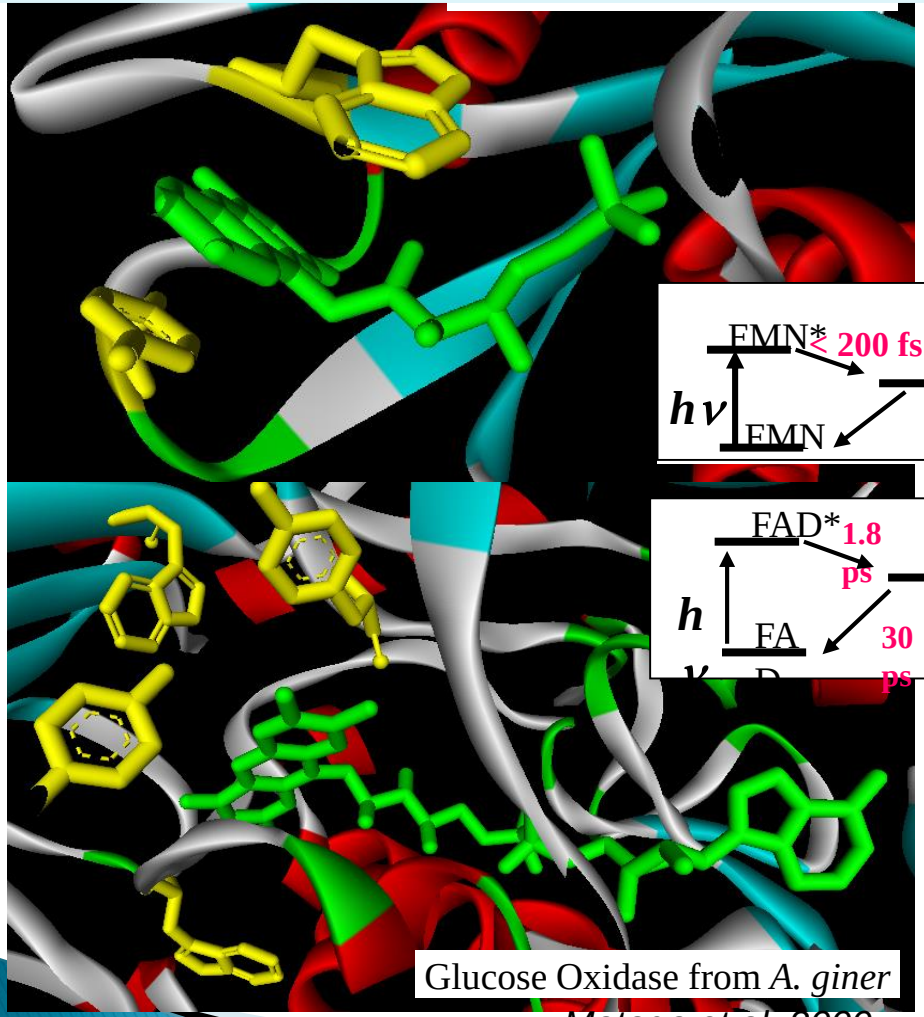
- There are many processes which take place on the femtosecond-picosecond timescale
- The primary steps of the photocycle in photolyase and cryptochrome family take place on the fs-ps timescale.
- The primary steps of the photocycle in BLUF domain proteins takes place in tens of picoseconds

Why do we need ultrafast spectroscopy?



Bernard Valeur, Molecular Fluorescence, 2001, Wiley

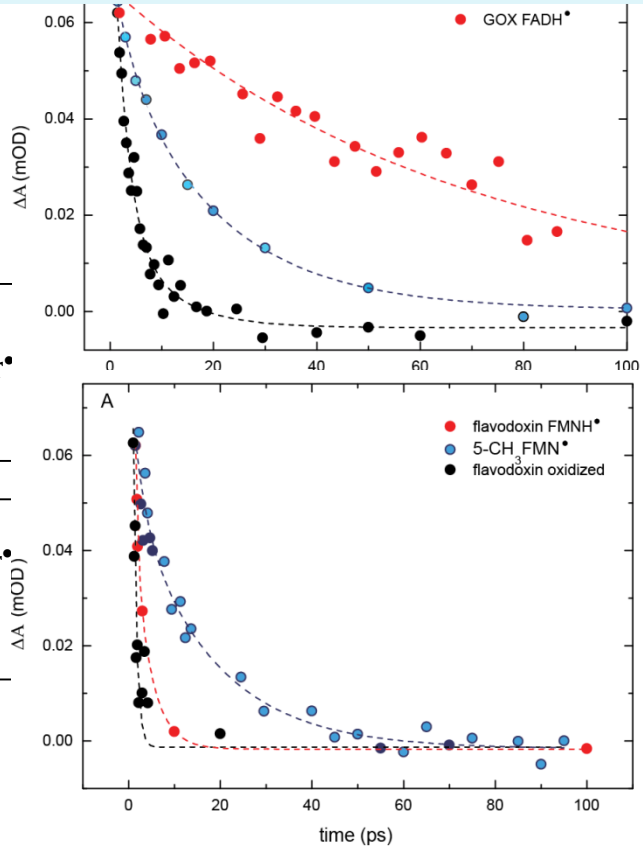
Flavin photochemistry



Glucose Oxidase from *A. giner*

Mataga et al, 2000

Zhong & Zewail, 2001

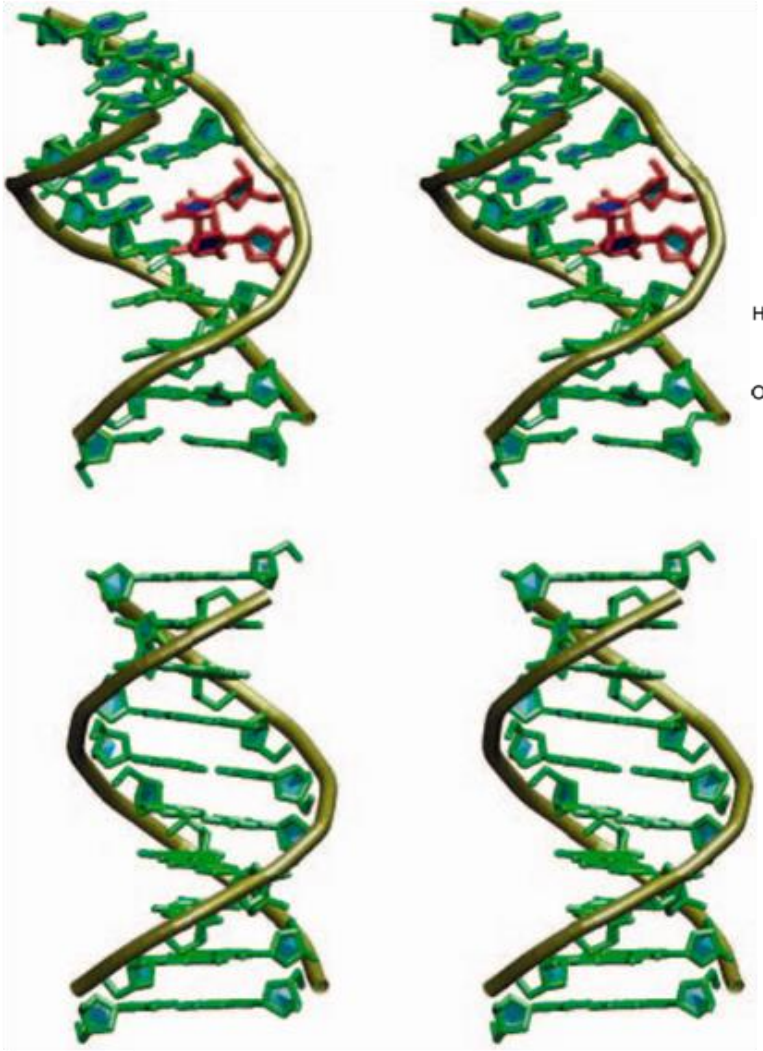


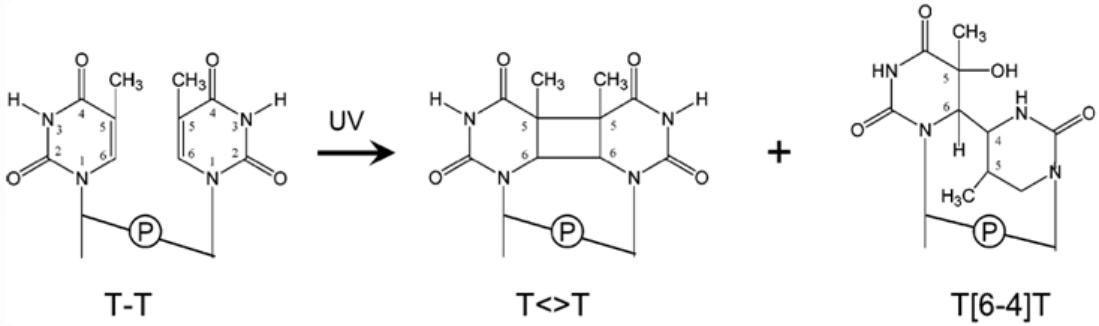
Lukacs et al., 2012

-FAD* lifetime in solution ~4 ns

In proteins: quenching by ET from aromatic residues and subsequent (sub-)picosecond recombination:

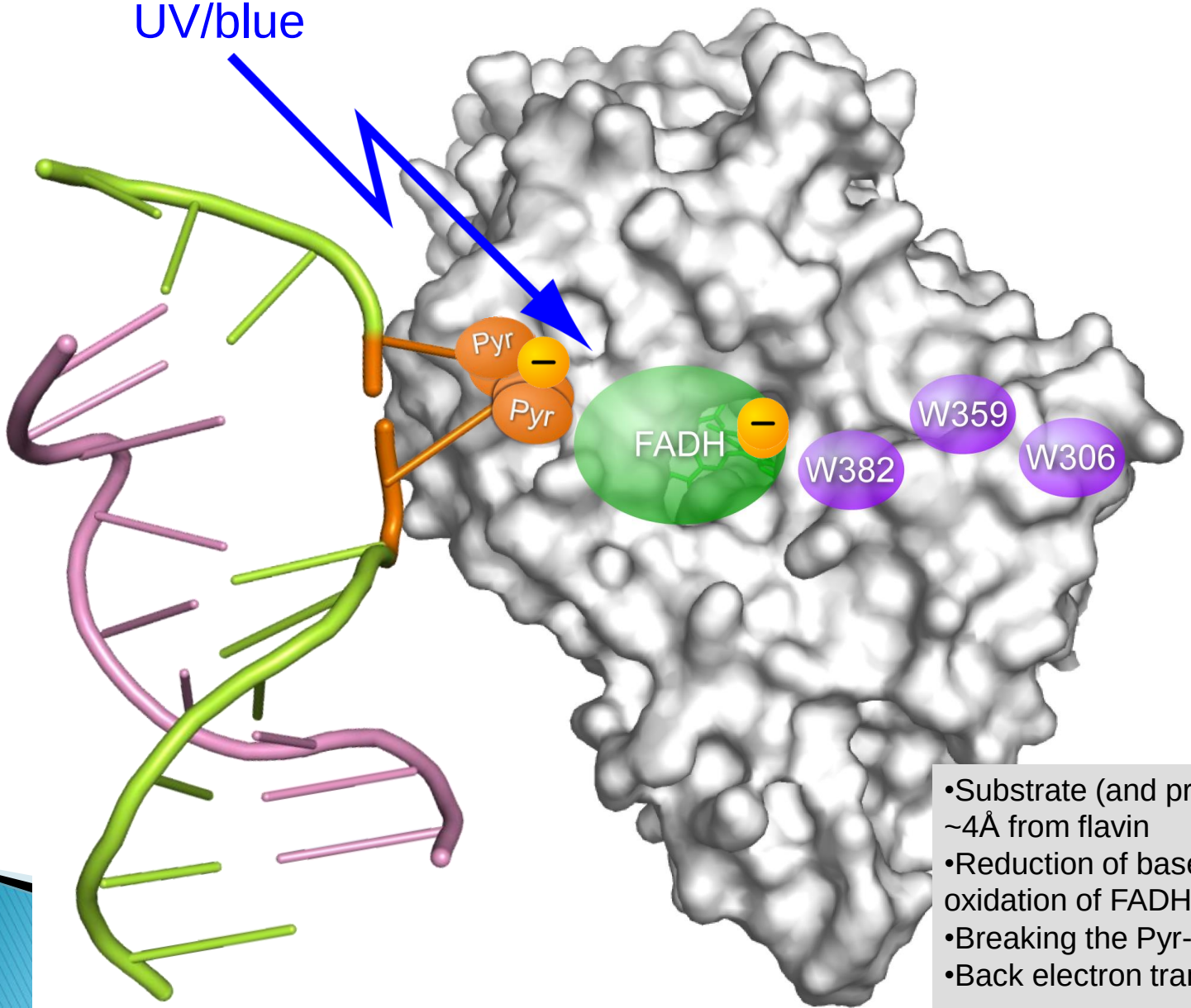
UV induced DNA damage





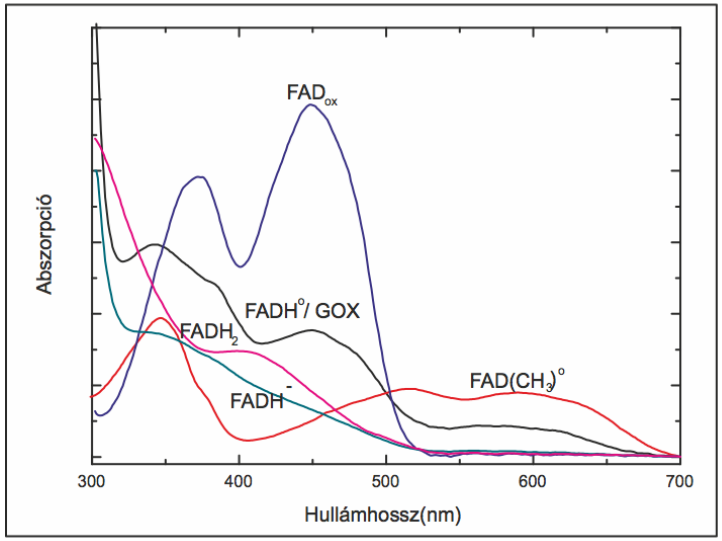
UV light induce two major lesions in DNA: cyclobutane pyrimidine dimers (Pyr<>Pyr) and the pyrimidine-pyrimidone (6-4) photoproduct (Pyr [6-4] Pyr)

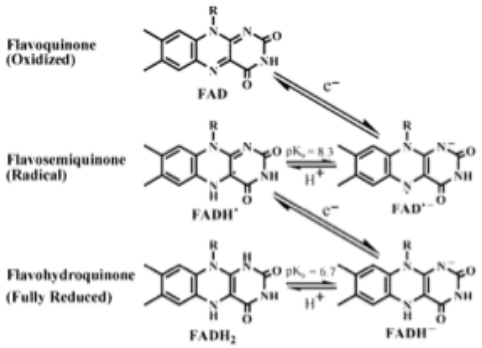
Photorepair in photolyase

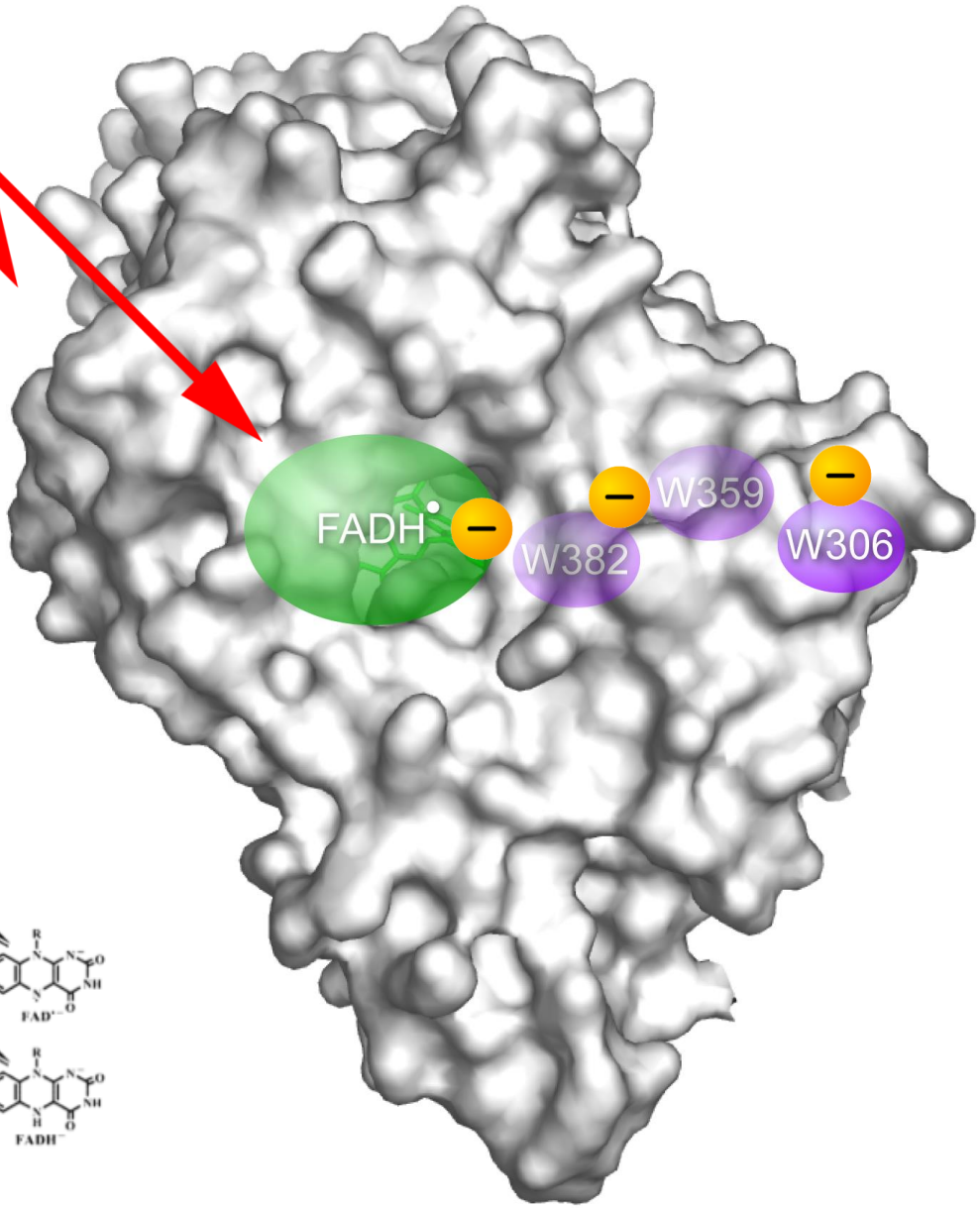


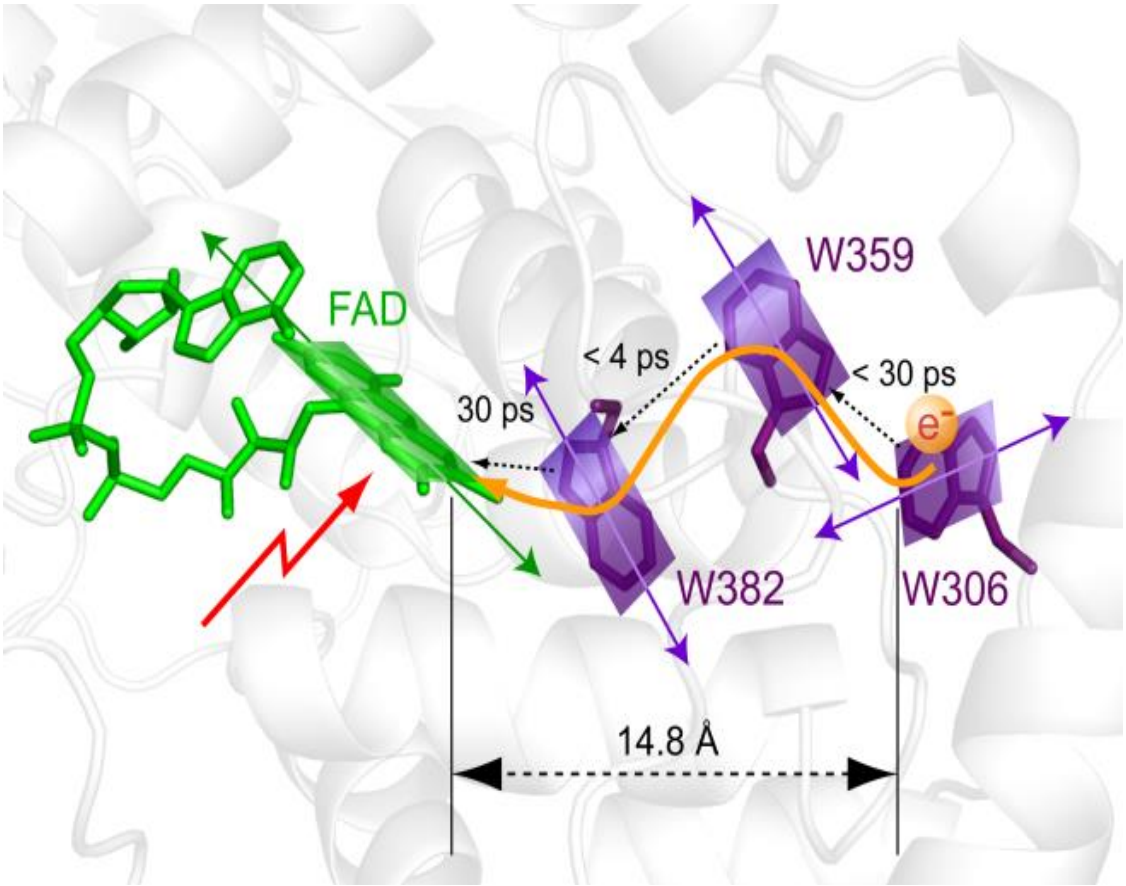
- Substrate (and product) binds at ~4Å from flavin
- Reduction of base pair by photo-oxidation of FADH- to FADH°
- Breaking the Pyr-Pyr bond
- Back electron transfer to flavin

Photoactivation in photolyase



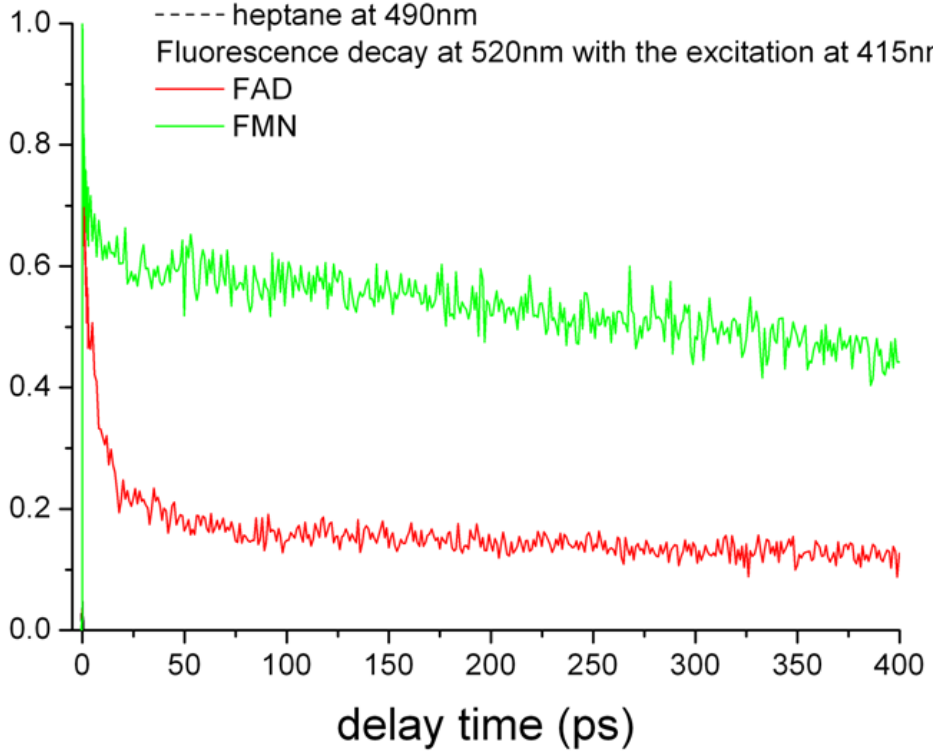


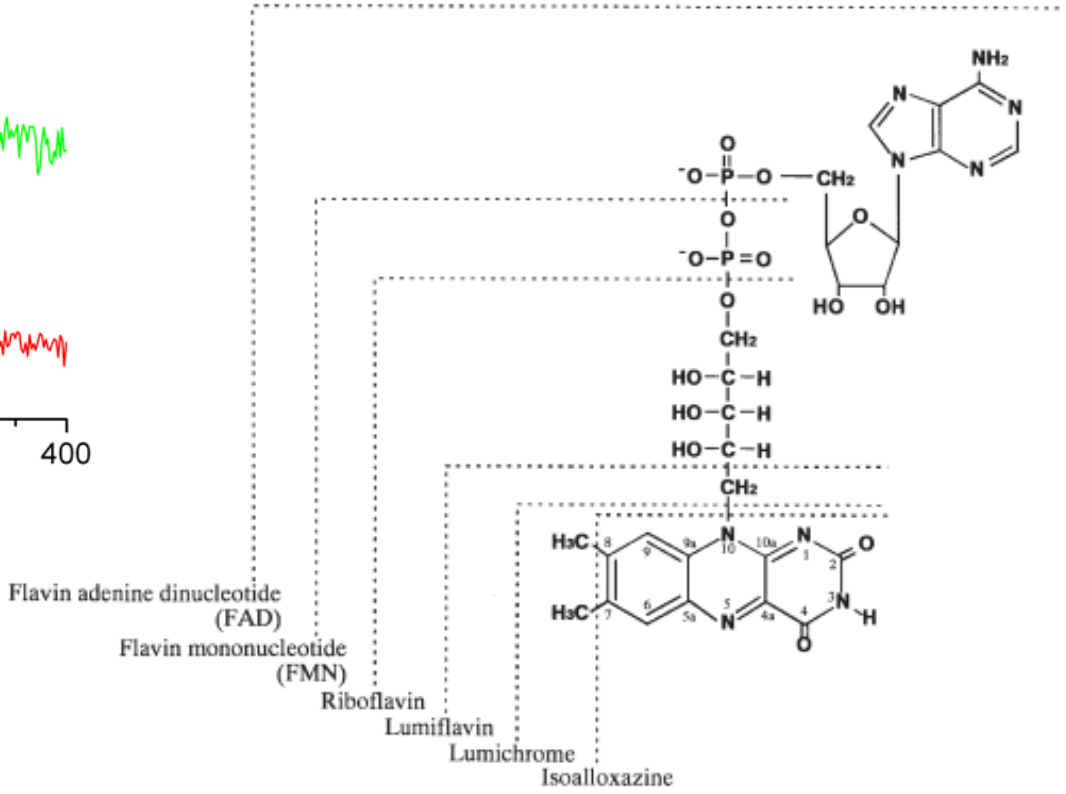


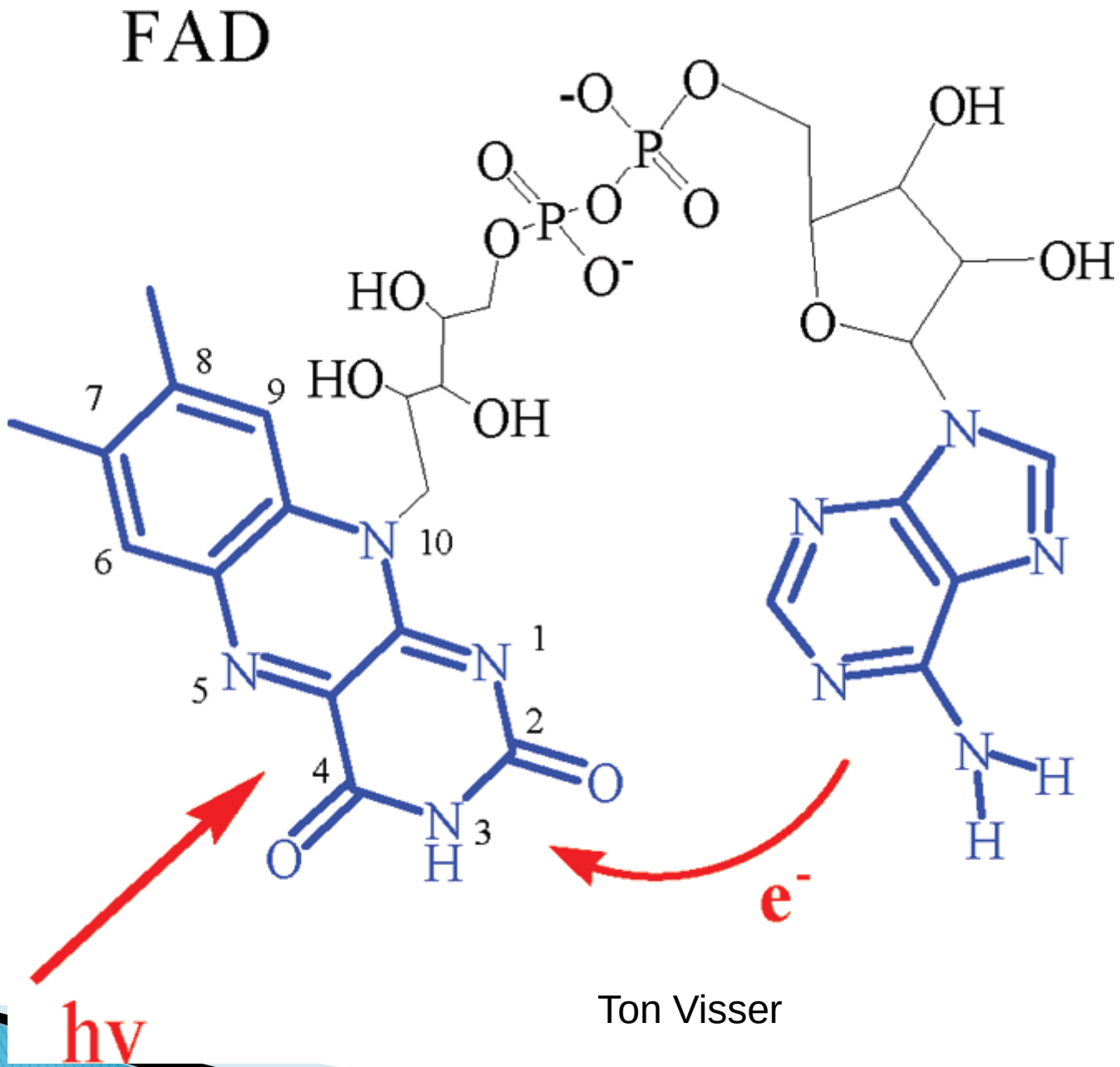


Photoreduction in photolyase (Lukacs et al, *JACS* , 2008)

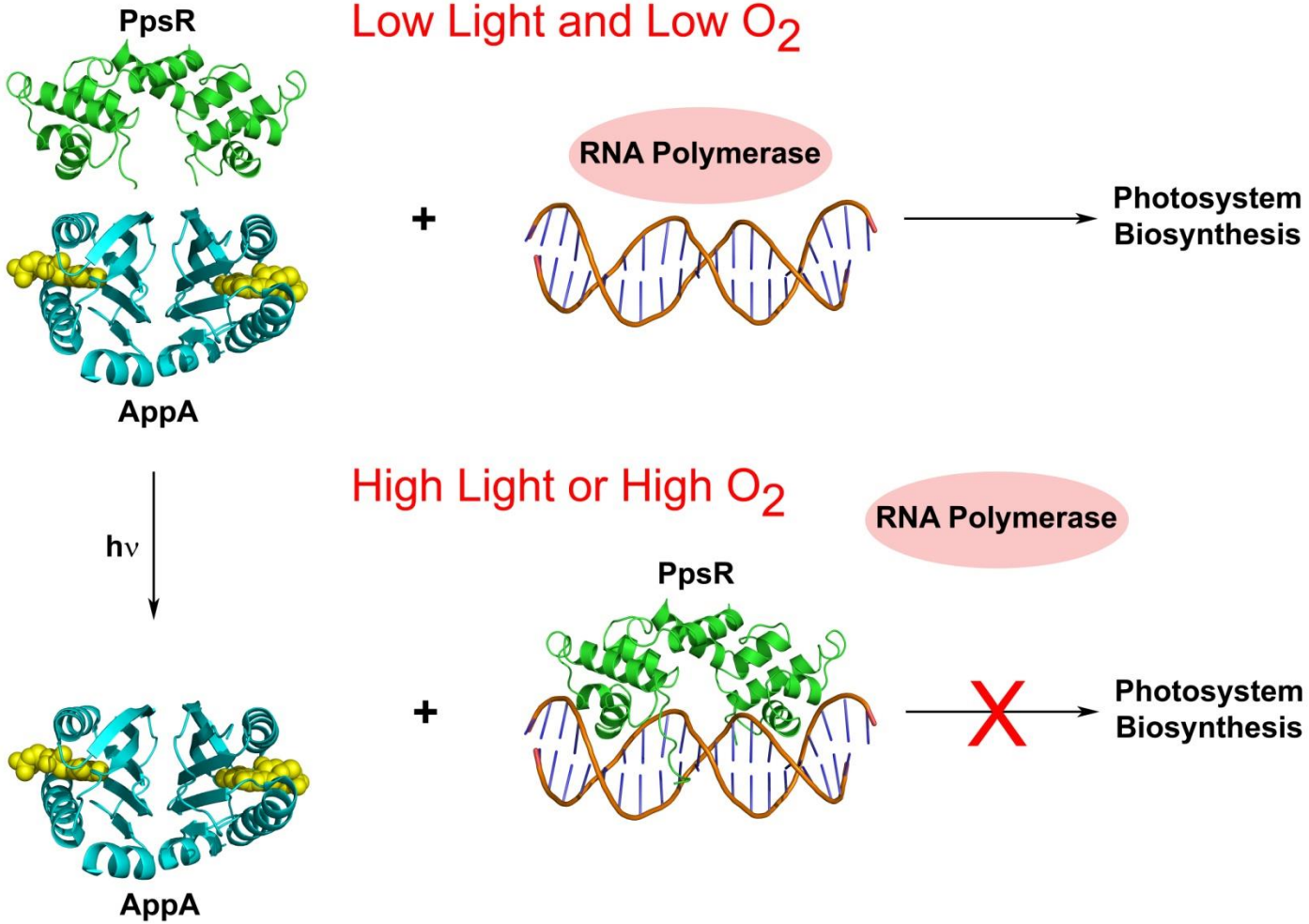
Fluorescence lifetime of FAD and FMN






FAD
hν
e-
Ton Visser

Biological Response of the AppA BLUF Domain



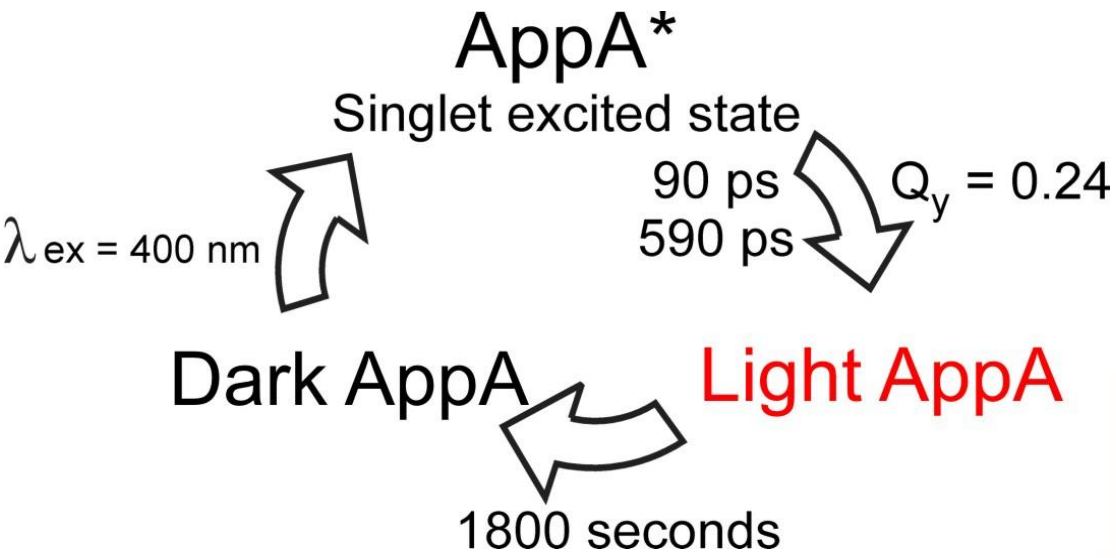
Masuda, S., and Bauer, C. E. Cell, 2002. 110: p. 613-623.

How Does Light Absorption Lead to the Release of PpsR?

AppA is a Multidomain Complex

- N-terminal BLUF domain
- C-terminal oxygen sensing domain that binds PpsR
- AppA acts as a dual sensor : two signals give the same response
  - How are the signals related?
  - How does light absorption by the N-terminal BLUF domain cause a structural change in the C-terminus, leading to the release of PpsR?

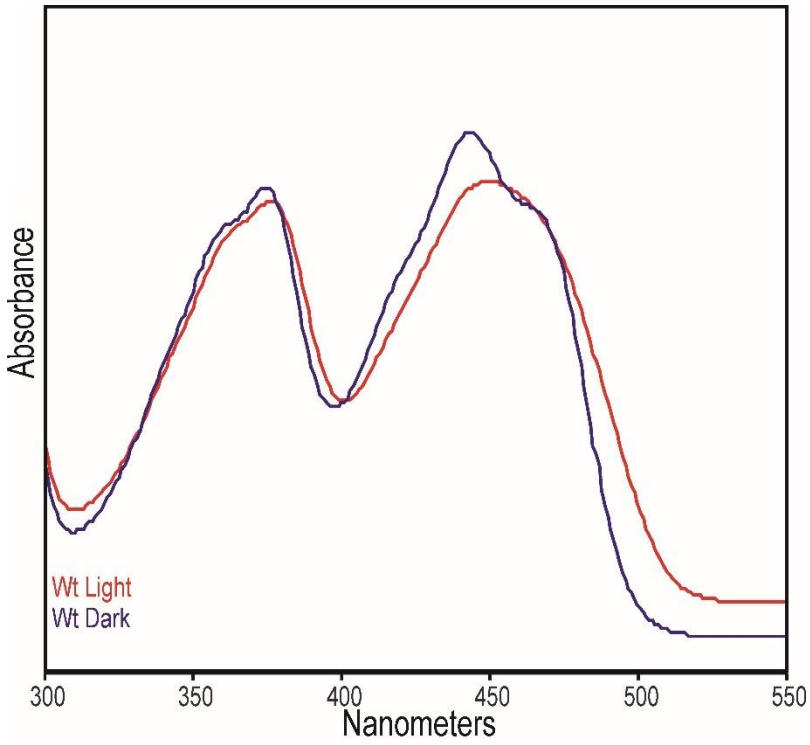
The BLUF Domain of AppA Has Two States: Dark AppA and Light AppA



Gauden M, Y.S., et al. Biochemistry, 2005. 44(10): p. 3653-62.

Formation of the long lived Light state on a picosecond timescale

After excitation with 400 nm light 10 nm red-shifted electronic spectrum



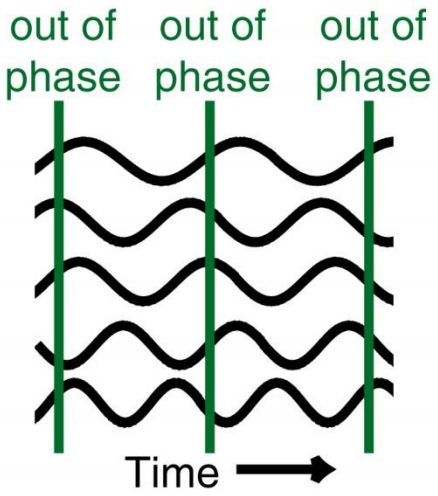
What do we need for ultrafast spectroscopy?

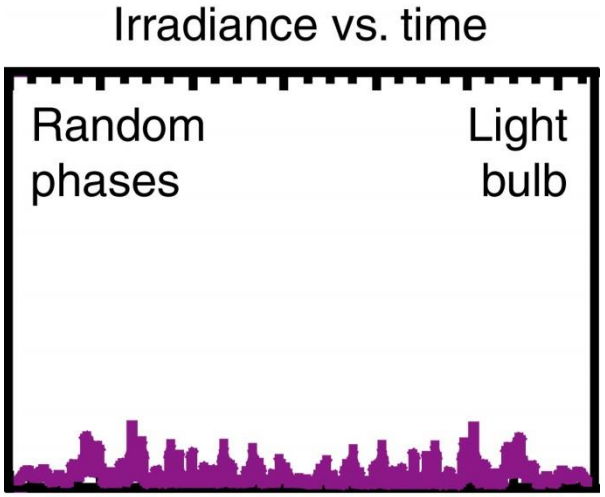
- Ultrafast lasers (oscillator or amplifier)
- Ti:Sa oscillator high repetition rate (100 MHz) and lower energy (1nJ)
- Regenerative amplifier (1-10 kHz) but high (1-5 mJ) energy
- The resolution of the system is limited by the pulse with of the laser
- Typical resolution is between 100-200 fs. There are system (UEA, RIKEN) less than 50 fs

Mode-locking

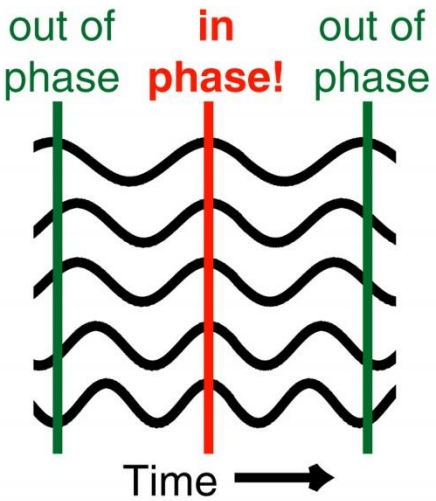
- Locking the phases of the laser modes yields an ultrashort pulse.

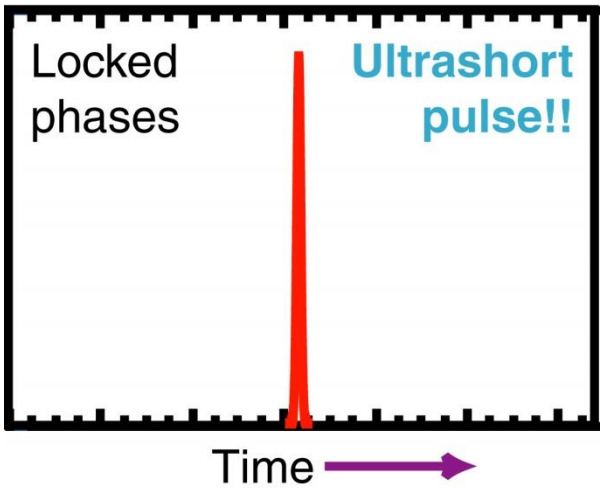
Random phases of all laser modes





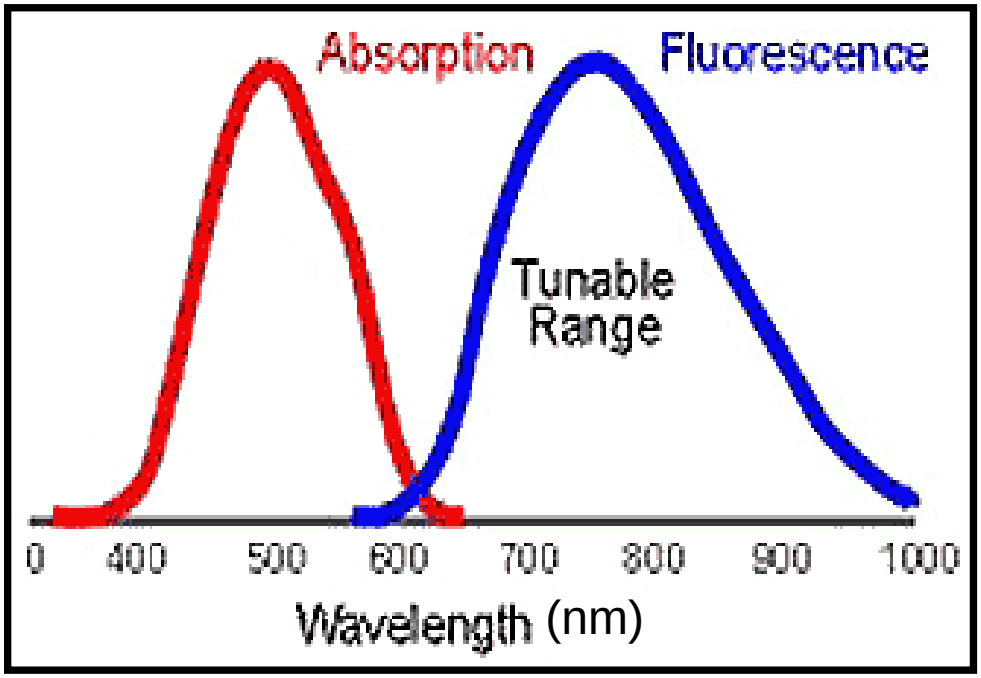
Locked phases of all laser modes





Ti:sapphire laser

It can be pumped with a (continuous) Argon laser (~450-515 nm) or a doubled-Nd laser (~532 nm).

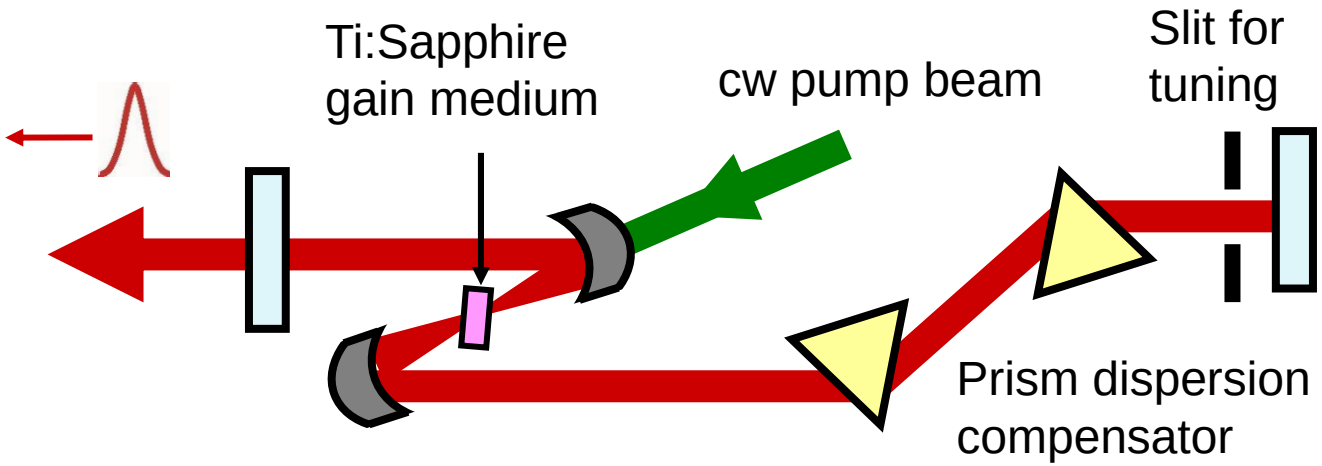


Upper level lifetime:
3.2 μsec

Ti:Sapphire lases from ~700 nm to ~1000 nm.

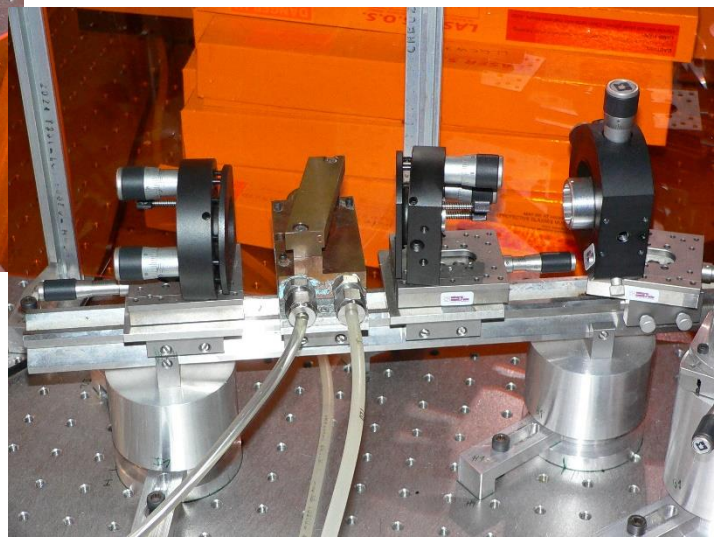
Ti:sapphire laser

Adding two prisms compensates for dispersion in the Ti:Sapphire crystal and mirrors.



This is currently the workhorse laser of the ultrafast optics community.

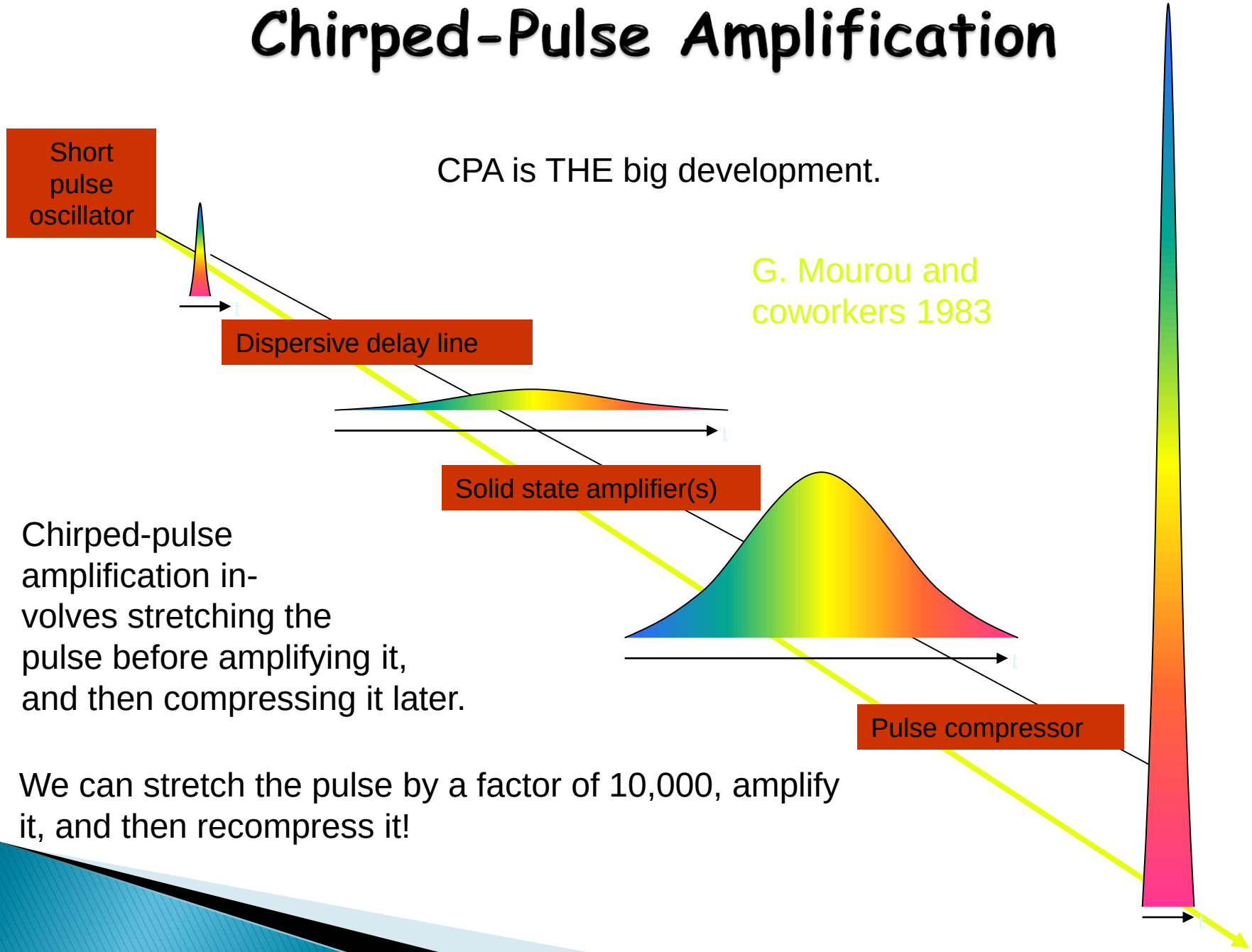
Ti:sapphire laser



Chirped-Pulse Amplification

CPA is THE big development.

G. Mourou and coworkers 1983



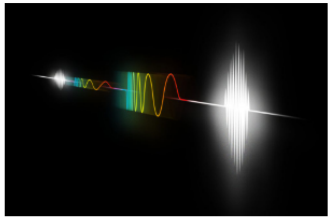
Chirped-pulse amplification involves stretching the pulse before amplifying it, and then compressing it later.

We can stretch the pulse by a factor of 10,000, amplify it, and then recompress it!

Nobel Prize in Physics 2018

Tools made of light

The 2018 Nobel Prize in Physics was awarded with one half to Arthur Ashkin "for the optical tweezers and their application to biological systems" and the other half jointly to Gérard Mourou and Donna Strickland "for their method of generating high-intensity, ultra-short optical pulses".



© Johan Jarnestad / The Royal Swedish Academy of Sciences

"When I described catching living things with light people said: 'Don't exaggerate Ashkin'"

Arthur Ashkin took a break from his current research to talk about the "old research" that led to a Nobel Prize. Listen to our interview with him.

Arthur Ashkin
Ill. Niklas Elmehed. © Nobel Media

"It's an amazing moment"

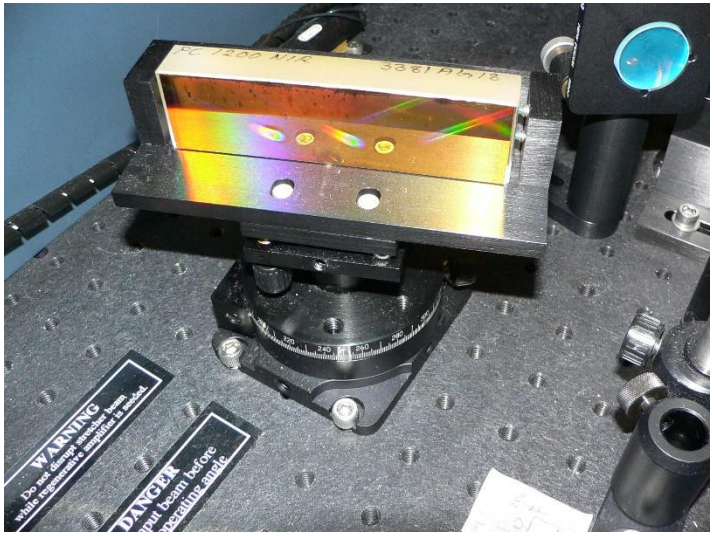
"Nobody is prepared for that kind of moment." Gérard Mourou shares his reaction to being awarded the Nobel Prize when we reached him for to a telephone interview following the announcement of the 2018 Physics Prize.

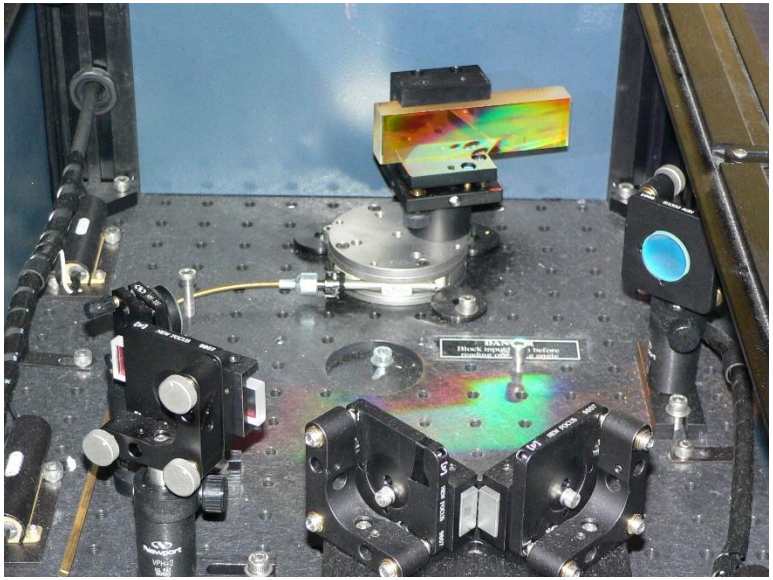
Gérard Mourou
Ill. Niklas Elmehed. © Nobel Media

"We scientists like to puzzle as to why something is working"

Donna Strickland never worked as hard or had as much fun as when she was performing the research that led her to the 2018 Nobel Prize in Physics. We talked to her after the prize had been announced.

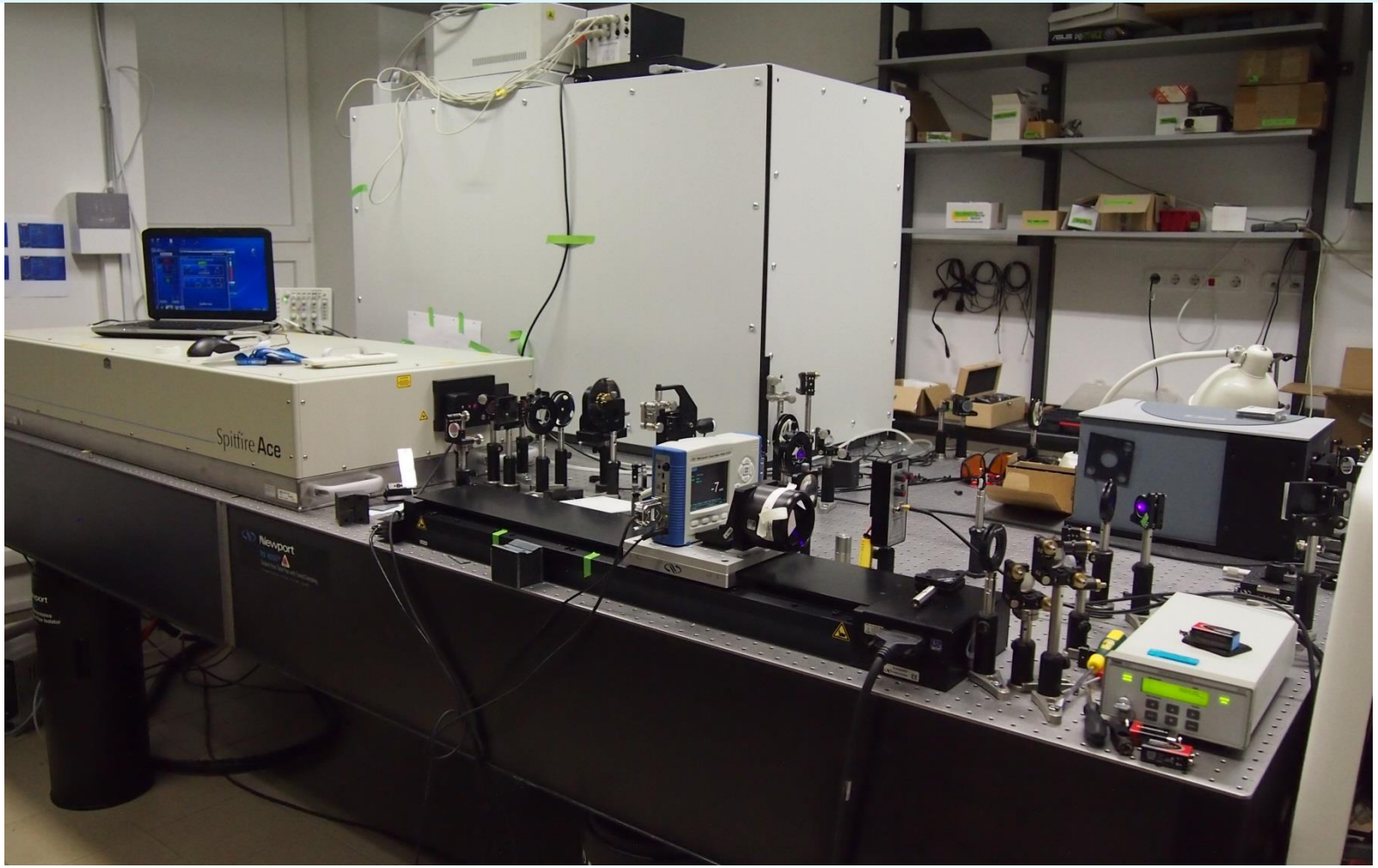
Donna Strickland
Ill. Niklas Elmehed. © Nobel Media


WARNING
Do not damage seeder beam
while regenerative amplifier is locked.
DANGER



What do we need for ultrafast spectroscopy?

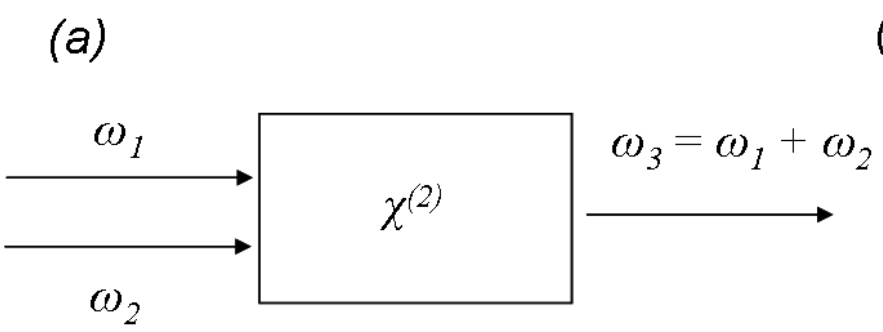
What do we need for ultrafast spectroscopy?

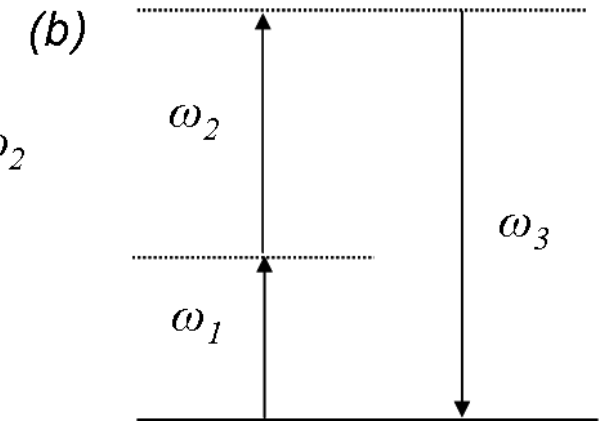


Ultrafast laser spectroscopy

I. Fluorescence upconversion

Frequency mixing





Minako Kondo, PhD thesis, UEA, 2011

ω3 = ω1 + ω2

$$\vec{k}_3 = \vec{k}_2 + \vec{k}_1$$

$$\Delta k = \vec{k}_3 - \vec{k}_2 - \vec{k}_1$$

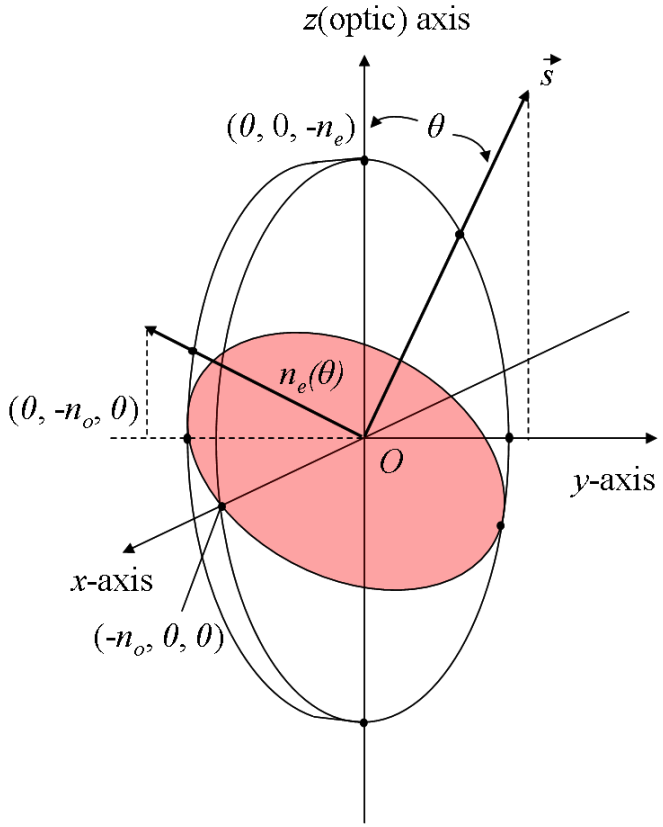
conservation of energy

Conservation of momentum

Phase mismatch

Frequency mixing

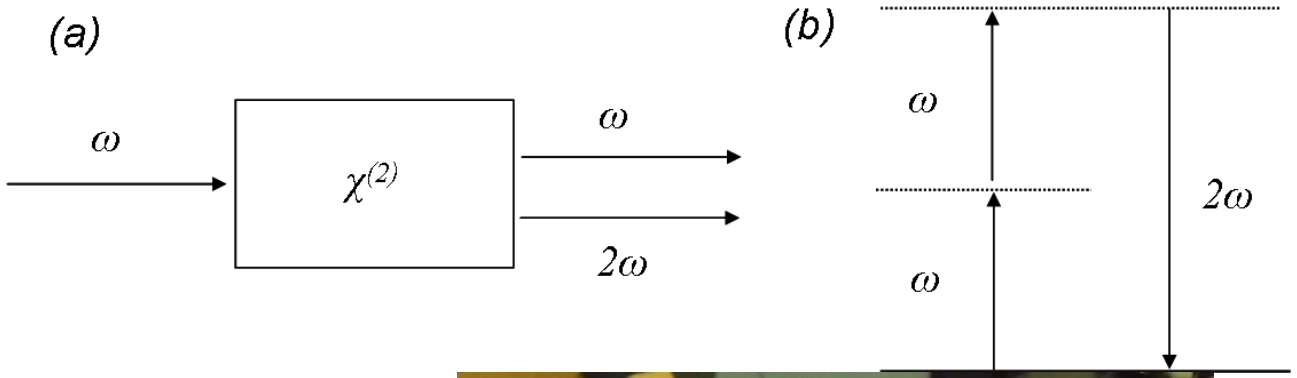
Phase matching in a birefringent crystal (BBO)



$$n_3^e \omega_3 = n_1^o \omega_1 + n_2^o \omega_2$$

$$\sin^2 \theta_m = \frac{(1/n_3^2(\theta_m)) - (1/n_{o3}^2)}{(1/n_{e3}^2) - (1/n_{o3}^2)}$$

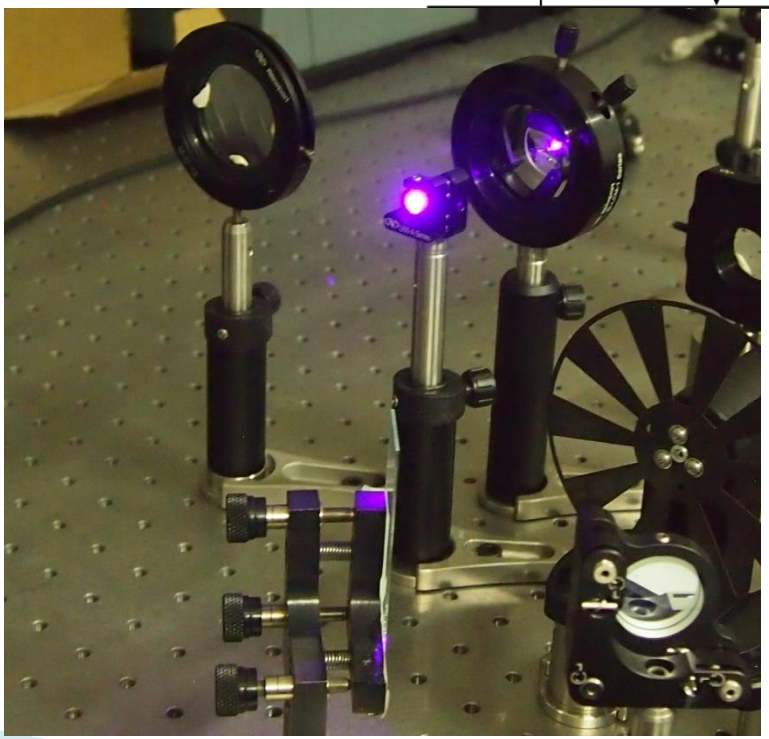
Frequency mixing



Type I. phase matching:

800.0(o)+ 800.0(o)= 400.0(e)

theta = 29.2 deg.



First demonstration of second-harmonic generation

- P.A. Franken, et al, Physical Review Letters 7, p. 118 (1961)

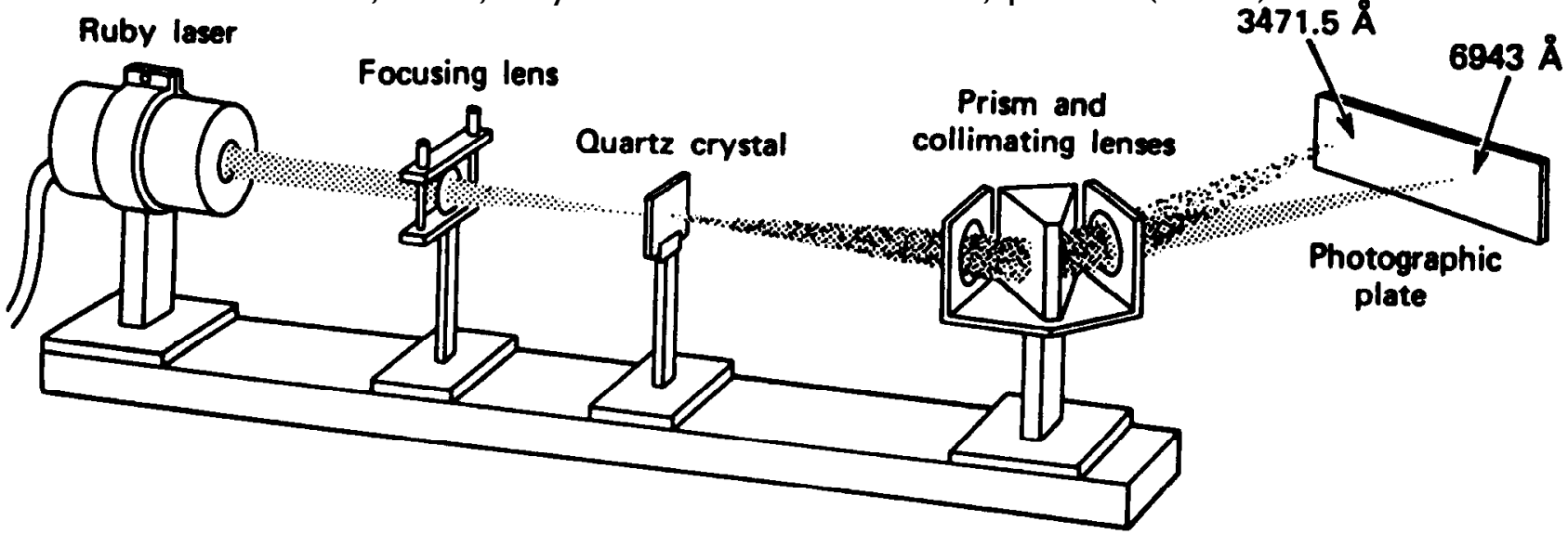
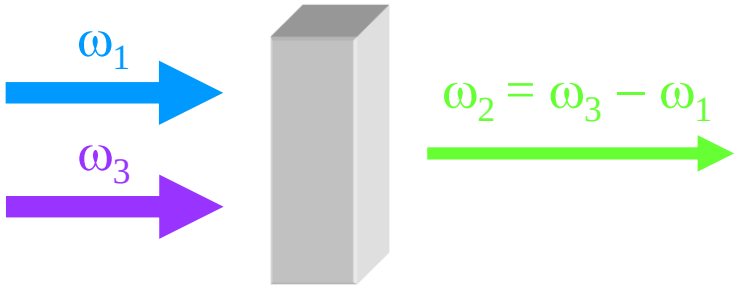


Figure 12.1. Arrangement used in the first experimental demonstration of second-harmonic generation [1]. A ruby-laser beam at $\lambda = 0.694\ \mu$m is focused on a quartz crystal, causing the generation of a (weak) beam at $\frac{1}{2}\lambda = 0.347\ \mu$m. The two beams are then separated by a prism and detected on a photographic plate.

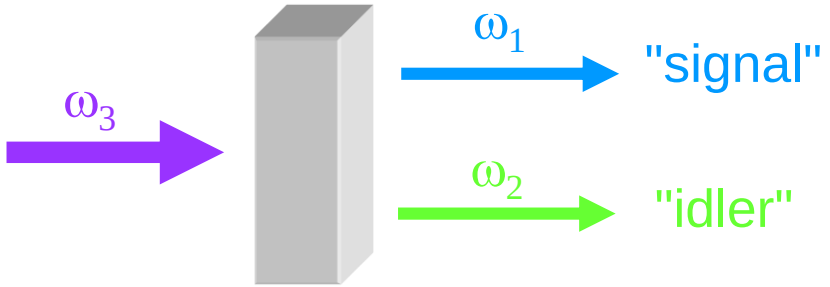
The second-harmonic beam was very weak because the process was not phase-matched.

Difference-Frequency Generation: Optical Parametric Generation, Amplification, Oscillation

Difference-frequency generation takes many useful forms.

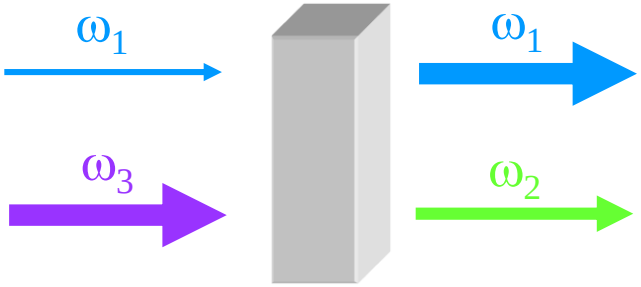


Parametric Down-Conversion
(Difference-frequency generation)

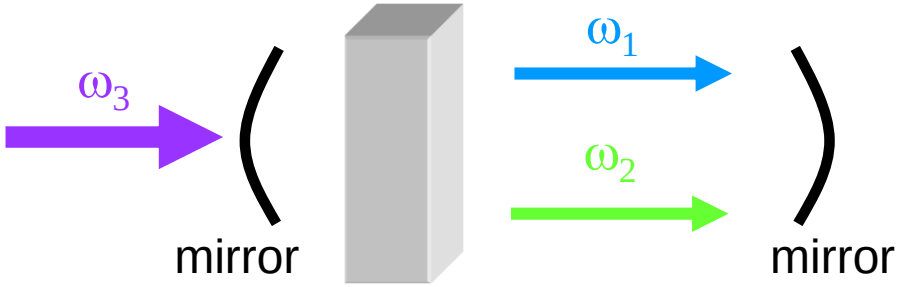


Optical Parametric
Generation (OPG)

By convention:
$\omega_{signal} > \omega_{idler}$



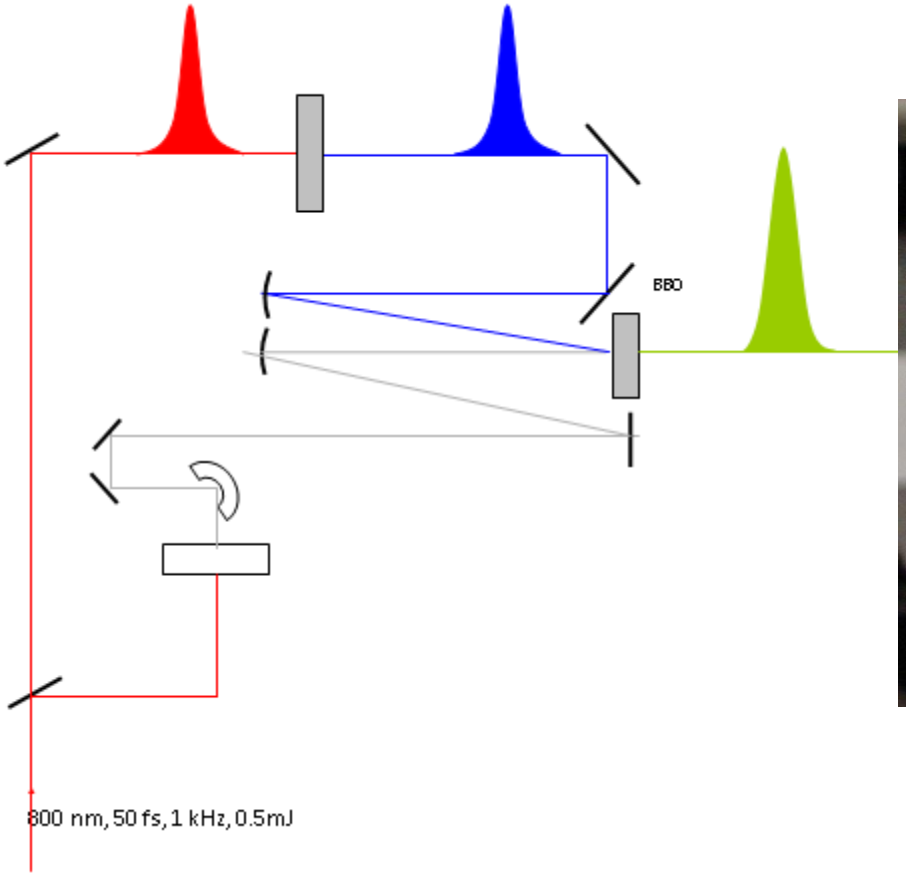
Optical Parametric
Amplification (OPA)

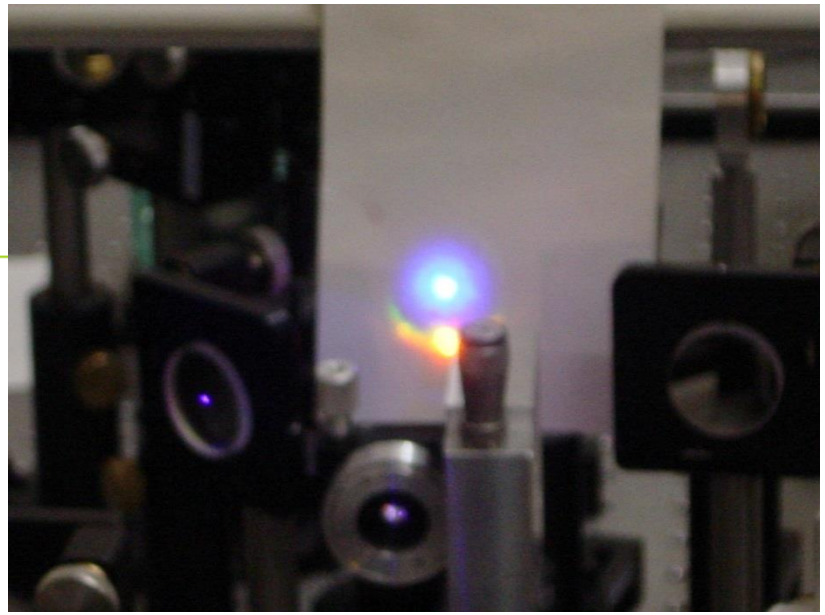


Optical Parametric
Oscillation (OPO)

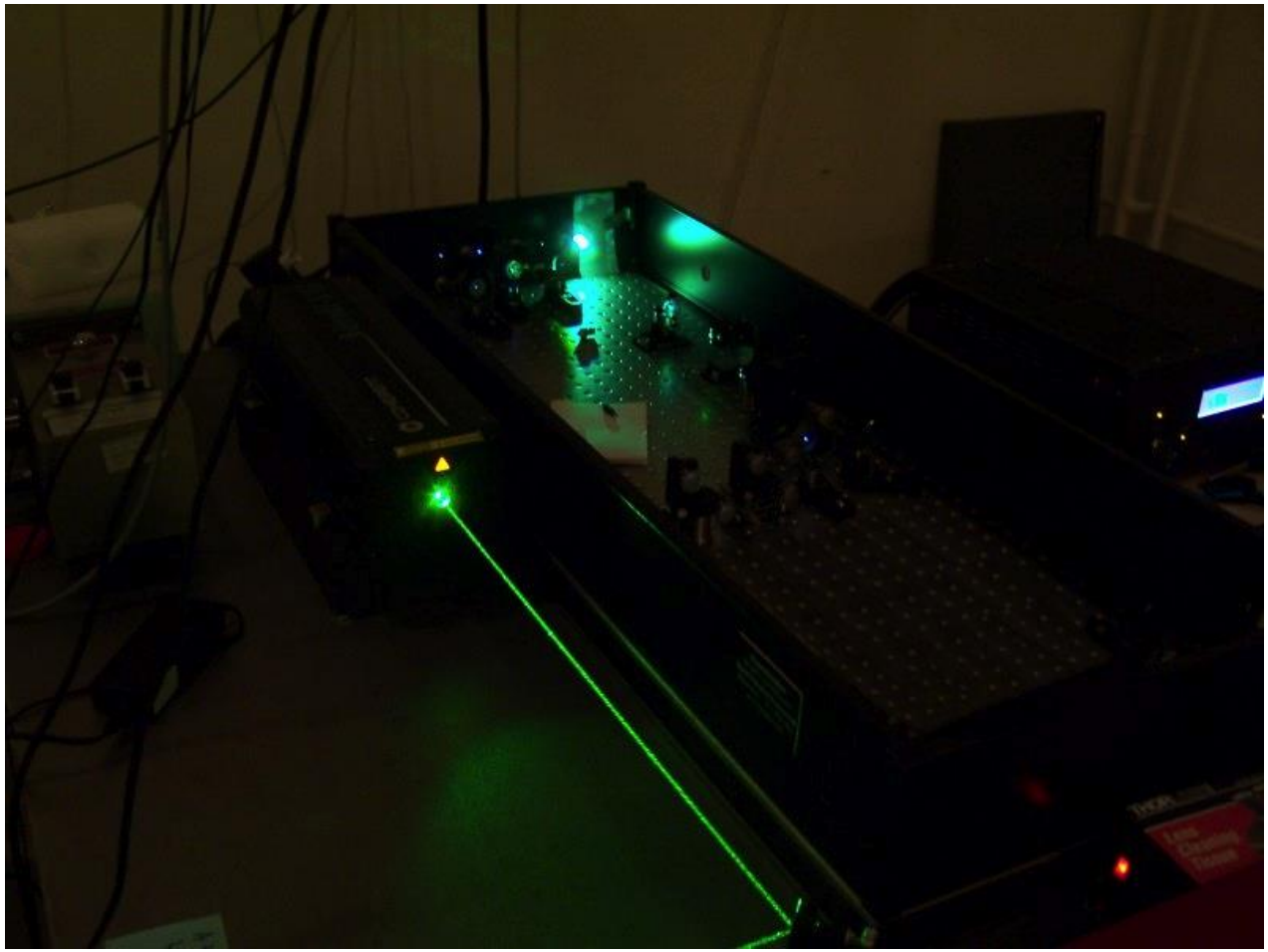
Frequency mixing

Noncollinear optical amplification



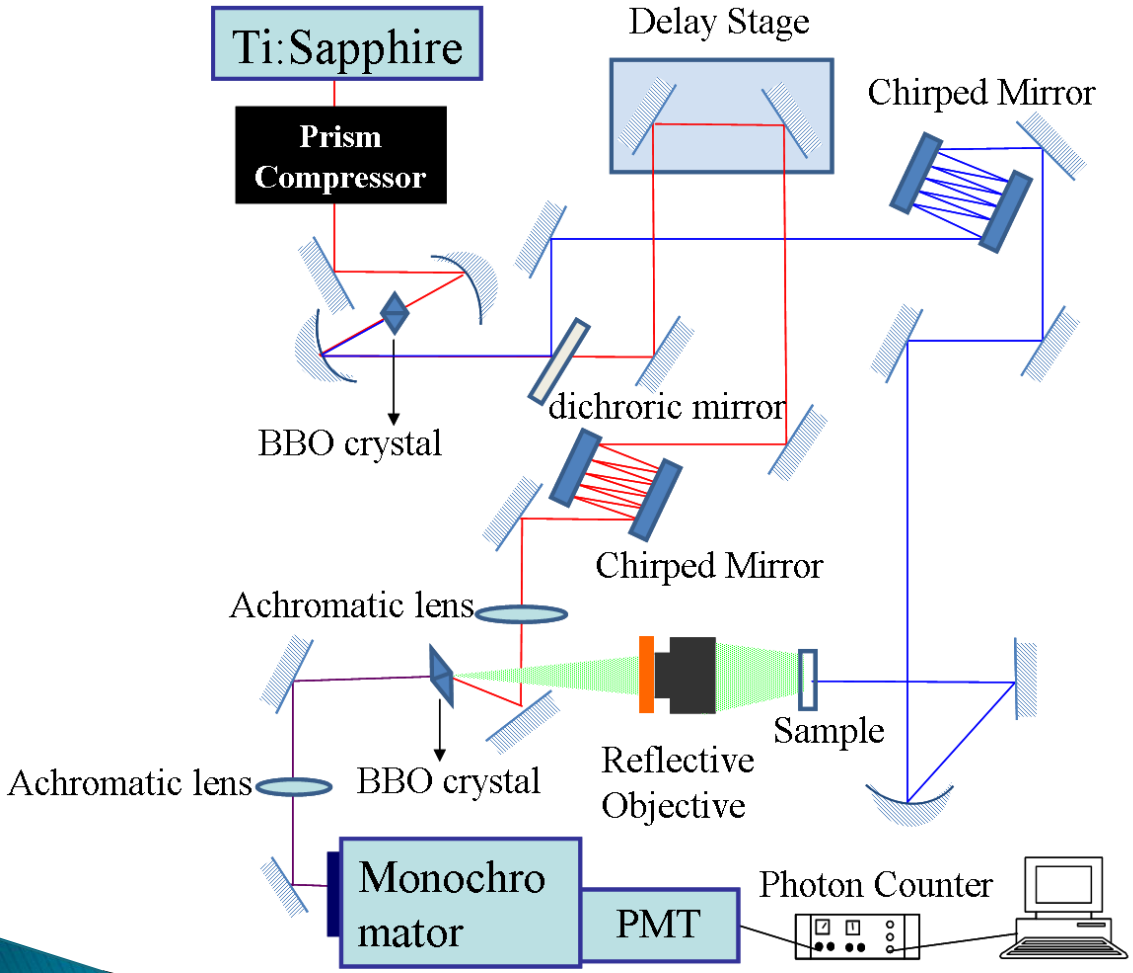


Frequency mixing (OPOs)



Optical parametric oscillators are tunable femtosecond light sources. They are working at the same repetition rate as the oscillator

Fluorescence upconversion

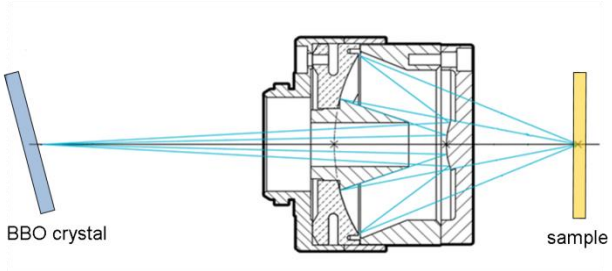


Type I. phase matching:

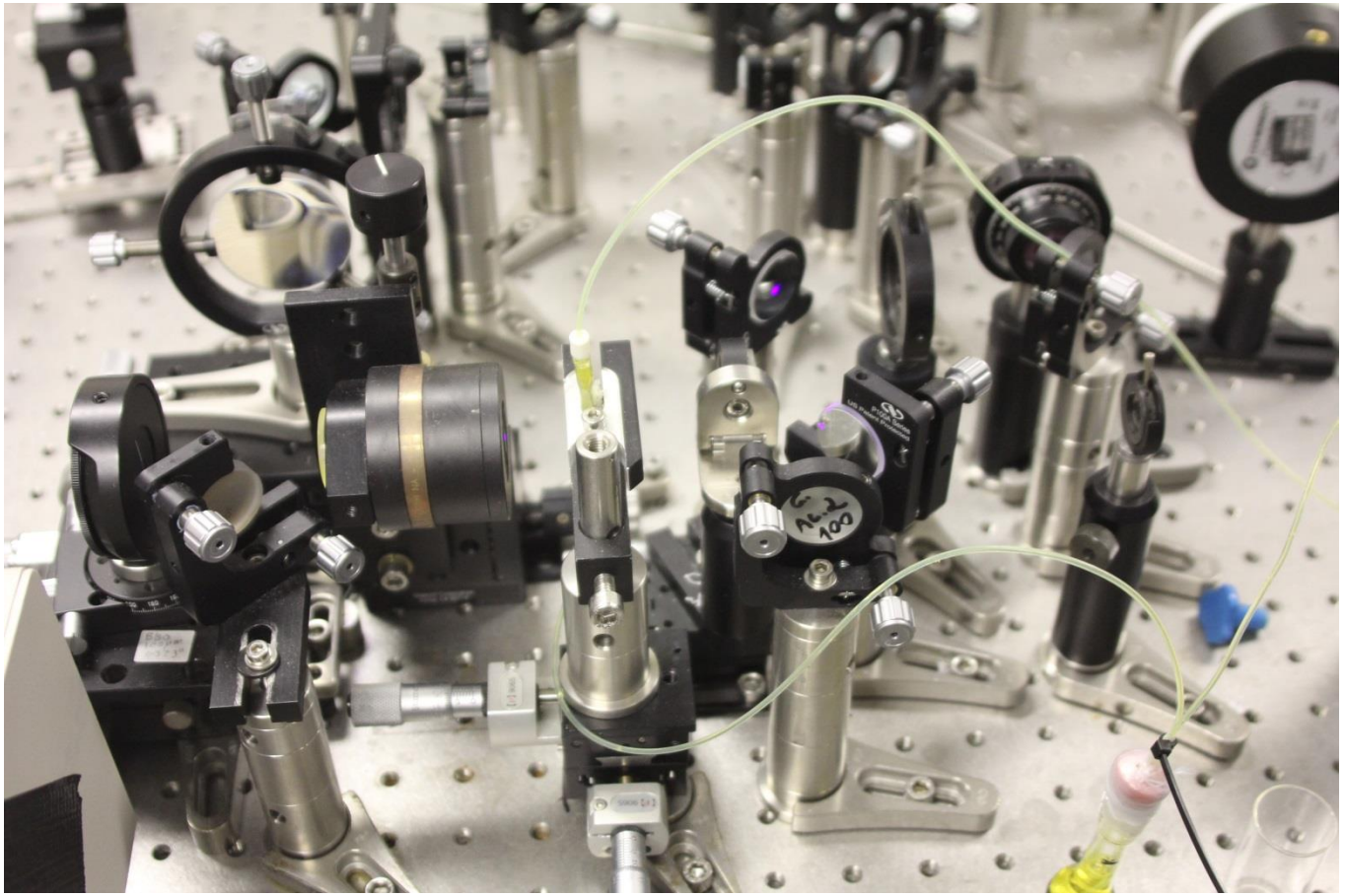
800.0(o)+ 520.0(o)= 315.2 (e)

Theta = 37.2 deg

Cassegrain objective

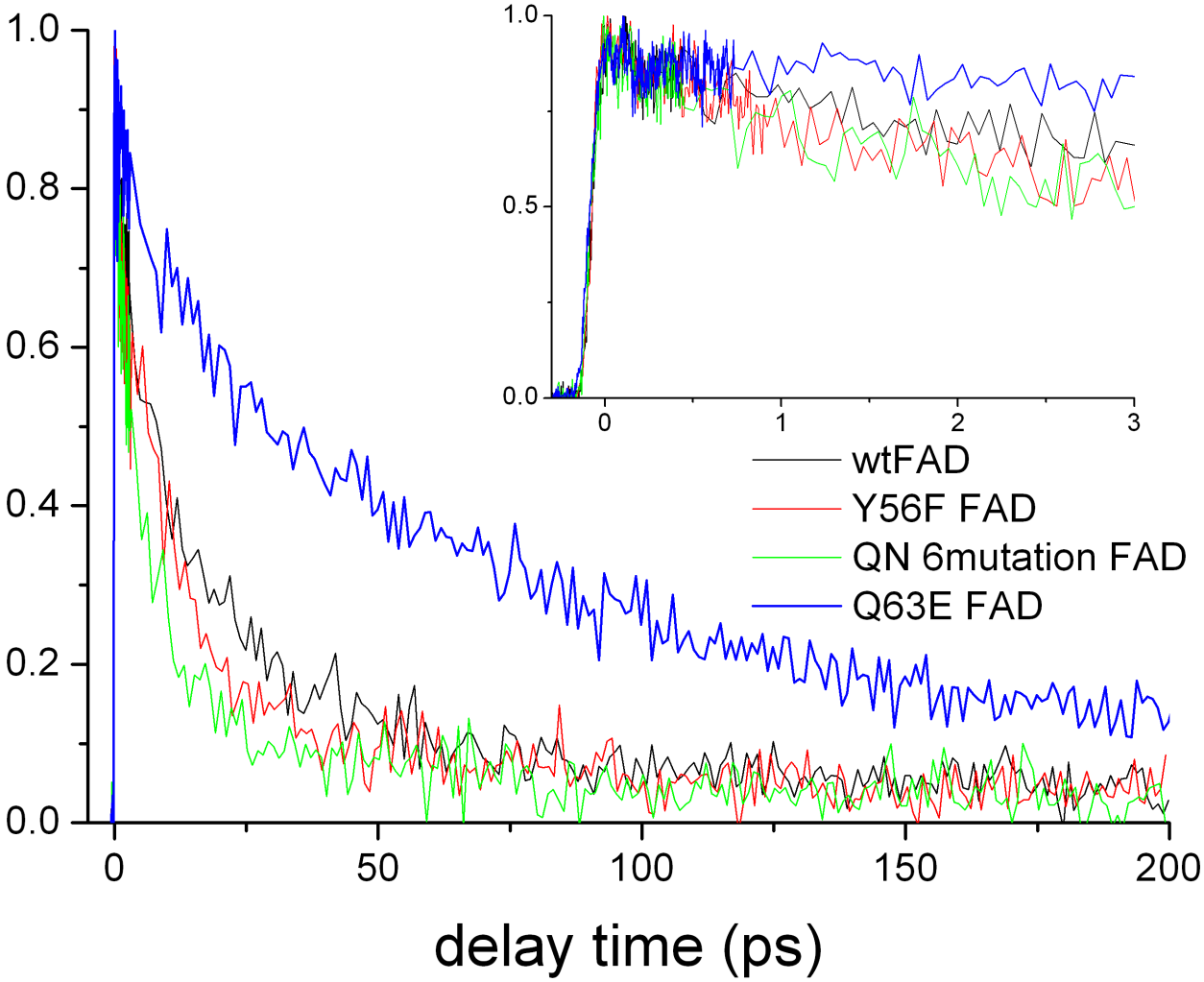


Fluorescence upconversion



Upconversion fluorescence setup, UEA, 2012

Fluorescence lifetime of WT AppA and Y56F, Q63E mutants



Fluorescence lifetime of WT AppA and Y56F, Q63E mutants

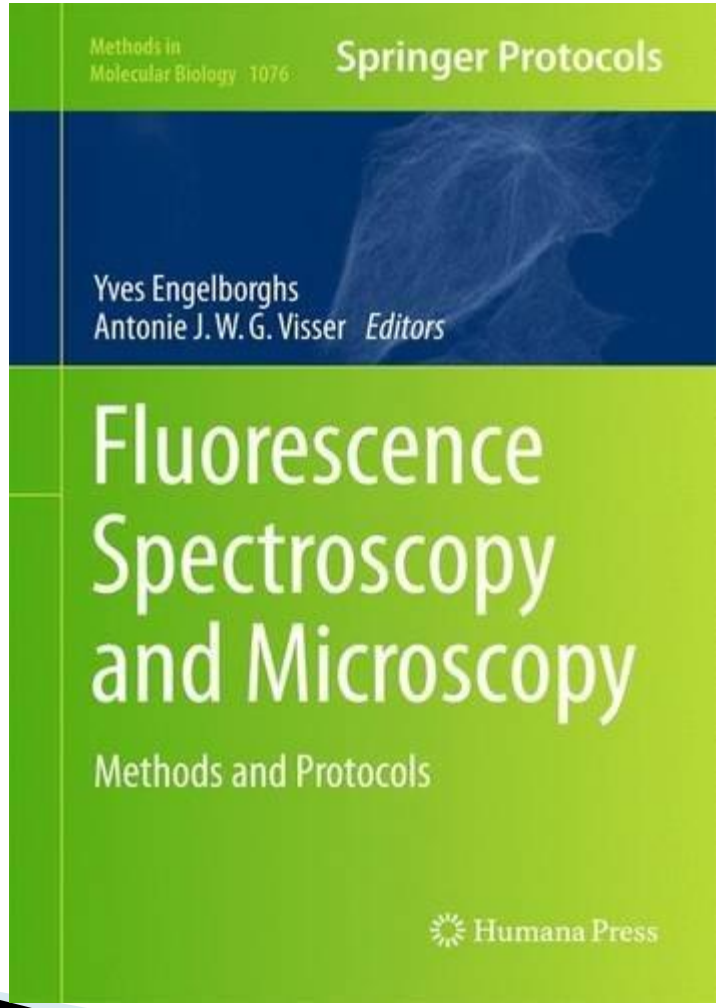
	wtFAD	Y56F FAD	QN 6mutation FAD	Q63E FAD
t_1	1.44	0.39	1.91	5.53
a_1	0.22	0.27	0.29	0.13
t_2	15.17	11.26	8.64	35.52
a_2	0.57	0.65	0.52	0.32
t_3	148.96	156.56	147.66	159.38
a_3	0.15	0.12	0.10	0.44
A_1	0.23	0.26	0.32	0.15
A_2	0.61	0.63	0.57	0.36
A_3	0.16	0.11	0.11	0.49
$\langle\tau\rangle$ /ps	32.95	24.73	21.82	92.12

	Q63E Rf	wtRf
t_1	1.44	0.70
a_1	0.11	0.32
t_2	26.62	15.71
a_2	0.39	0.54
t_3	153.16	133.02
a_3	0.40	0.07
A_1	0.13	0.34
A_2	0.43	0.58
A_3	0.45	0.08
$\langle\tau\rangle$ /ps	80.05	19.44

Ultrafast laser spectroscopy

II. Kerr-gate fluorescence spectroscopy

Kerr-gate fluorescence spectroscopy

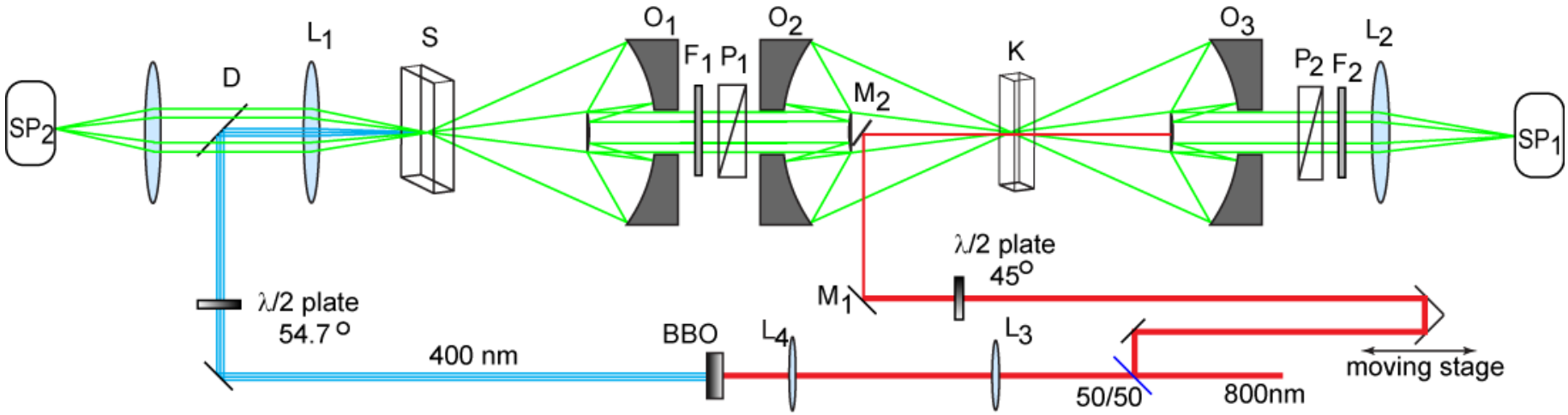


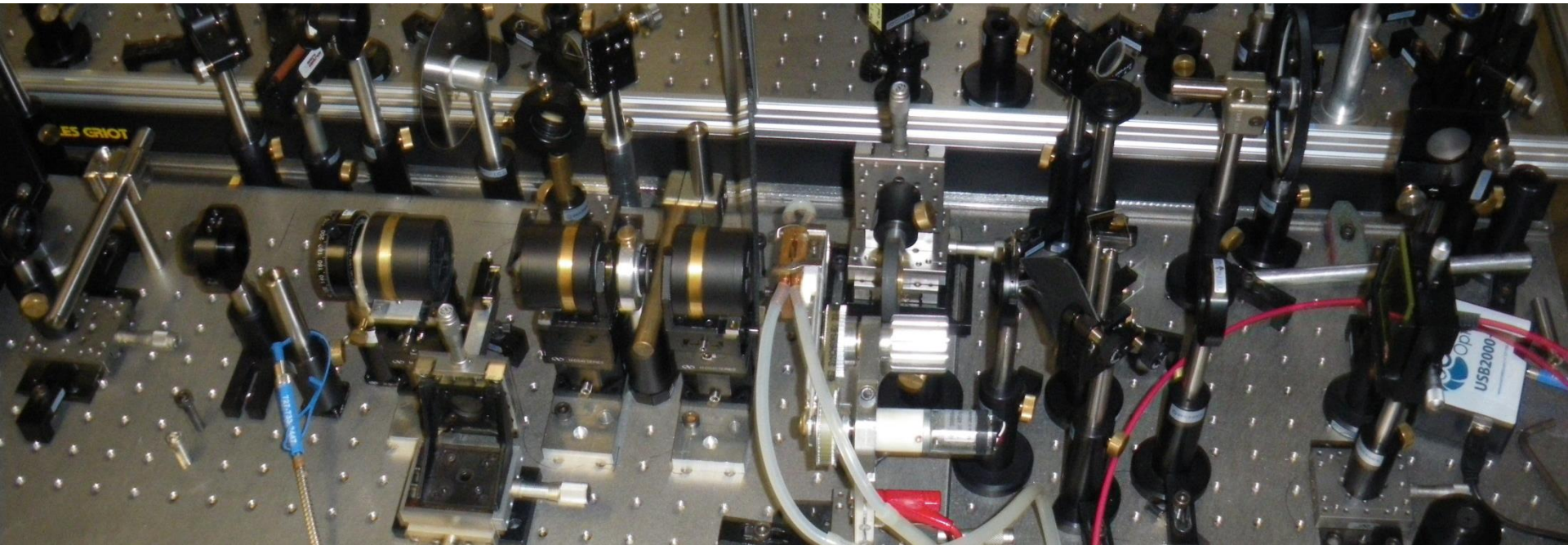
Laptenok SP, Nuernberger P, Lukacs A, Vos MH:
Subpicosecond Kerr-Gate Spectrofluorometry.

in Fluorescence Spectroscopy and Microscopy Methods in Molecular Biology Volume 1076, 2014, pp 321-336 (Springer Protocols)

- Optical Kerr-effect: high laser intensity will change the refractive index of the optical material
- $n=n_0+n_2I$ if the intensity (I) of the laser pulse is high
- The incident laser pulse will induce a change in polarisation

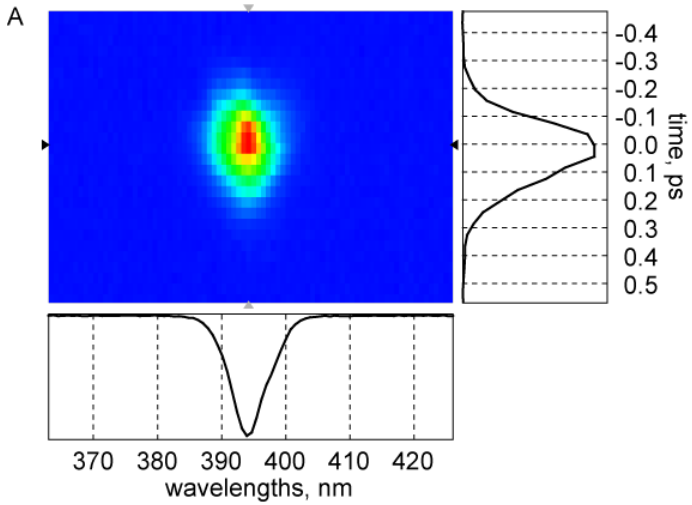
Kerr-gate setup

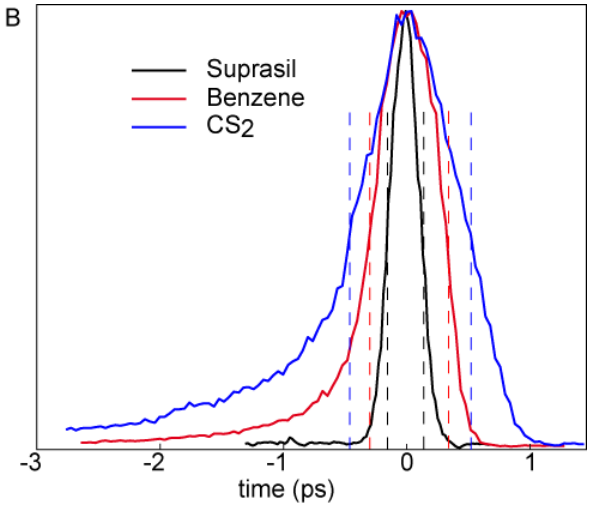




Kerr-gate setup

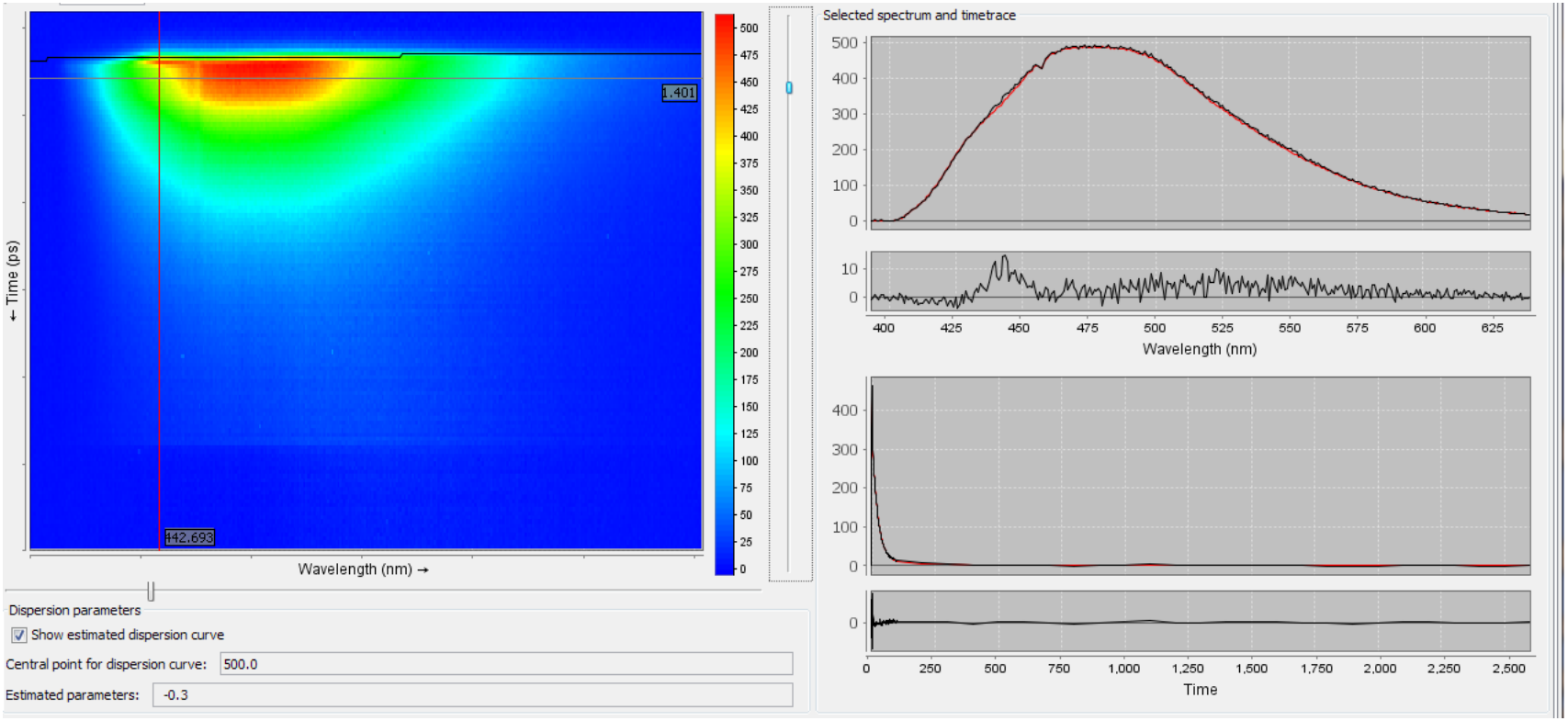
Pick-up mirror on the Cassegrain objective



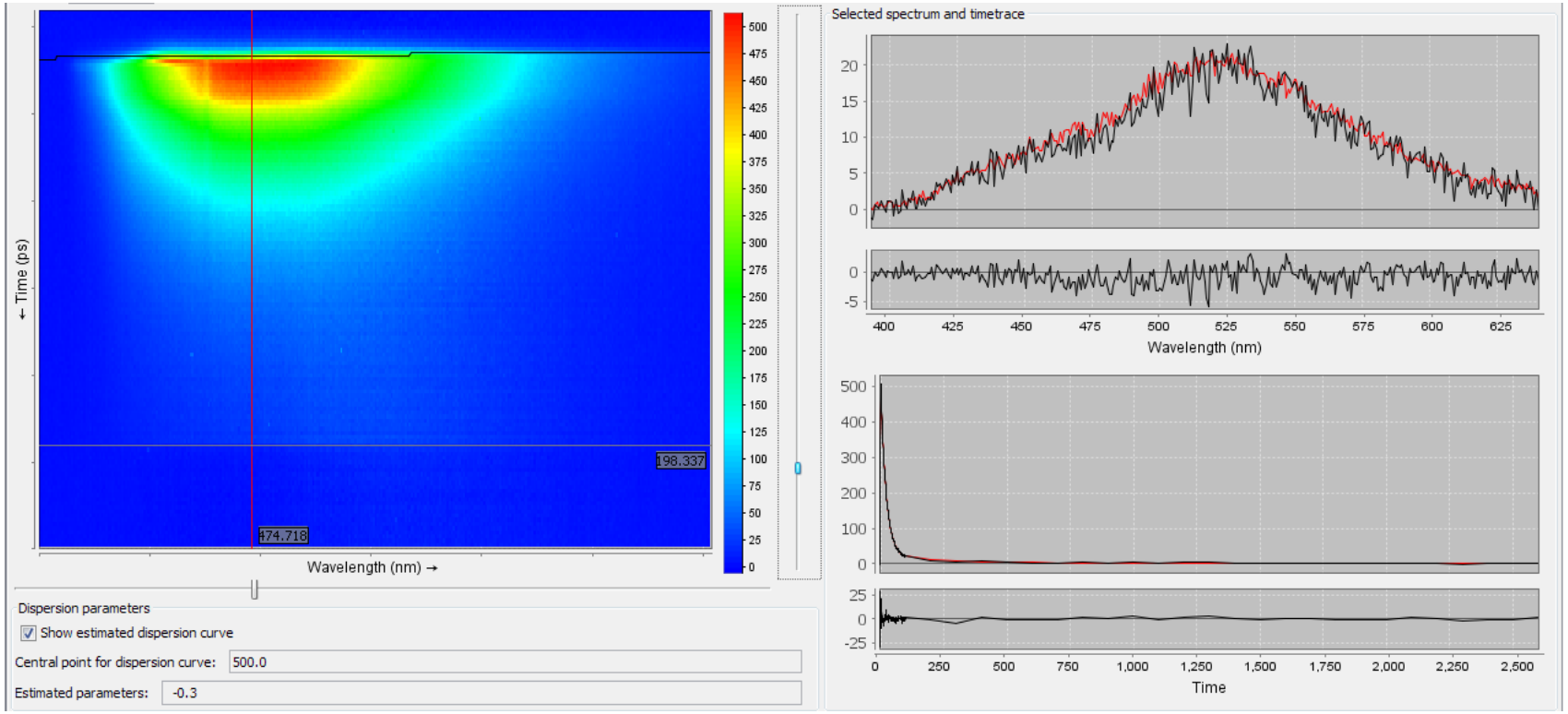


Comparison of Kerr-materials

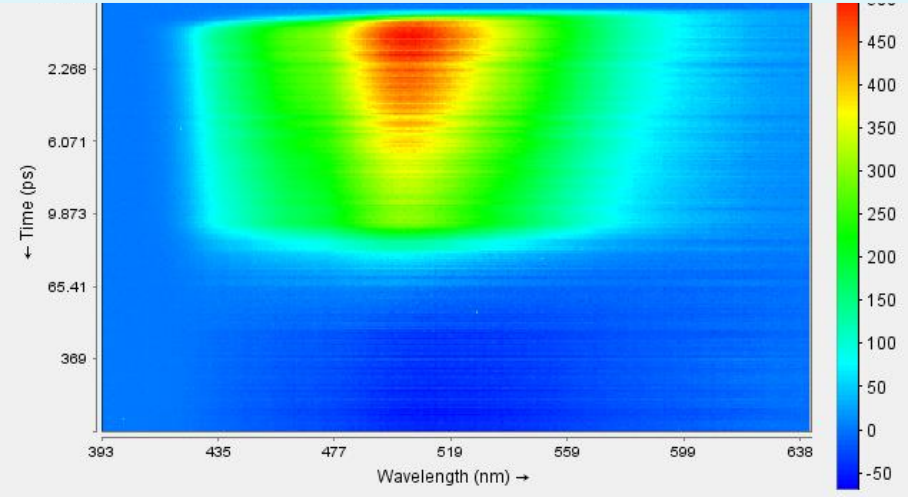
Fluorescence lifetime of MTHF in N378D photolyase

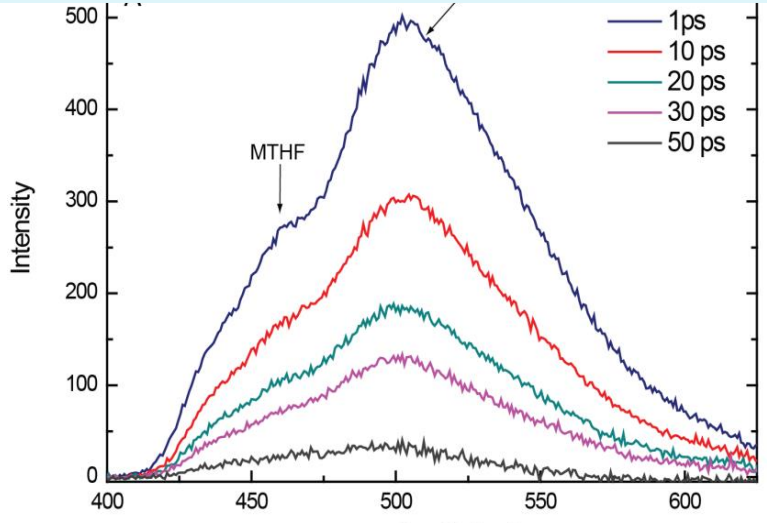


Fluorescence lifetime of MTHF in N378D photolyase



Fluorescence lifetime of MTHF in N378D photolyase





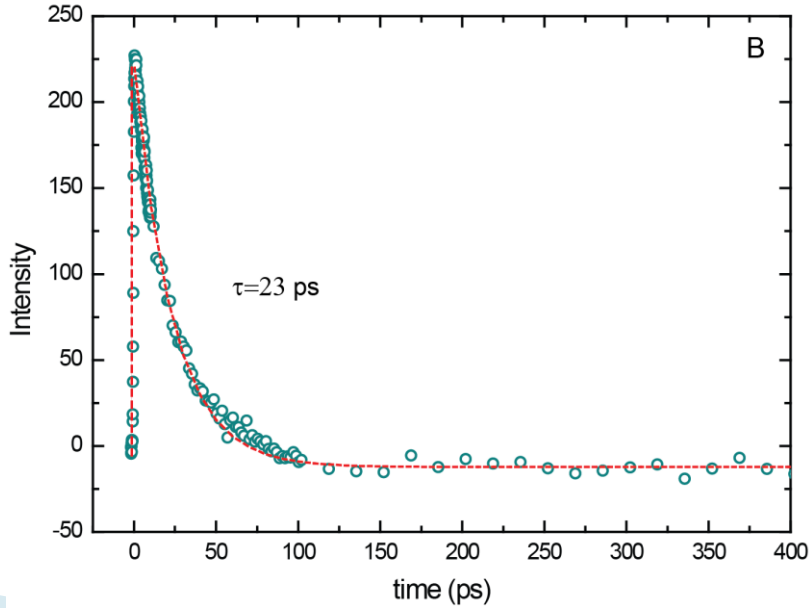
τ_{MTHF} = 354 ps

$\tau_{MTHF\text{-}FAD}$ = 23 ps

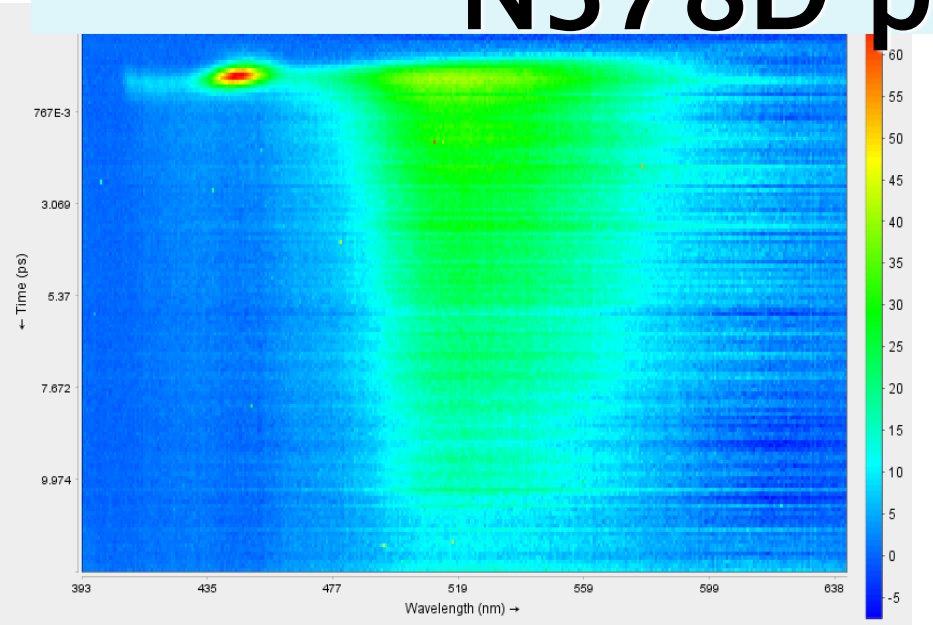
$E = 1 - \frac{\tau_{DA}}{\tau_D} = 0.935$

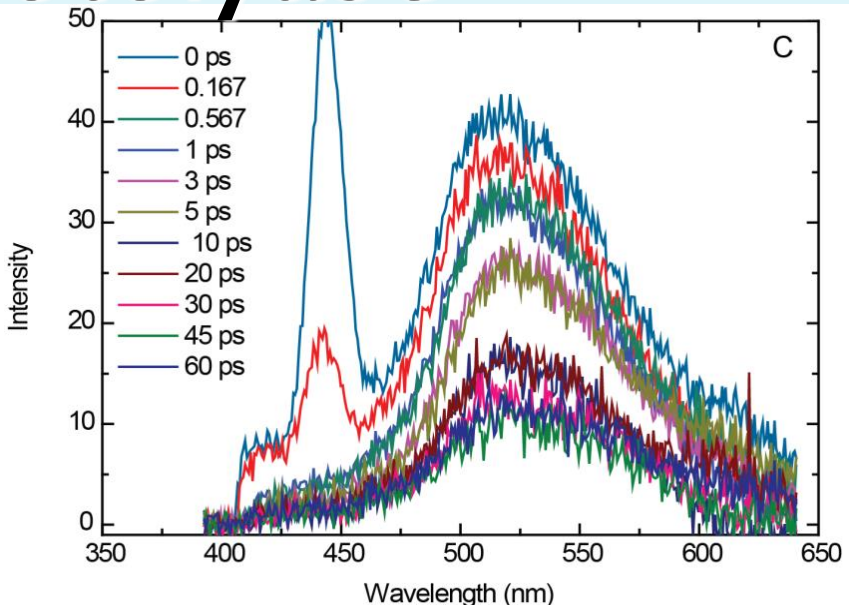
$E = \frac{R_0^6}{R_0^6 + R^6}$

R = 17.53Å



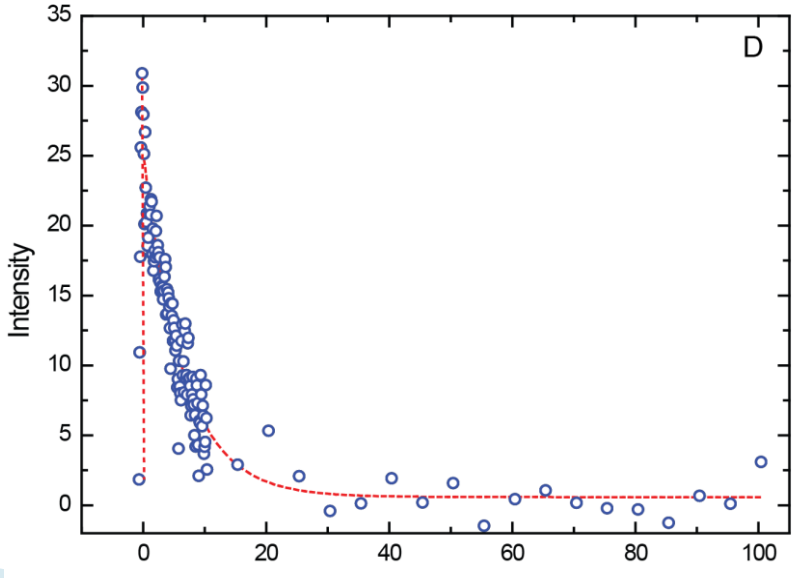
Fluorescence lifetime of FAD in N378D photolyase





$\tau_{N378D\text{-}FAD} \sim 6$ ps

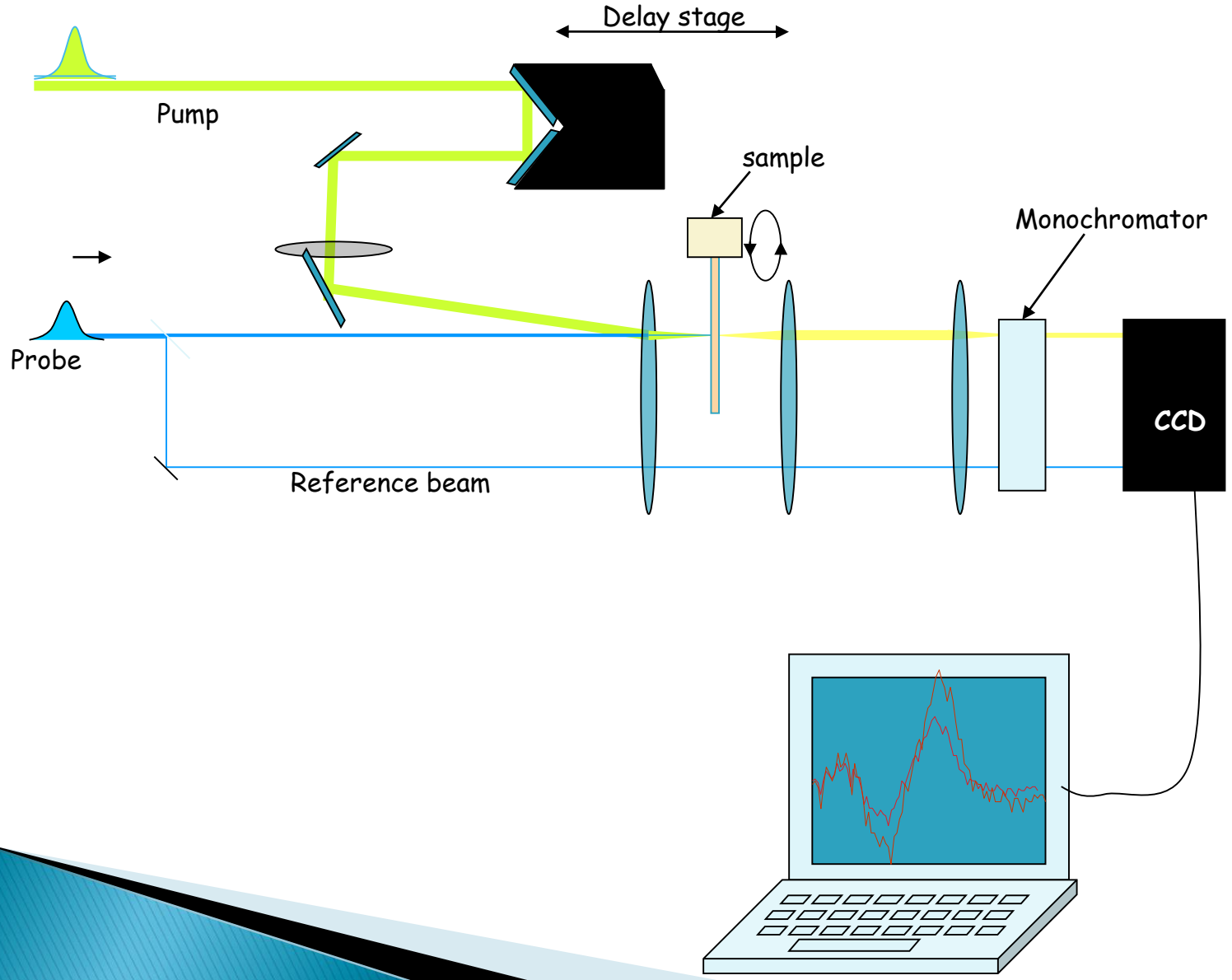
$\tau_{FAD} \sim 9$ ps



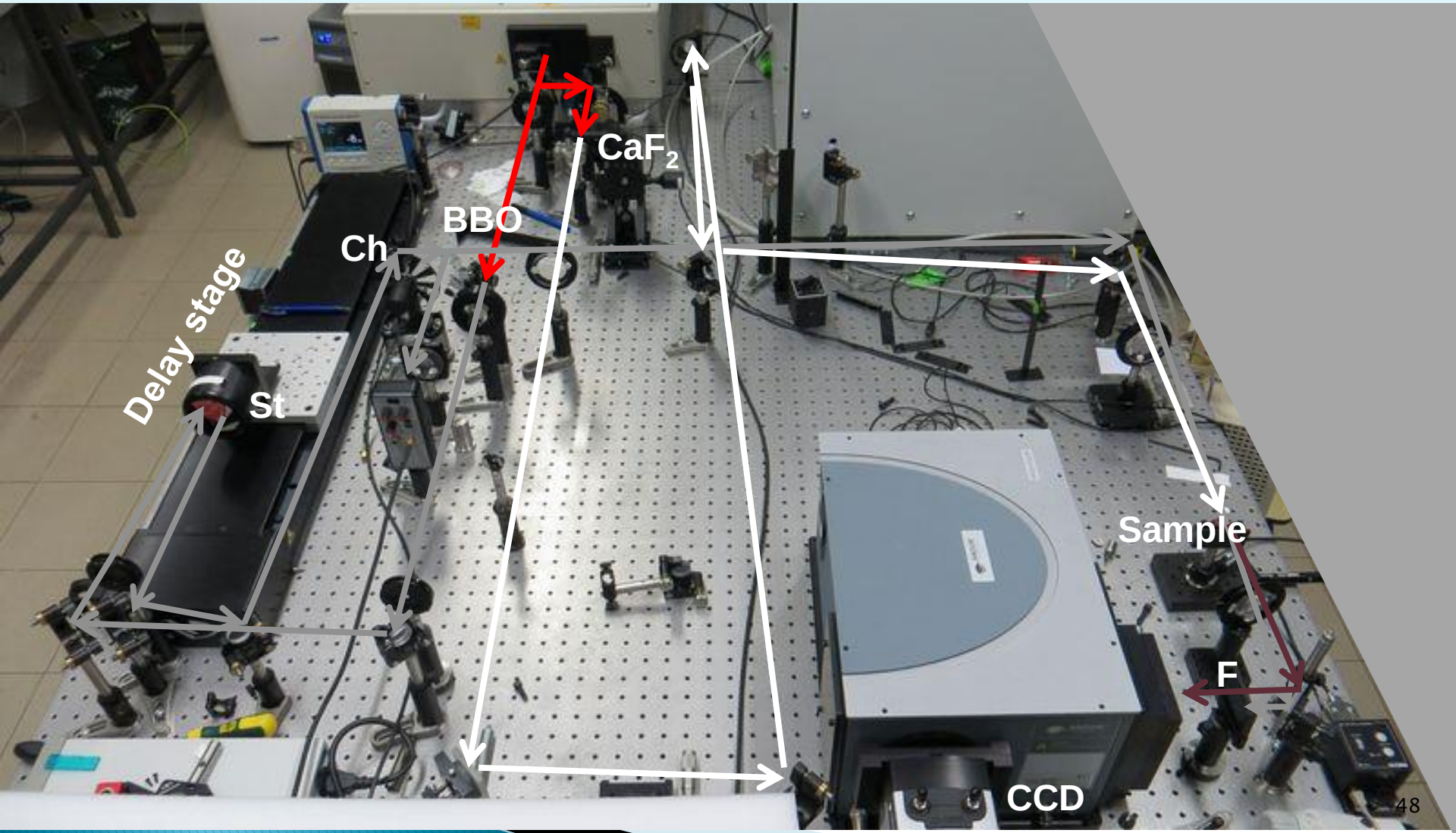
Ultrafast laser spectroscopy

III. Transient absorption spectroscopy

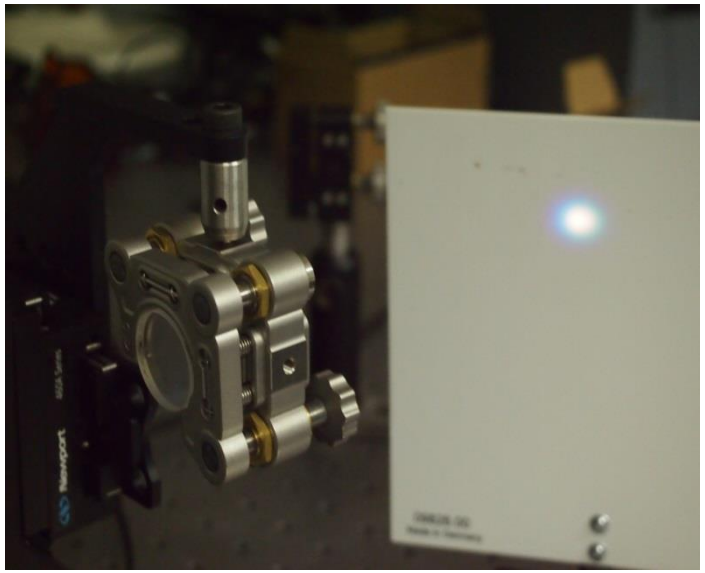
Transient absorption spectroscopy

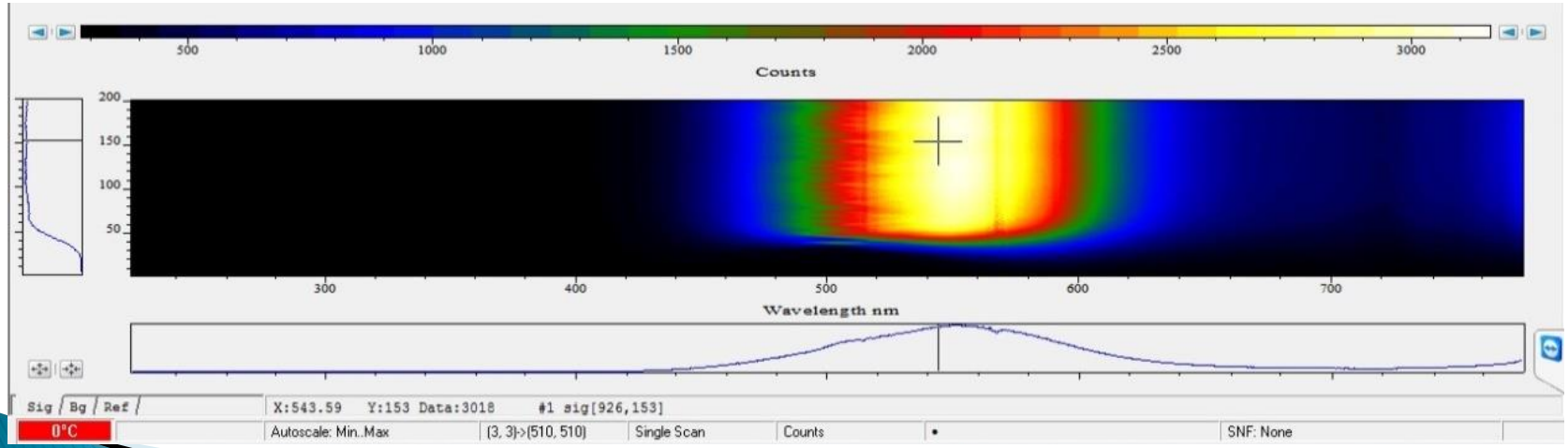


Transient absorption spectroscopy

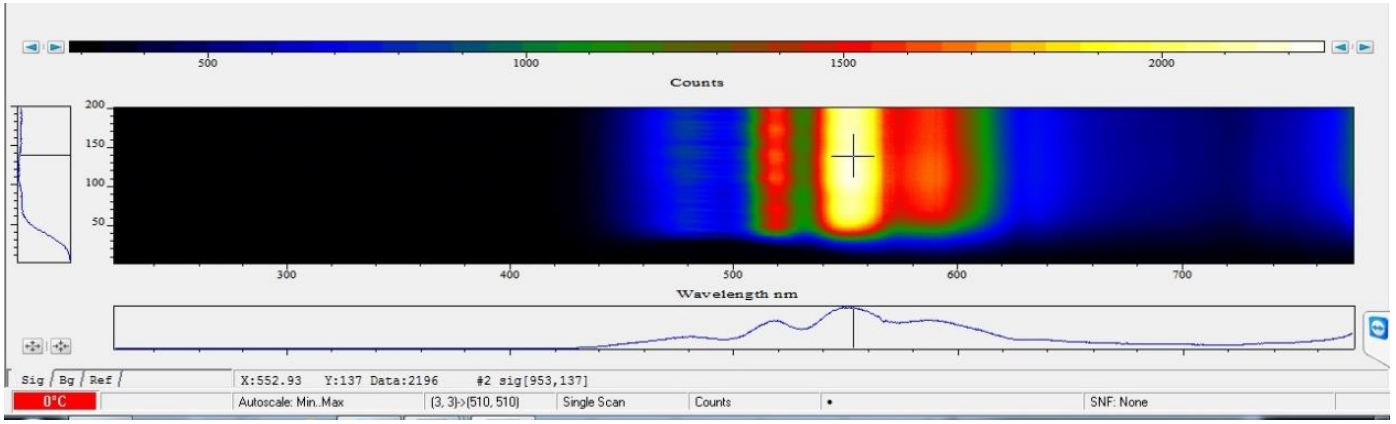


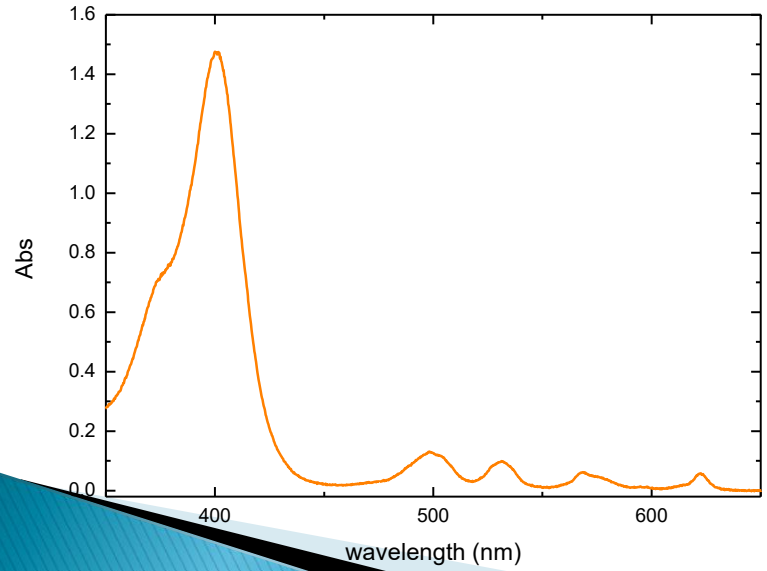
Transient absorption spectroscopy White light continuum

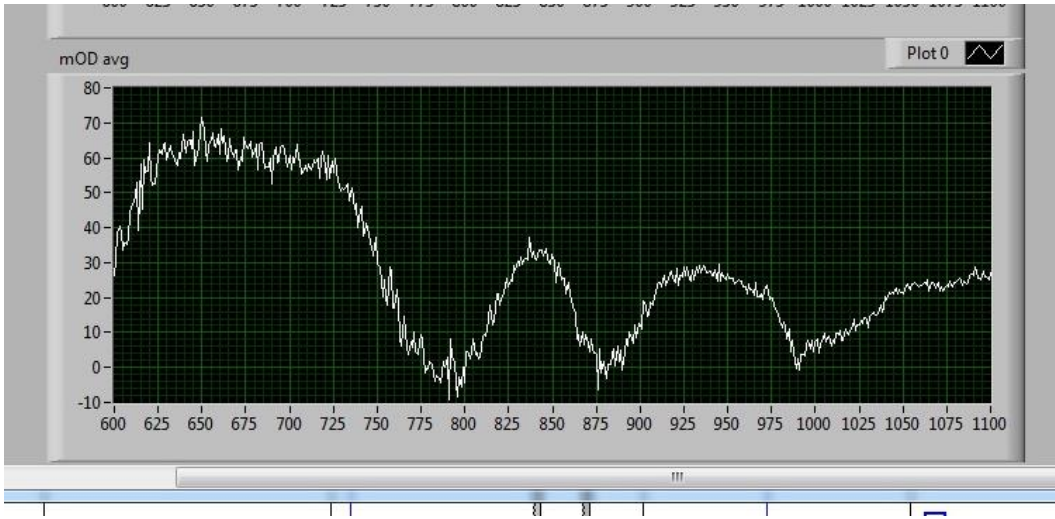




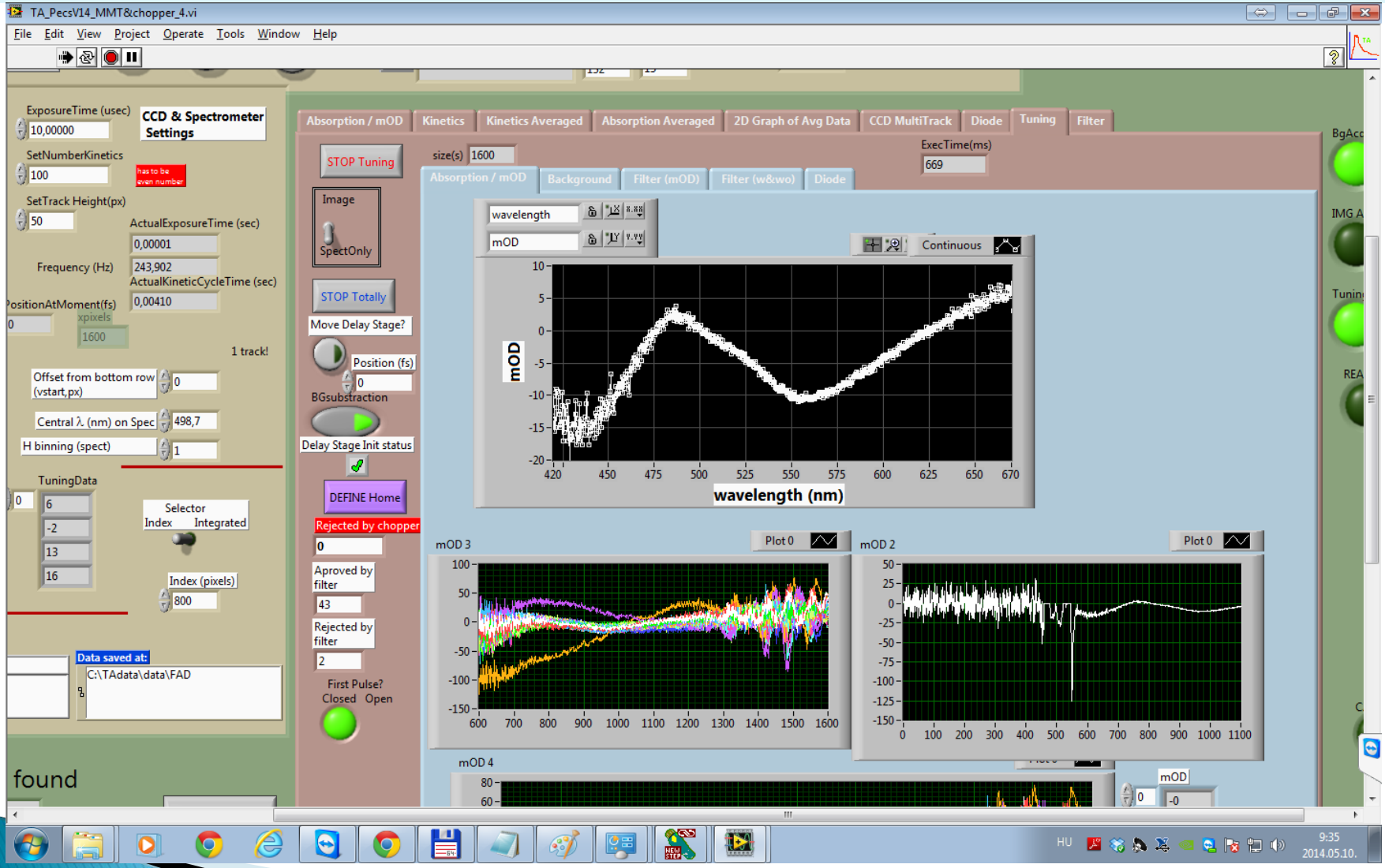
Transient absorption spectroscopy



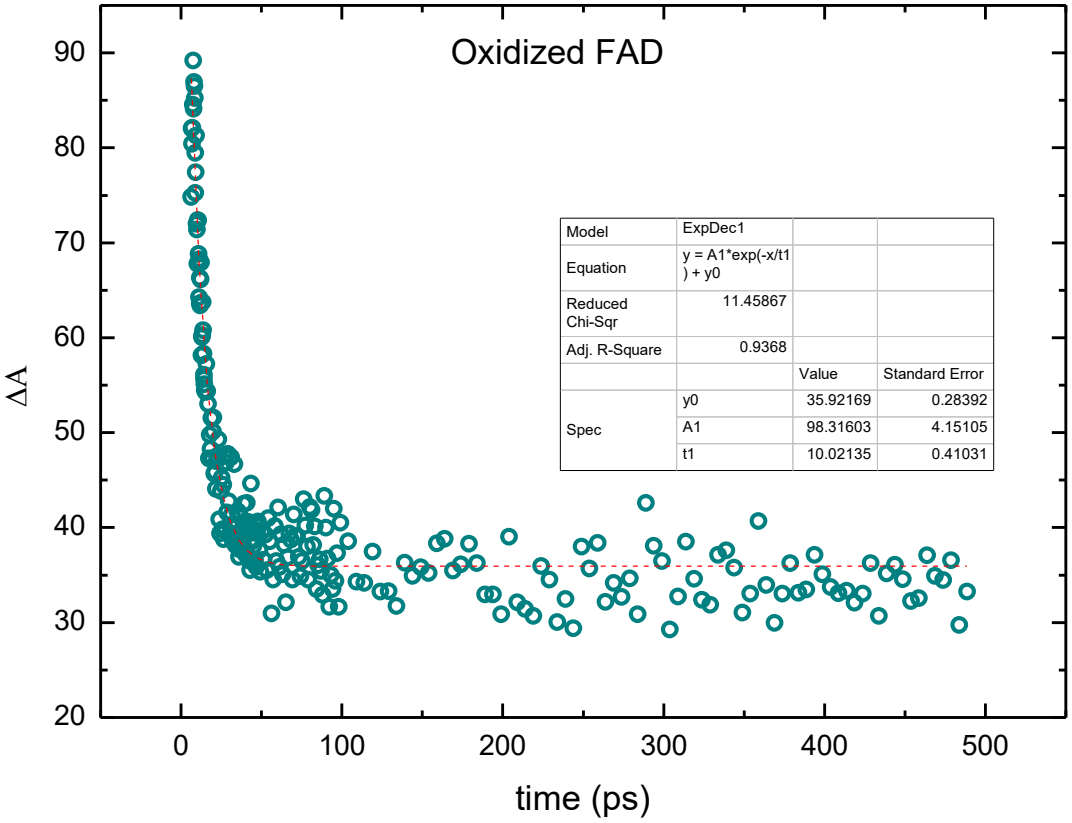




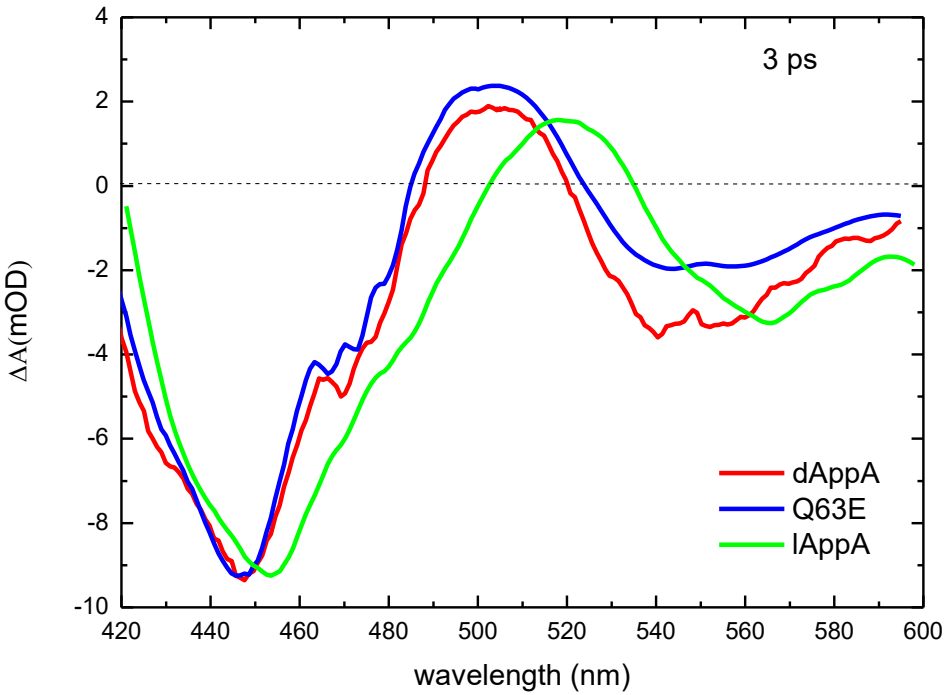
Transient absorption spectroscopy

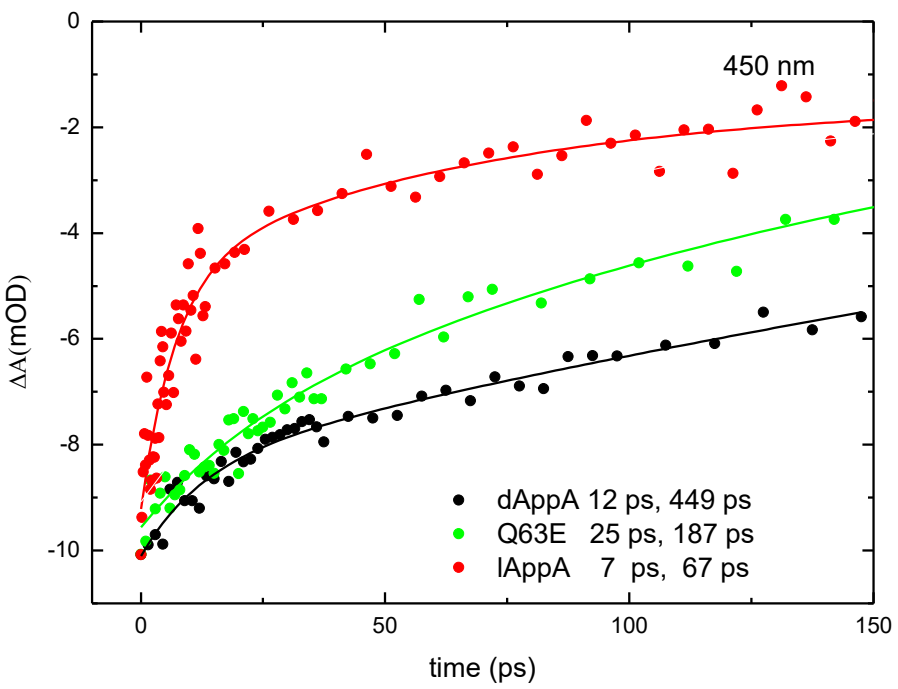


Transient absorption spectroscopy



Transient absorption spectroscopy





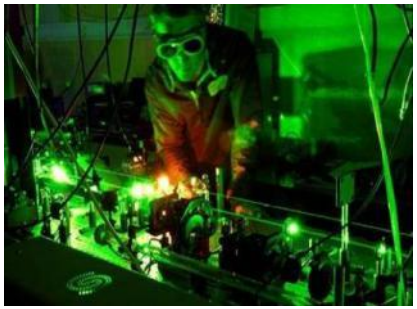
Ultrafast laser spectroscopy

IV. Transient infrared absorption spectroscopy

Rutherford Appleton Laboratory

Central Laser Facility

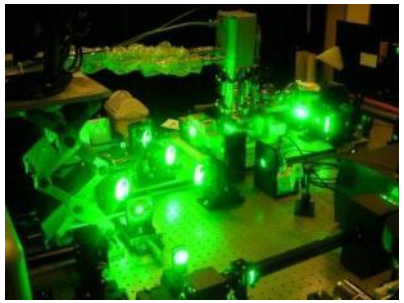
ASTRA



- Ultra-high intensity 2.6 kJ in sub-picosecond pulses, 100 TW → PW
- Fusion and Plasma Research
- Extreme UV generation
- Attosecond pulse generation research

VULCAN

LSF



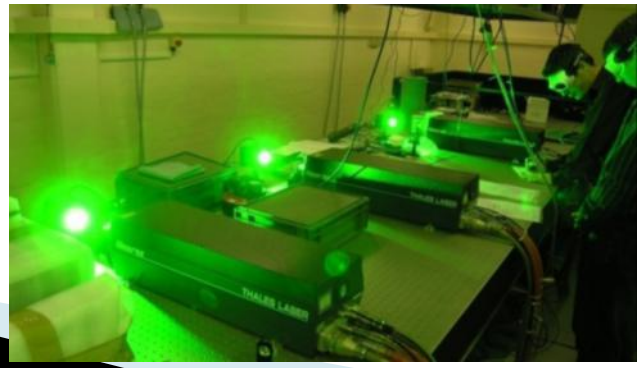
- Smaller scale lasers
- Vibrational spectroscopy
- Imaging; laser tweezers and microscopy

LSF User community

Research Complex

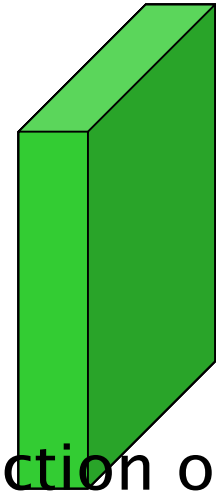
- RCUK Building
- Activity Across “Life and Physical Sciences Interface”
- Short and Long term Research Visitors using facilities across RAL site (Diamond, ISIS CLF)

Research Complex at Harwell



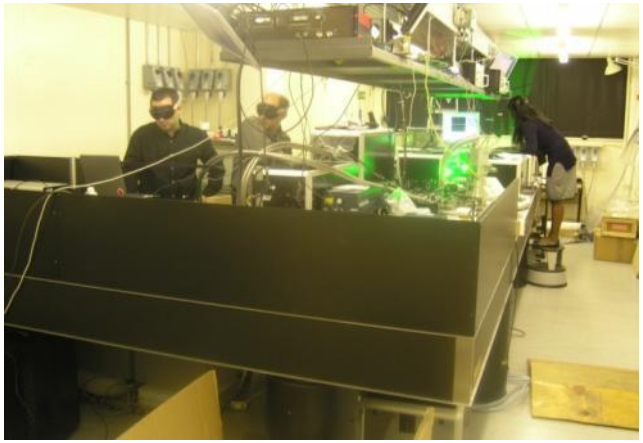
Time-Resolved Spectroscopy

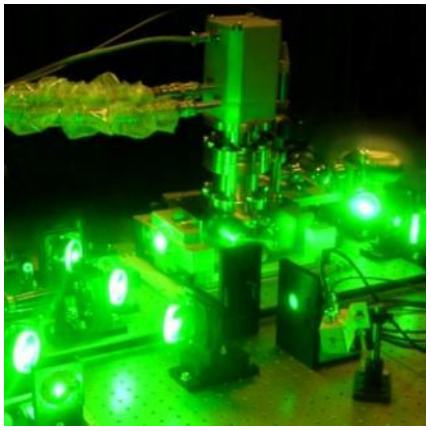
- **Pump – probe** scheme with variable time delays

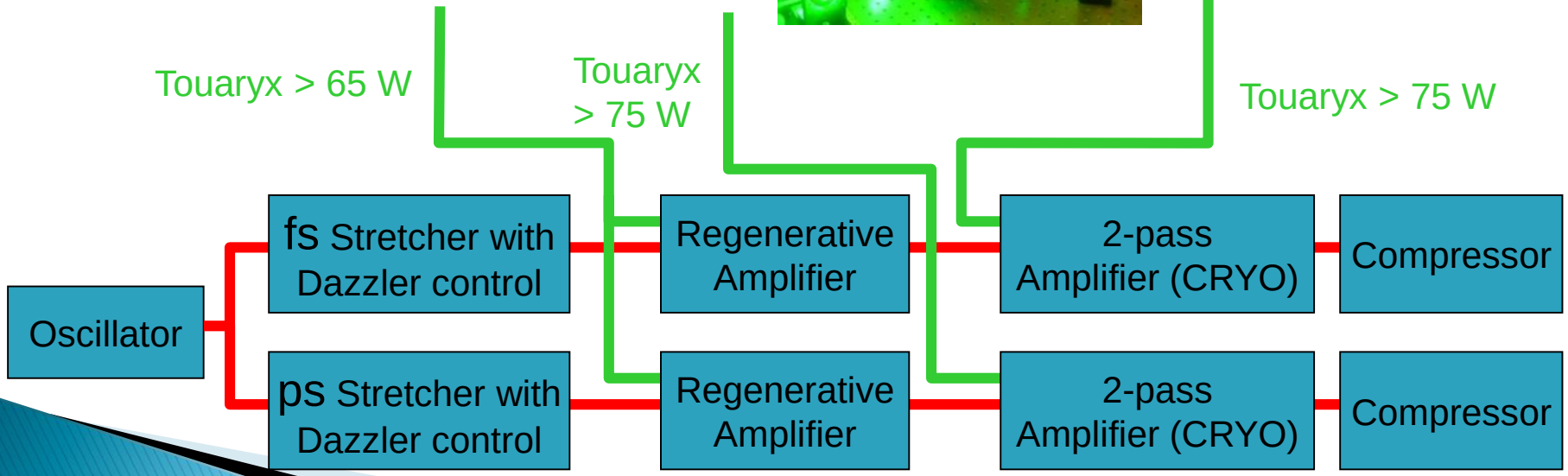


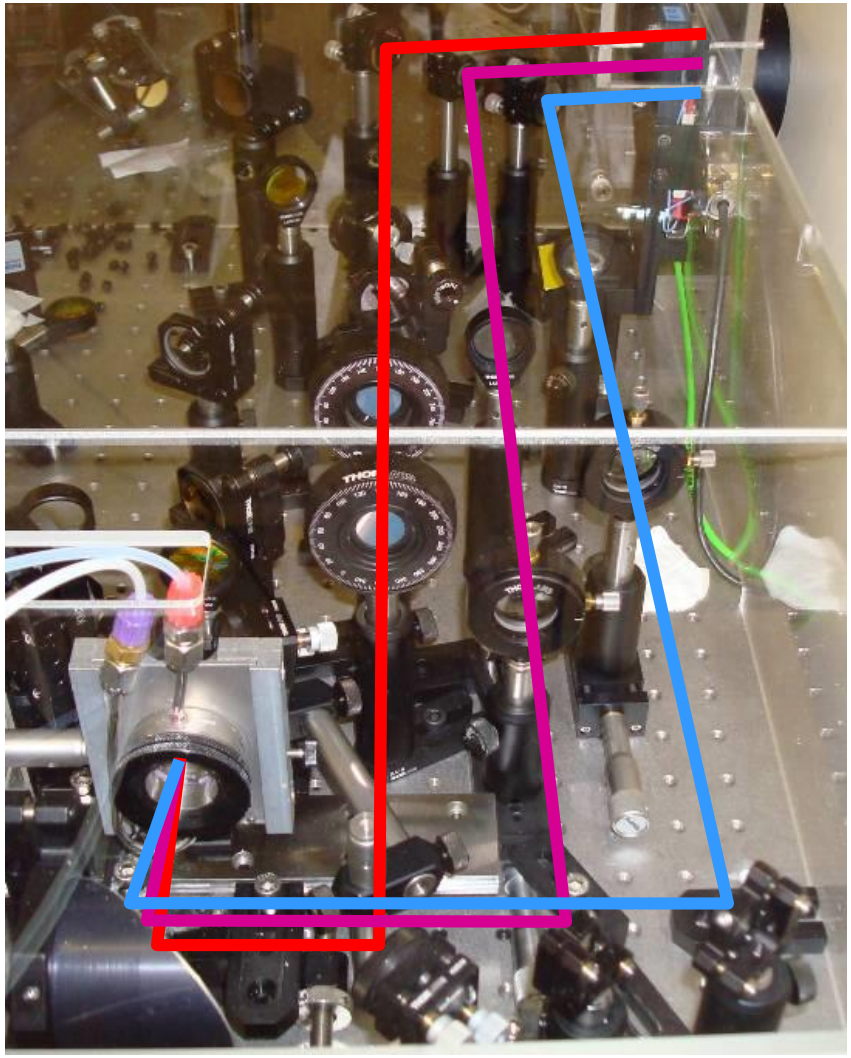
- Pump pulse drives a chemical reaction or energy transfer
- Probe pulse may observe UV – IR absorption spectrum or Raman spectrum

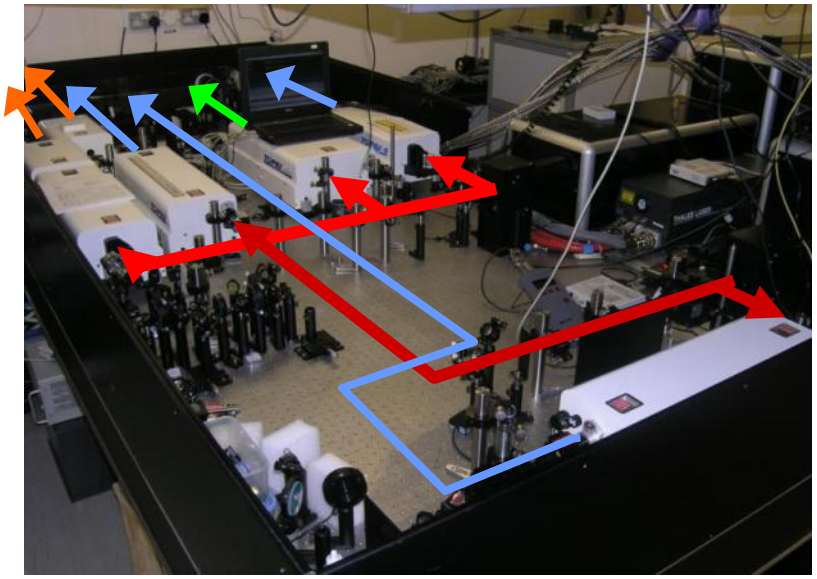
Dual 10 kHz Ti:S Amp



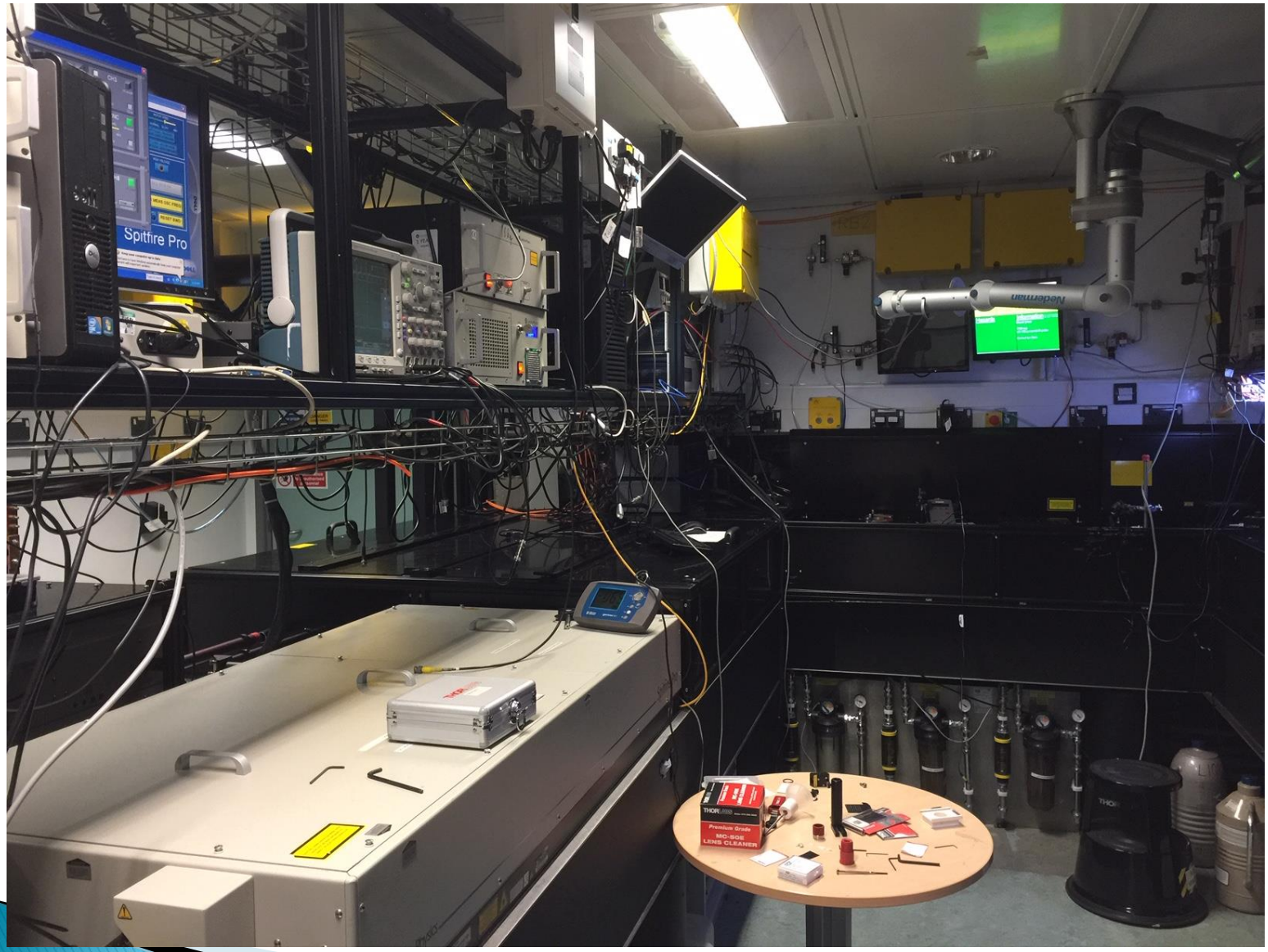






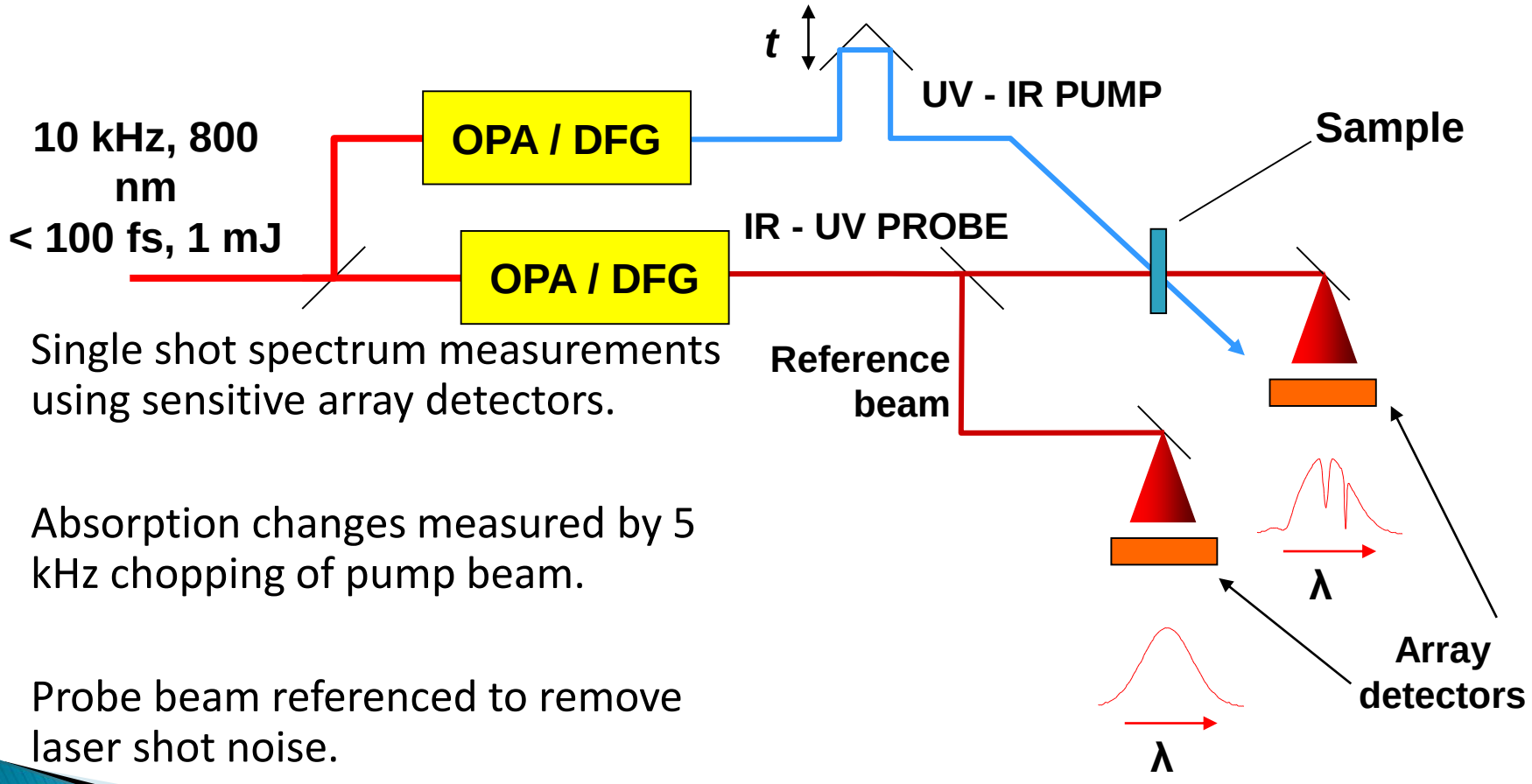


Tunability: 200 – 20000 nm
Pulse durations: 40 fs – 1 nm
Bandwidth: 5 – 600 cm^{-1}


Spitfire Pro
Premium Grade
MC-80E
LENS CLEANER

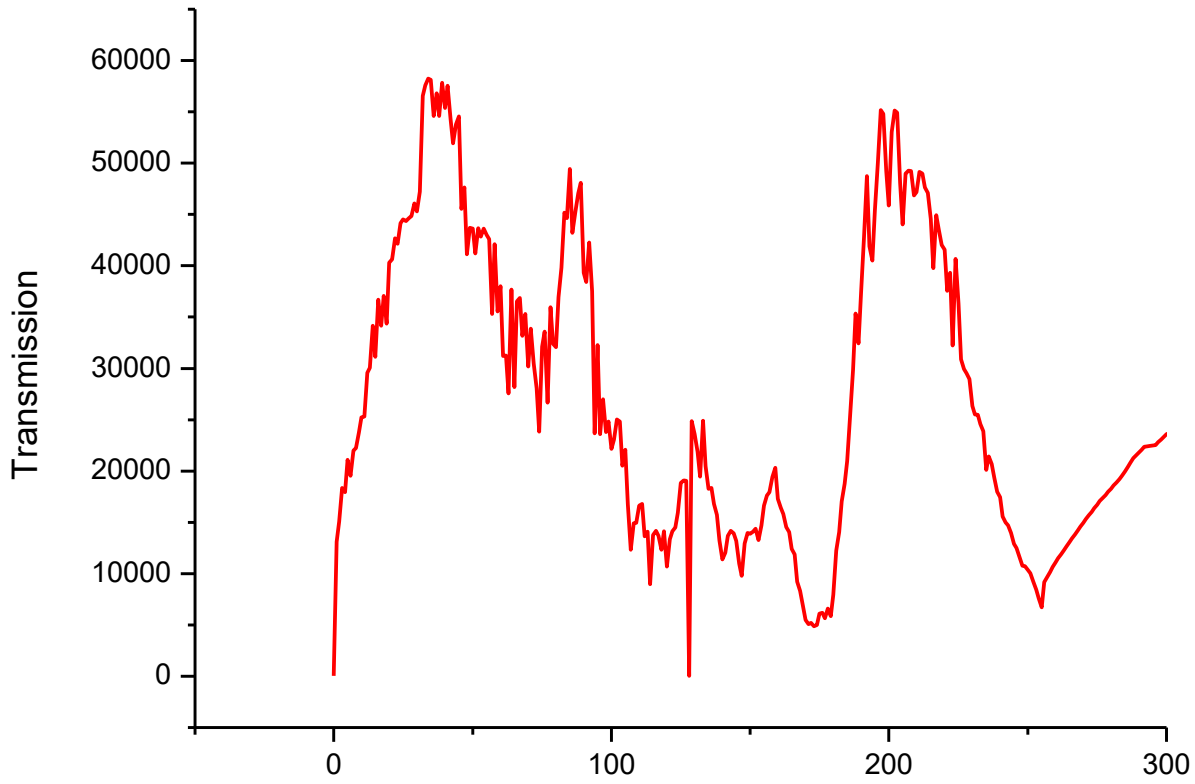

TRANSFER
ENCLOSURE
1

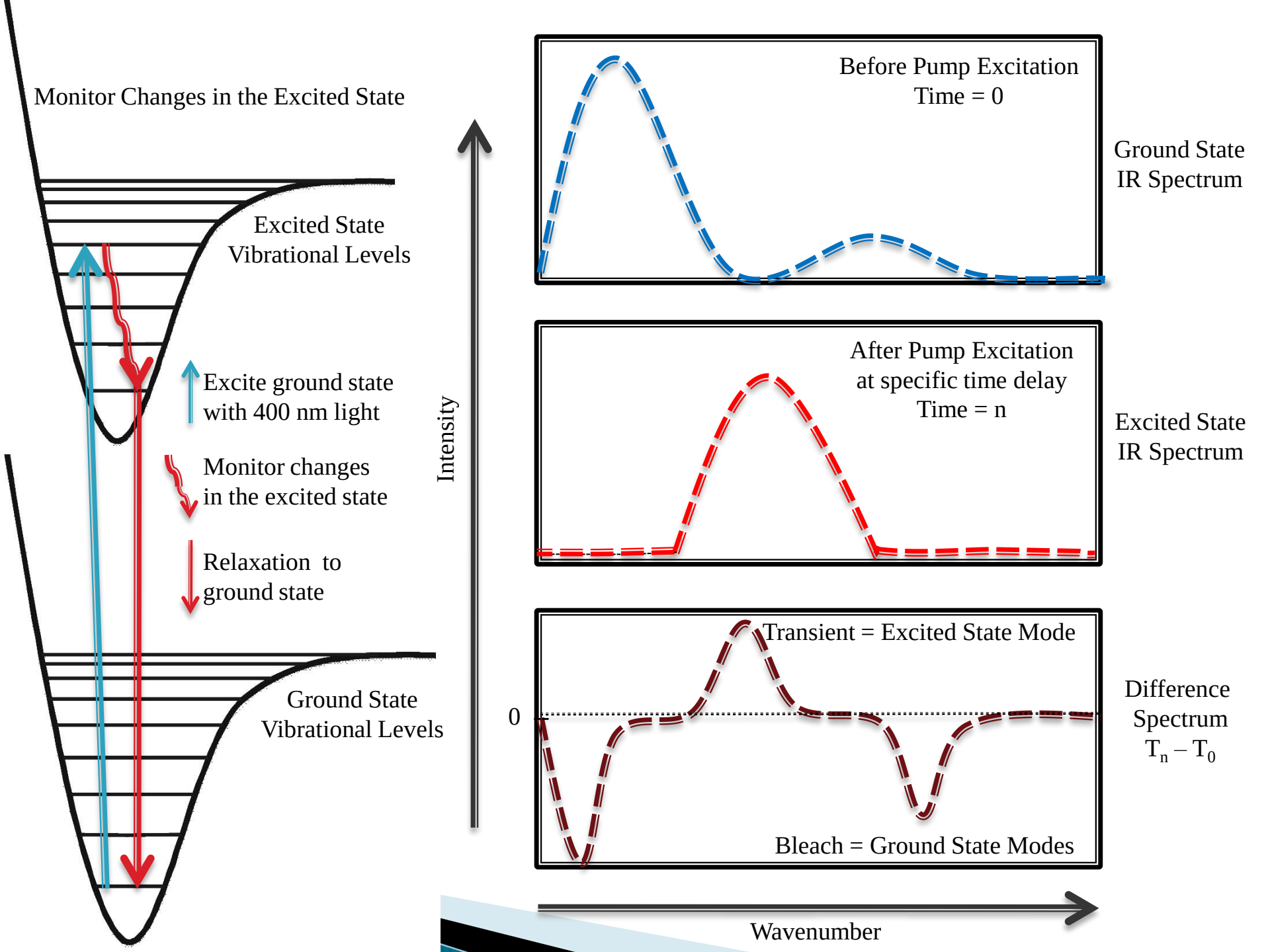
Time-Resolved Absorption Spectroscopy



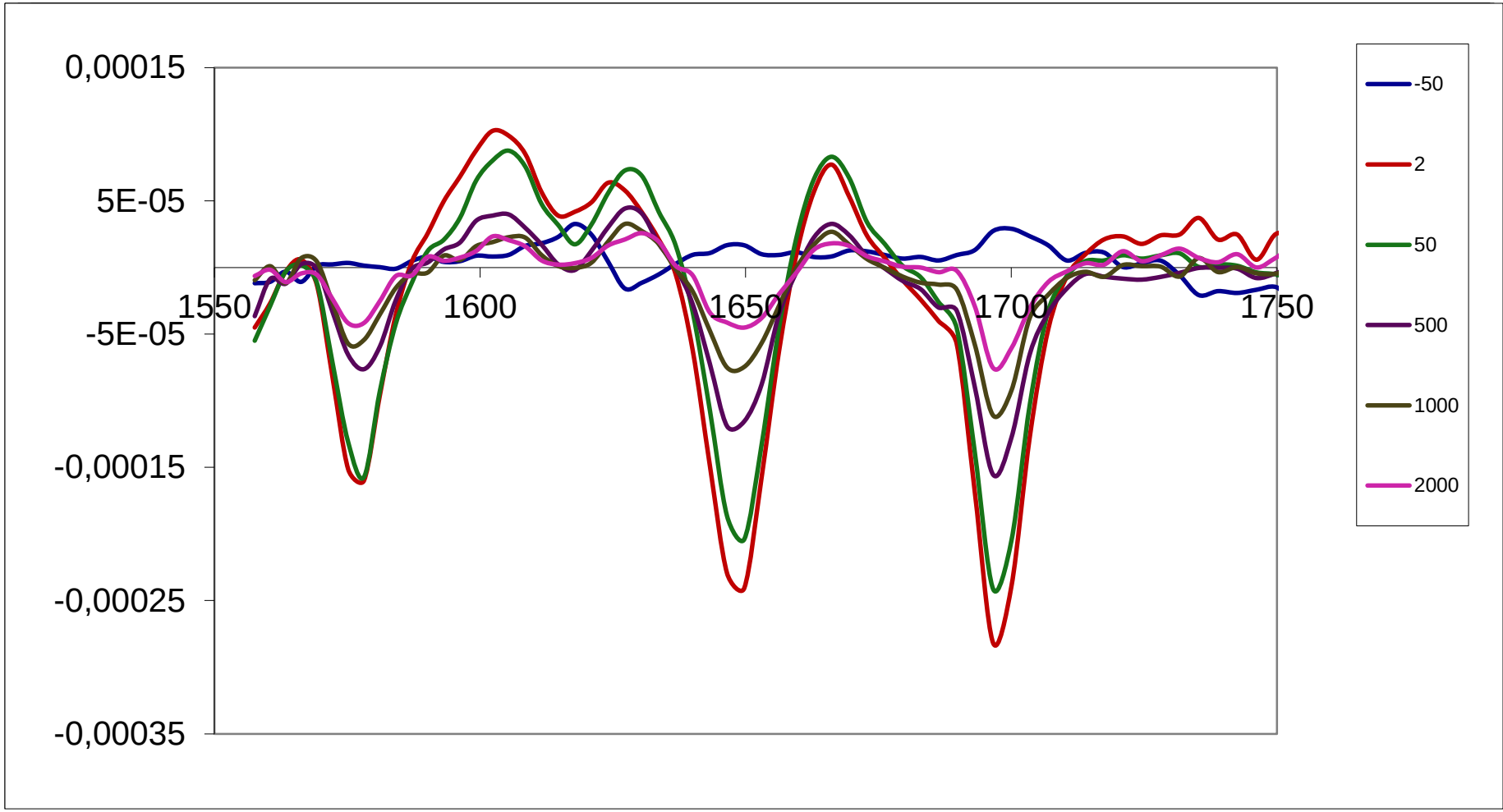
- Single shot spectrum measurements using sensitive array detectors.
- Absorption changes measured by 5 kHz chopping of pump beam.
- Probe beam referenced to remove laser shot noise.

Primary data

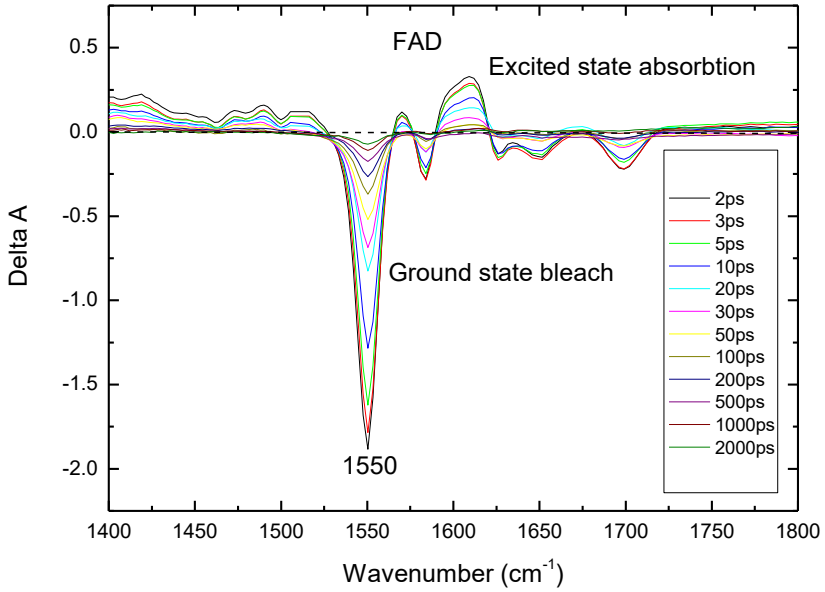


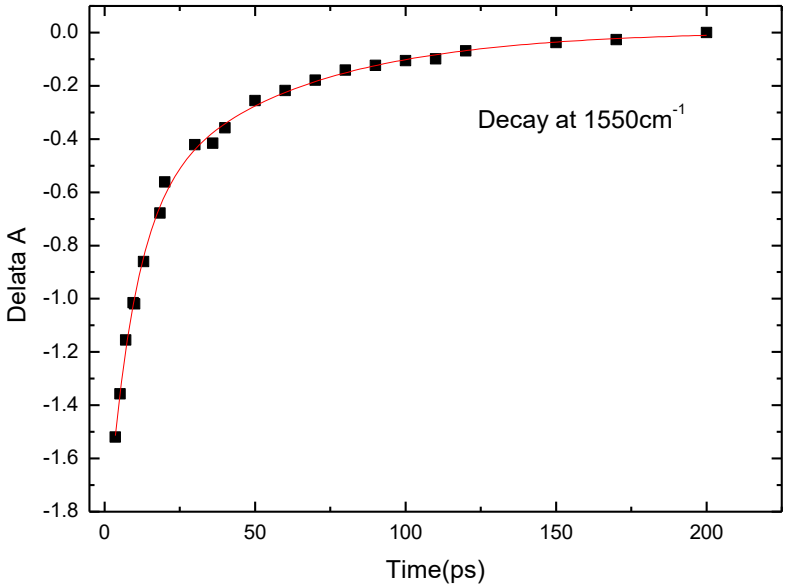

Monitor Changes in the Excited State
Excited State
Vibrational Levels
Excite ground state
with 400 nm light
Monitor changes
in the excited state
Relaxation to
ground state
Ground State
Vibrational Levels
Intensity
Before Pump Excitation
Time = 0
Ground State
IR Spectrum
After Pump Excitation
at specific time delay
Time = n
Excited State
IR Spectrum
Transient = Excited State Mode
0
Bleach = Ground State Modes
Difference
Spectrum
$T_n - T_0$
Wavenumber

Transient infrared spectra



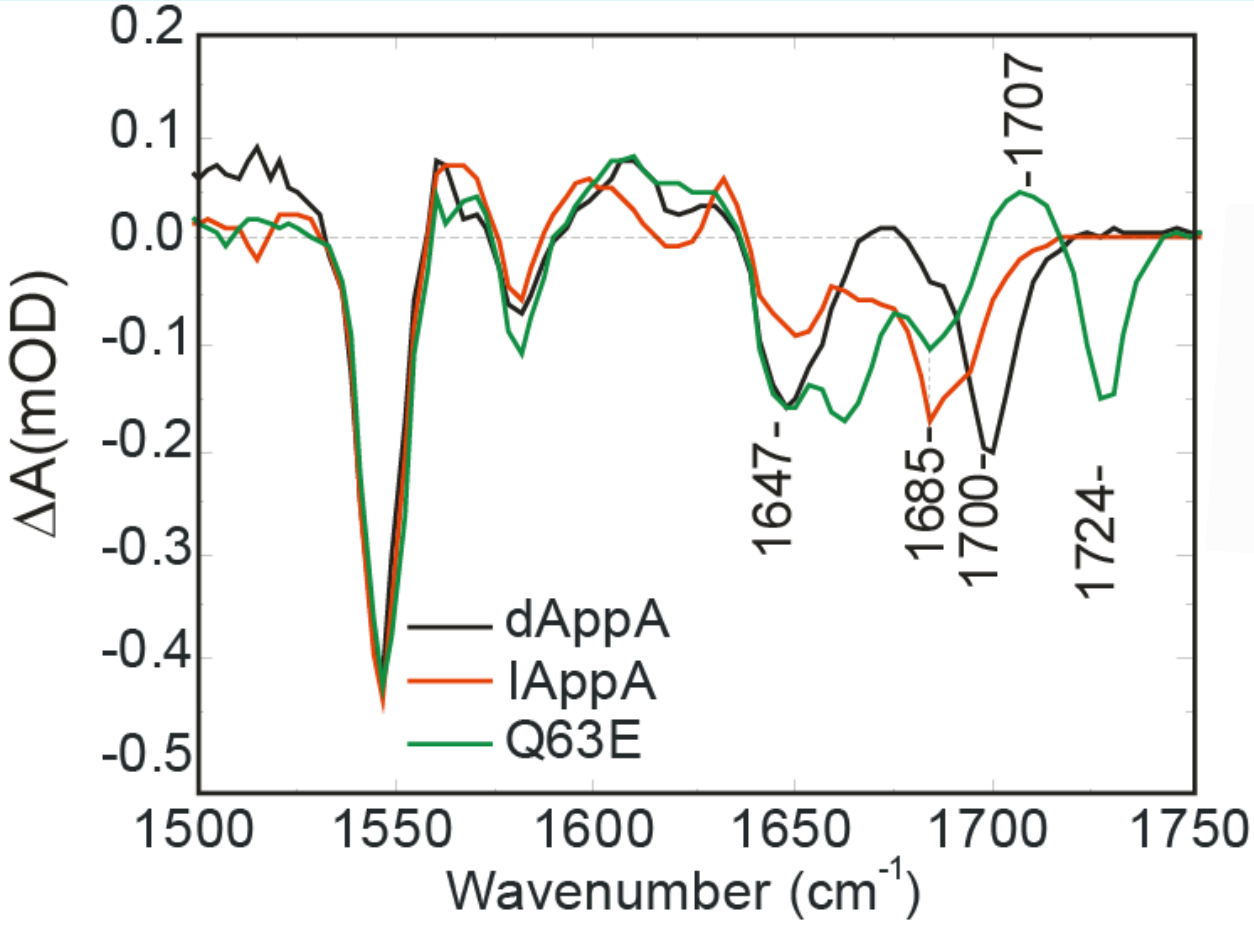
Transient infrared spectra

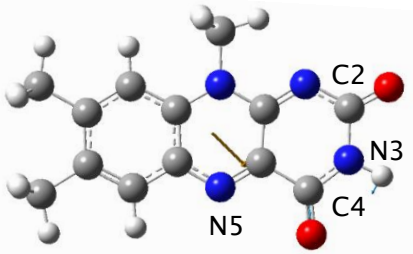




a) TRIR spectra of FAD at various time delays b) Decay curve at 1550 cm^{-1} band

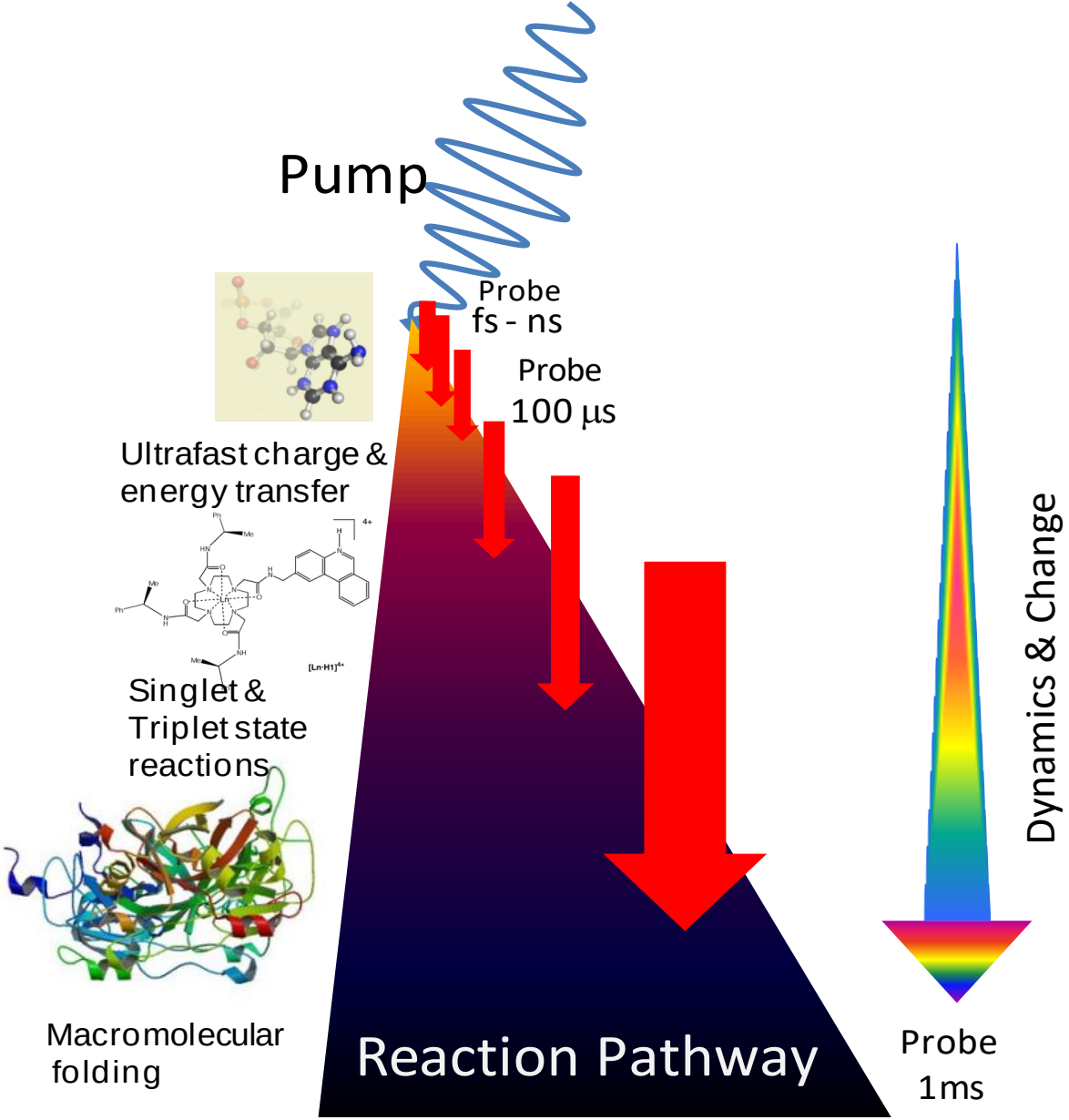
TRIR measurements on AppA



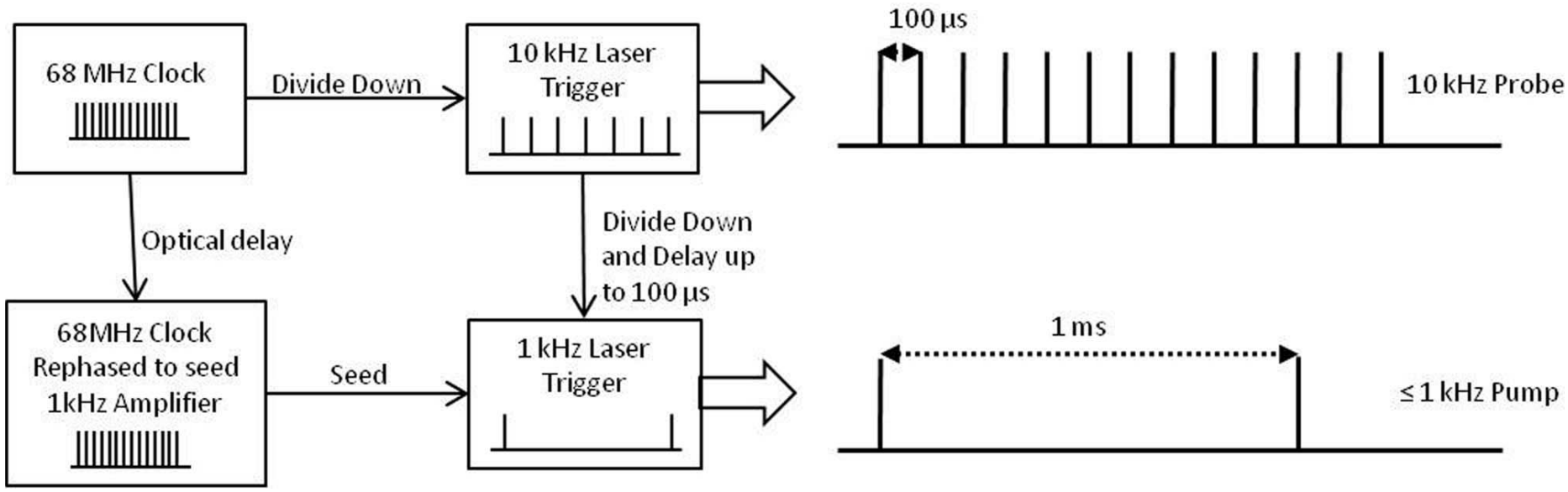


Flavin chromophore

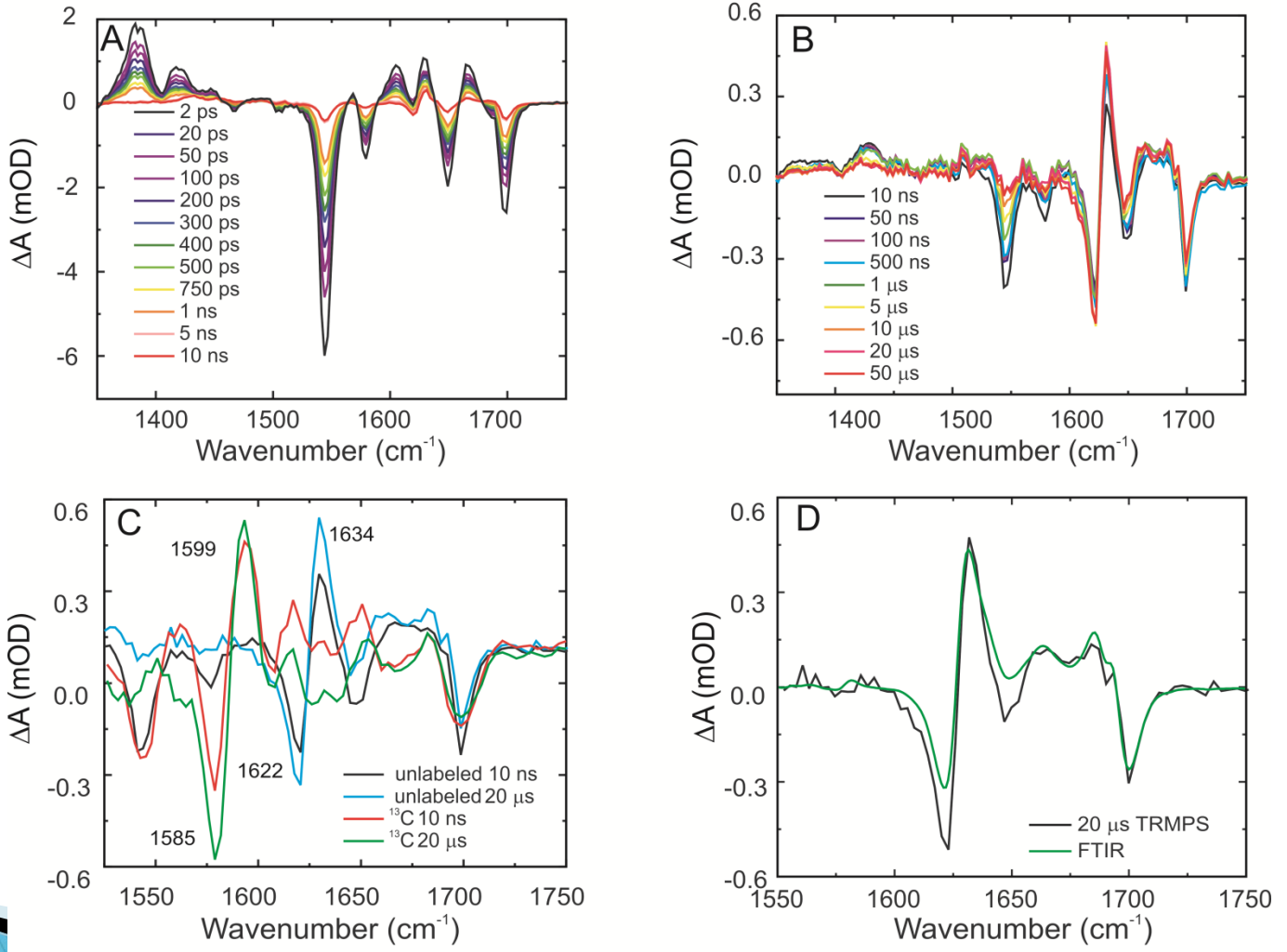
Time Resolved Multiple Probe Spectroscopy



TRMPS concept

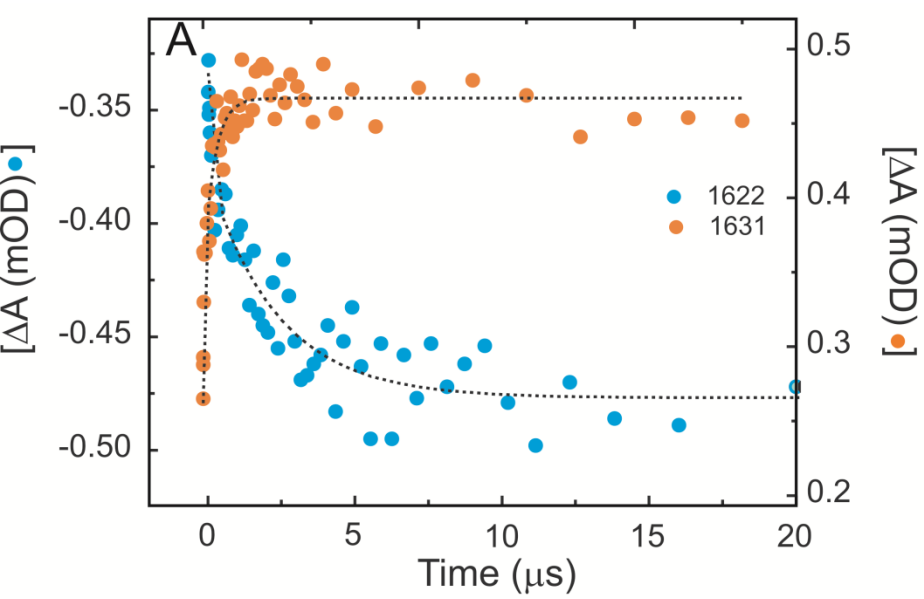


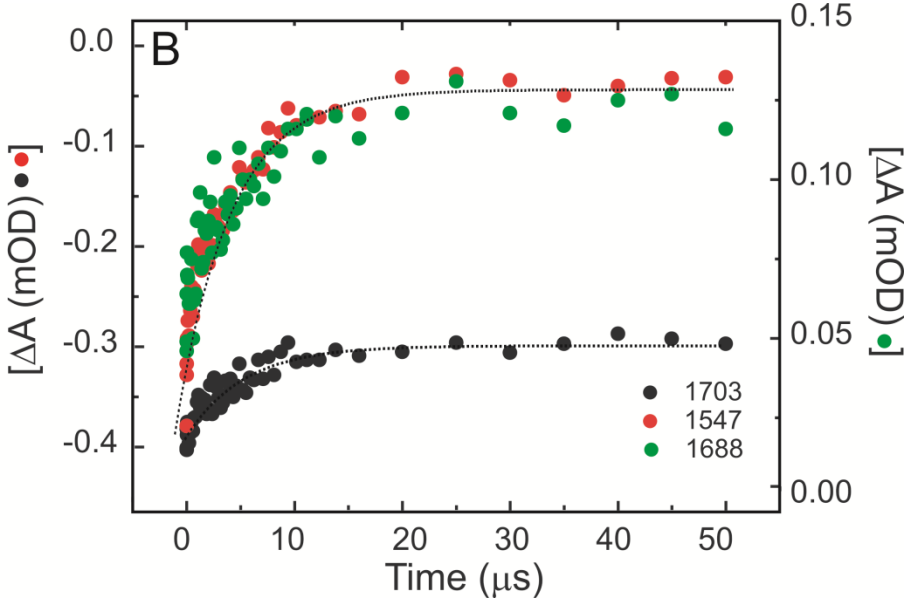
TRMPS on WT AppA



R. Brust , A. Lukacs,et al., 2013, JACS

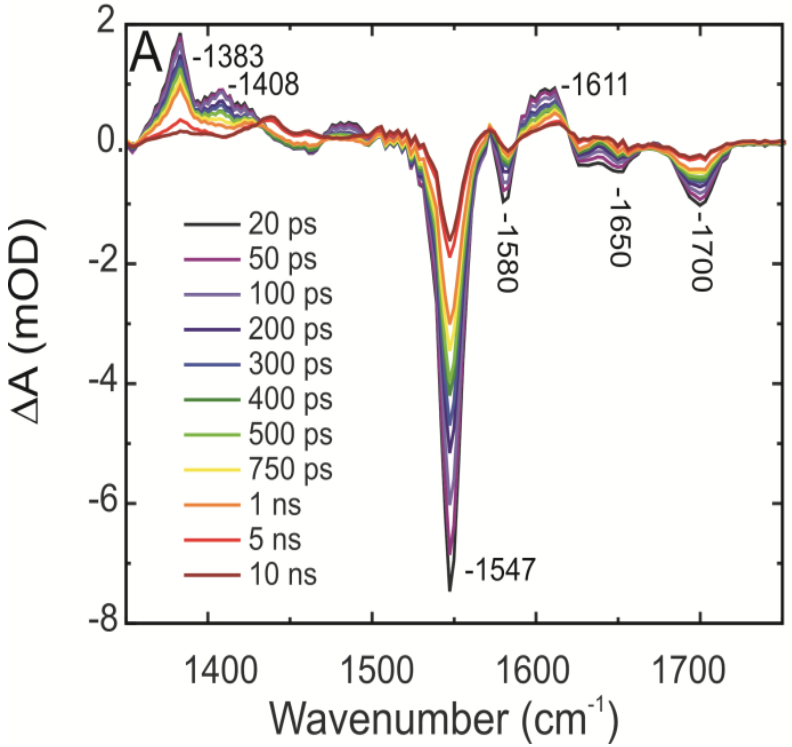
TRMPS on WT AppA

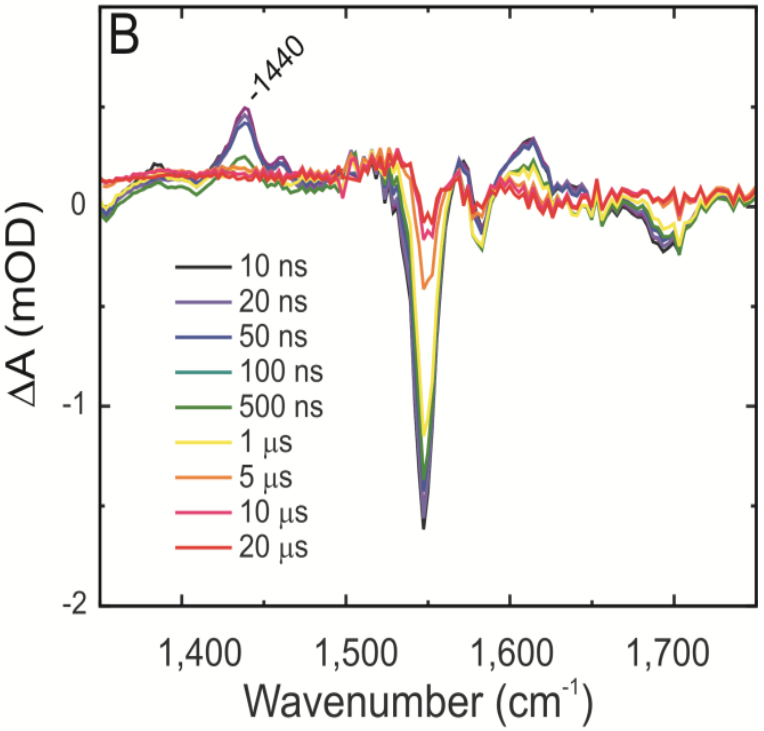




R. Brust , A. Lukacs,et al., 2013, JACS

TRMPS on FMN





R. Brust , A. Lukacs,et al., 2013, JACS

JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Spotlights on Recent *JACS* Publications

2D π-CONJUGATED POLYMERS: LARGER SIZE, SMALLER BAND GAP

A new computational study brings researchers one step closer to designing tailor-made polymers with desirable electronic properties. The study, performed by Rico Gutzler and Dmitrii Perepichka, sheds light on the relationship between the size of planar two-dimensional π-conjugated polymers and their semiconducting potential (DOI: 10.1021/ja408355p).

Due to a growing interest in 2D materials and their electronic properties, chemists are increasingly exploring new synthetic routes to create semiconducting organic analogues to graphene. To assist in this pursuit, the researchers performed density functional theory calculations to determine the structures and electronic properties of both theoretical and experimentally realized 2D π-conjugated polymers.

The team reports that, compared to their linear, 1D counterparts, 2D polymers that are grown in two directions as planar sheets have smaller band gaps, a quality that is favorable for most electronic device applications. Additionally, the band gap in 2D polymers decreases faster with the number of molecular repeat units in 2D than in 1D polymers. This information may help researchers in the field of band gap engineering design functional organic materials with desirable semiconducting and luminescent properties for applications ranging from optoelectronics to sensing.

Christine Herman, Ph.D.

SHINING A LIGHT ON PROTEIN DYNAMICS

Light can provoke changes in protein structure, which in turn can affect a cell's movement or gene expression. Now a team led by Peter J. Tonge and Stephen R. Meech uses high-speed spectroscopy to reveal how certain proteins change their structure on time scales ranging from 100 fs to 1 ms after the short pulse of light arrives (DOI: 10.1021/ja407265p).

Light is a useful tool for studying protein dynamics because its arrival at the protein is easier to control than other stimuli, such as small molecules. And unlike well-established X-ray studies, infrared observations do not require the proteins to be in crystalline form, so they offer a more natural glimpse of how proteins change their shape in real time.

In this study, the researchers bombard a bacterial protein that naturally responds to light and changing oxygen levels with pulses of blue light. Using a tool called time-resolved multiple probe spectroscopy, the team identifies a hierarchy of activity in the protein's structural changes: residues close to the site of light absorption respond first and then activate more remote parts of the protein. In a mutant version of the protein, the team finds that the response to light is short-circuited. The ability to observe a protein's dynamics across multiple time domains will help test and improve models of protein function and lead to the development of new tools for controlling gene expression with light, the authors say.

Lucas Laursen

NEW CLASS OF HYDROGELS FALLS APART WITH LIGHT

"Smart" biomaterials, which undergo a physical change in response to external stimuli, are of enormous interest to researchers in cellular and biomolecular engineering. A new class of photoresponsive hydrogels, designed by Yan Zhang and co-workers, represents a significant step toward the goal of controlling both the structure and assembly of cellular microenvironments (DOI: 10.1021/ja409000b).

The team synthesizes peptides modified with a small-molecule phototrigger, known as a biaryl-substituted tetrazole. The peptides self-assemble to form a hydrogel that can be used to culture cells in either a 2D or 3D environment. In the presence of UV light, the tetrazole-based phototrigger undergoes an intramolecular ligation that causes the hydrogel to partially disassemble, presumably because the new slightly tilted ring system interrupts the hydrophobic π−π stacking.

To demonstrate the utility of the light-responsive hydrogels in biological applications, the researchers show that they can control the differentiation of a model cell line by using UV light to release a differentiation-inducing protein from within the hydrogel. Efforts to create photopatterned channels that induce different biological behaviors of cultured cells are currently underway, the researchers say.

Christine Herman, Ph.D.

BREAKING DNA, TWICE

A compelling therapeutic strategy for diseases characterized by out-of-control cell growth, like cancer and rheumatoid arthritis, is to kill the cells by inducing DNA damage. Numerous compounds damage DNA by breaking a single strand of the DNA double helix, but molecules that break both strands are more efficient damaging agents. Toward the design of compounds capable of inducing such double strand breaks, Marisa Taverna Porro and Marc Greenberg delineate a chemical pathway that leads to this deadly alteration (DOI: 10.1021/ja409513q).

The authors determine that, in the presence of oxygen, formation of a highly reactive free radical at a particular location within the DNA double-helix structure leads to a double strand break. Specifically, they find that the radical triggers breakage of the first strand, which generates a second radical. This second radical triggers the removal of a hydrogen atom from the double helix, which initiates breakage of the second strand.

These findings provide a starting point for the design of molecules that can produce double strand breaks for potential therapeutic applications. In addition, they may offer insight into the mechanisms by which certain natural products cause double strand breaks in DNA.

Eva J. Gordon, Ph.D.

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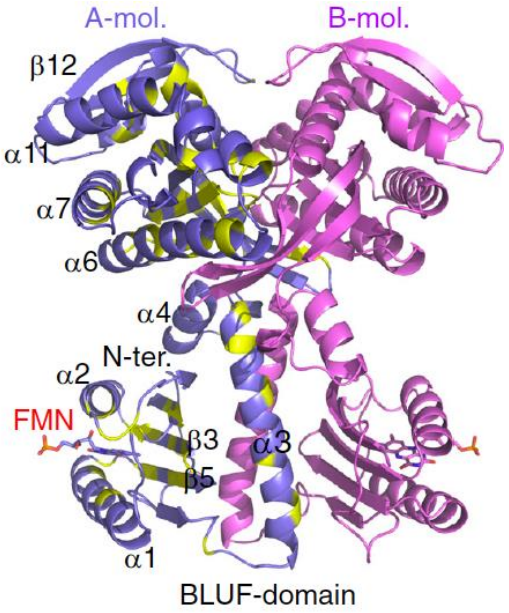
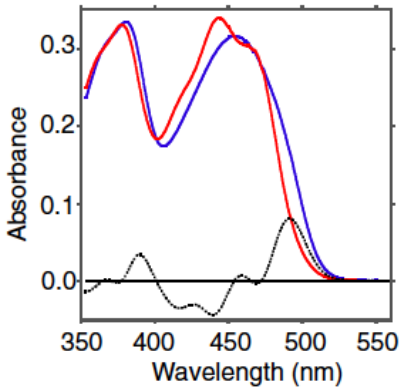
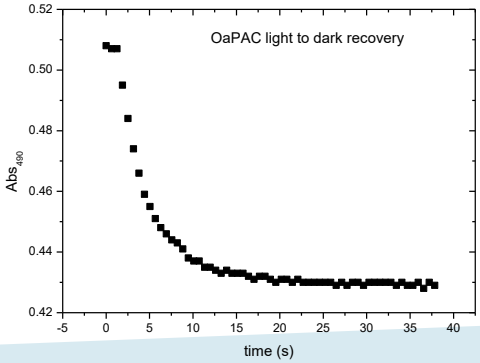
   dx.doi.org/10.1021/ja411266n | J. Am. Chem. Soc. 2013, 135, 16745−16745

Photoactivated adenylate cyclase from Oscillatoria acuminata (OaPAC)

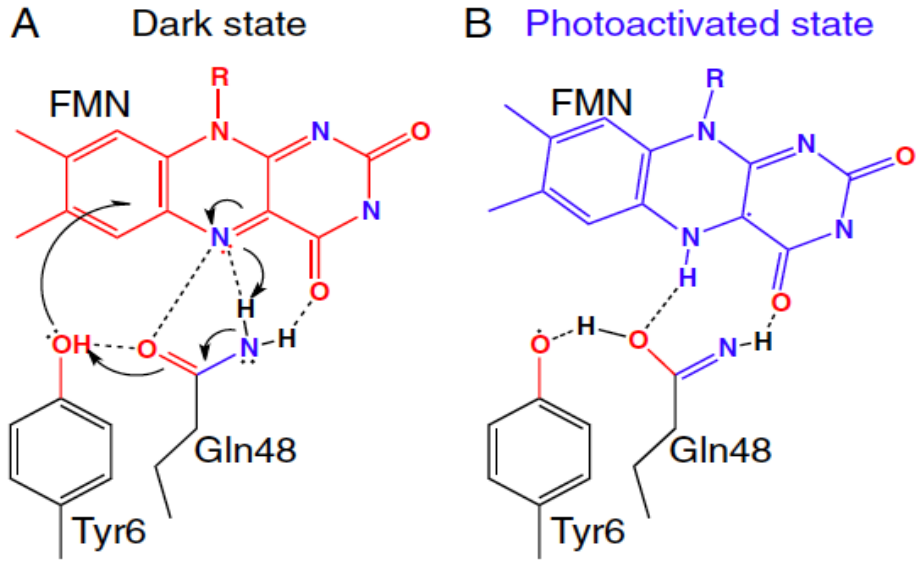






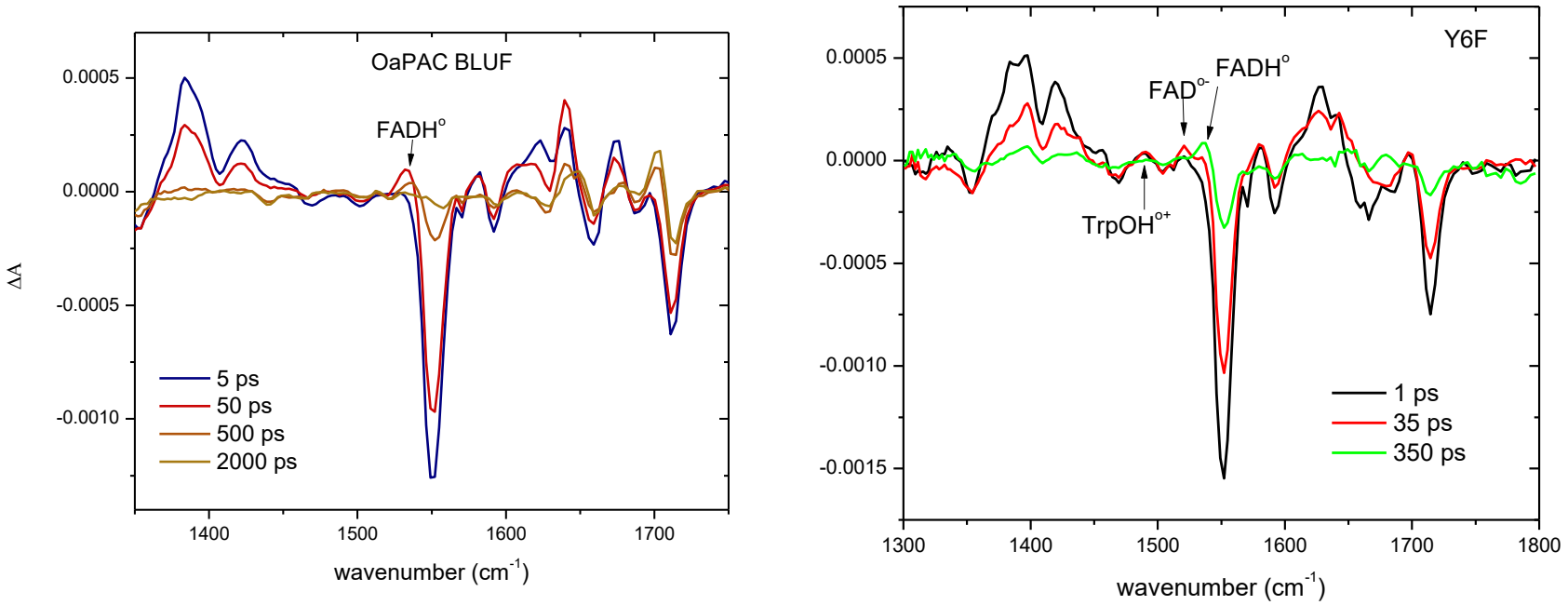
Ohki et al, PNAS, 2016

OaPAC is a BLUF (Blue Light sensing using FAD) protein wich controls the cAMP level in a light regulated manner



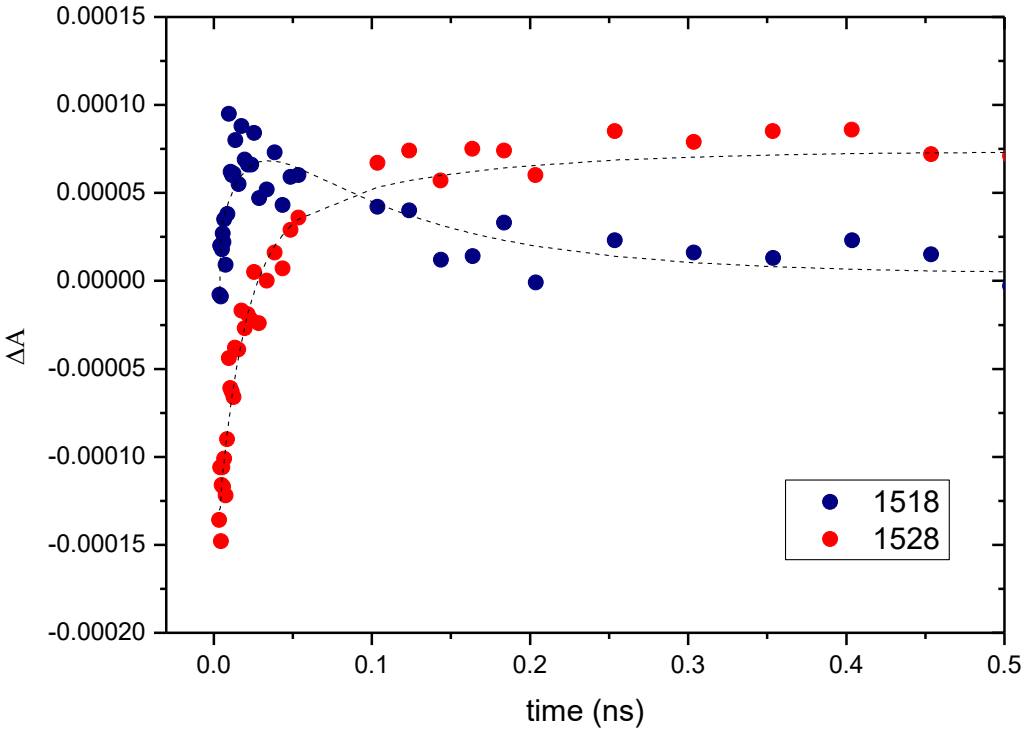
Ohki et al proposed that after excitation neutral tyrosine radical and neutral flavin radical is formed. Flavin is protonated via Gln48

TRIR on OaPAC



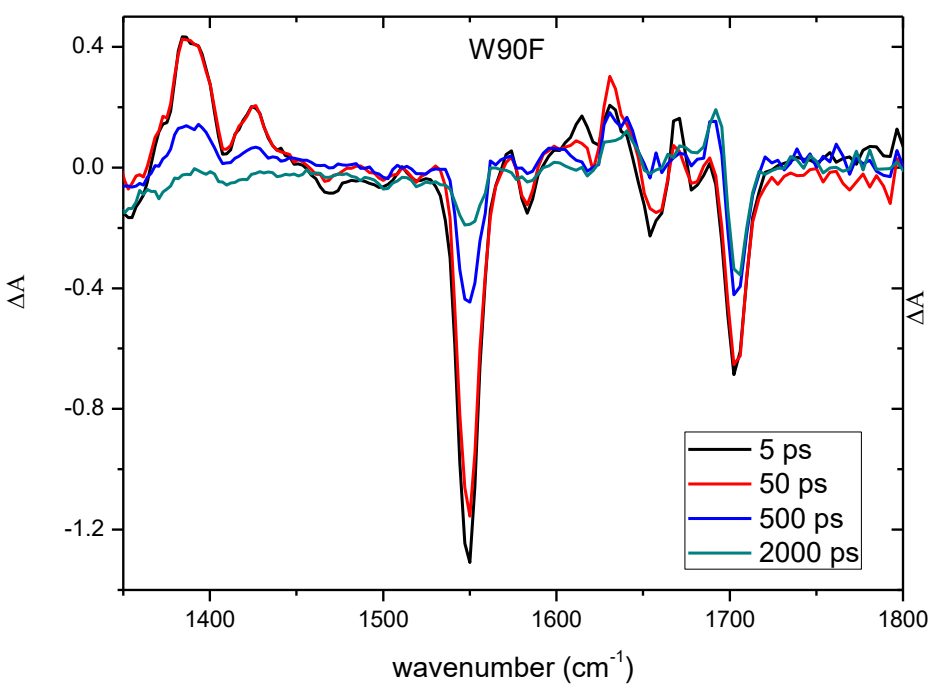
- TRIR data of WT shows the formation of the neutral semiquinone as proposed by Ohki et al,
- Y6F data shows the formation of the neutral semiquinone as well, and it is sequential as in PixD
- In the case of Y6F, W91 is the primary electron donor (one can see Trp cation radical formation at 1488 cm-1)
- Protonation of the flavin still happens, but the proton donor is another tyrosine or the W91 tryptophan (as the TrpOHo+ radical

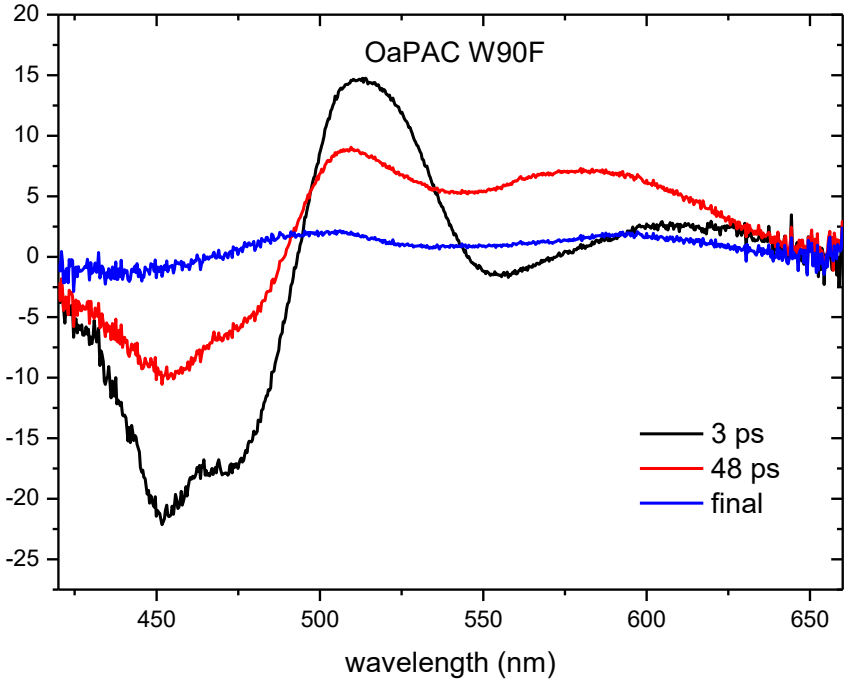
TRIR on Y6F



Flavin anionic radical is formed first, the protonation (formation of neutral radical) happens on a longer timescale

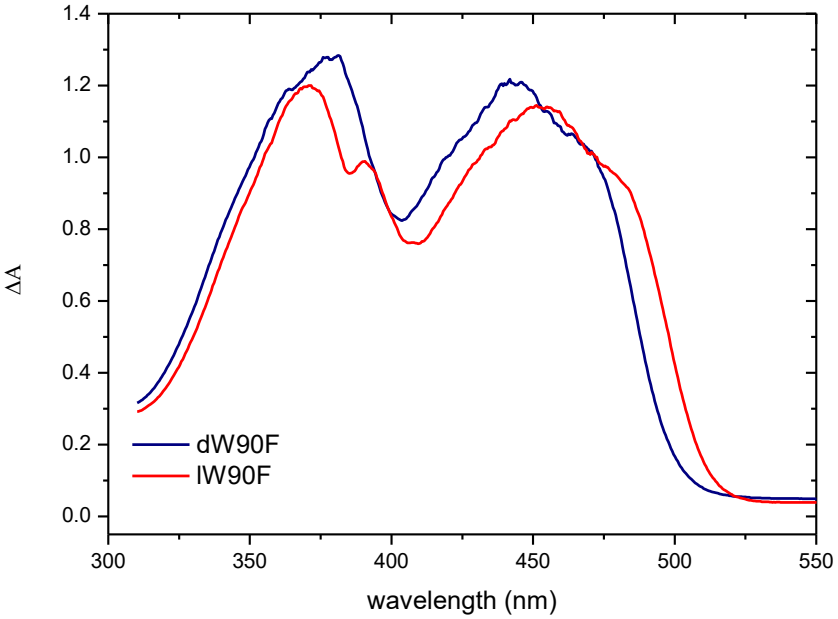
TRIR and TA measurements on W90F

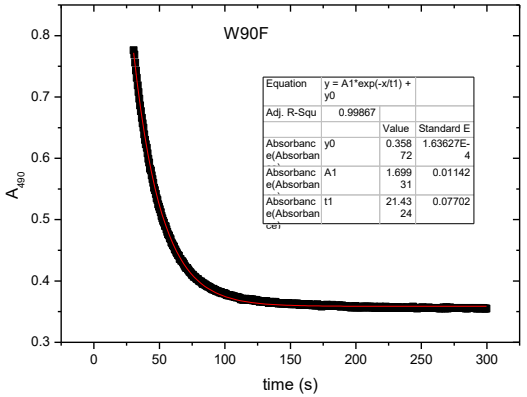


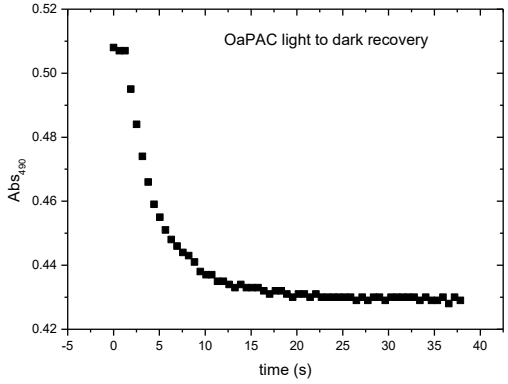


- Transient IR data still shows the formation of the neutral flavin radical (less obvious than in WT) and maybe the formation of the tyrosine cation radical (~ 1500 cm-1)
- From the TA measurements one can see that FADHo is formed -> the primary ET donor is Y6

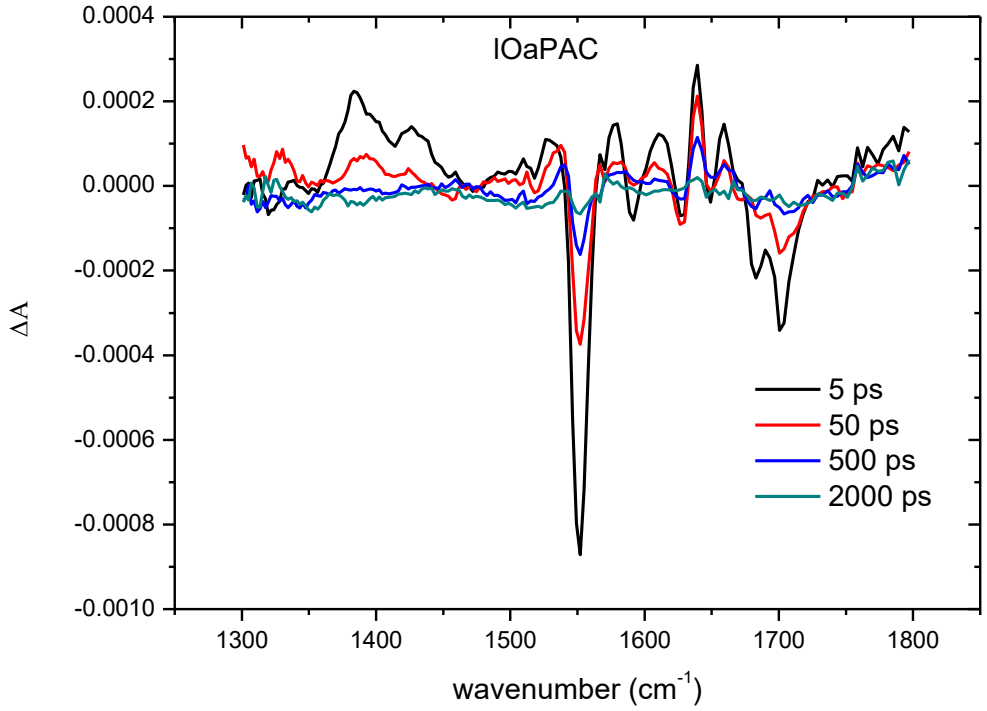
W90F mutant


ΔA
wavelength (nm)
dW90F
lW90F

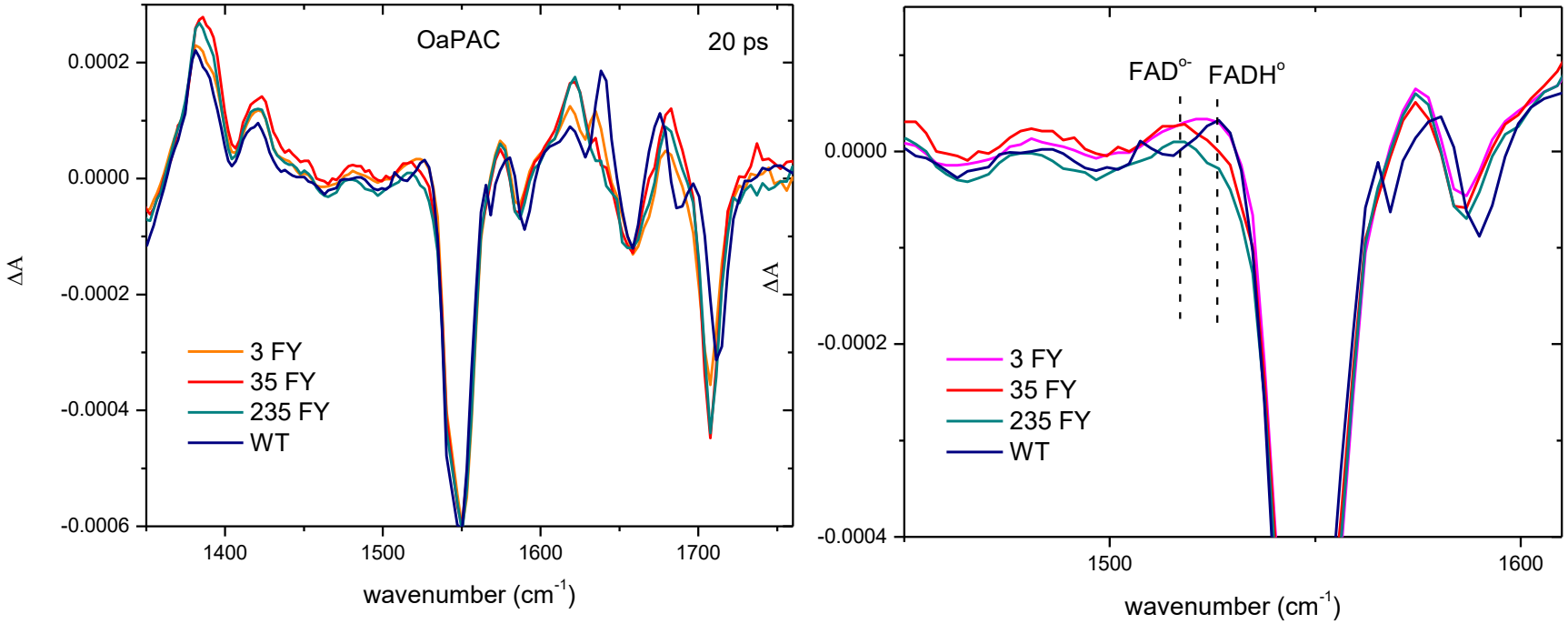

W90F
Equation
y = A1*exp(-x/t1) + y0
Adj. R-Squ
0.99867
Value
Standard E
Absorbance(Absorban
y0
0.35872
1.63627E-4
Absorbance(Absorban
A1
1.69931
0.01142
Absorbance(Absorban
t1
21.4324
0.07702
A490
time (s)


OaPAC light to dark recovery
Abs490
time (s)

TRIR on light OaPAC



Fluotyrosine TRIR measurements



- Lowering the pKa leads to the formation of the anionic radical, but the neutral radical is not formed
- Y6 is deprotonated -> the flavin cannot be protonated

Acknowledgements

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