Dr. Brand's impact on my research, from before we met to 2022 JACS paper

Dmitri Toptygin, Department of Biology (JHU)

Before I met Lenny Brand in 1990, I worked at the P. N. Lebedev Physics Institute of the Academy of Sciences of the Soviet Union.

My first project: Intracavity Laser Spectroscopy (1977-1982)

Multichannel recording of absorption spectra obtained by intracavity laser spectroscopy

V. M. Baev, S. A. Kovalenko, É. A. Sviridenkov, A. F. Suchkov, and D. D. Toptygin

P. N. Lebedev Physics Institute, Academy of Sciences of the USSR, Moscow
(Submitted December 8, 1979)
Kvantovaya Elektron. (Moscow) 7, 1112–1115 (May 1980)

A multichannel photoelectric system was developed for recording absorption spectra obtained by intracavity laser spectroscopy. Such a recording system was chosen because the features of the kinetics of the spectral distribution of a broad-band laser, having frequency-dependent losses in its resonator, make it impossible to use single-channel successive recording of the spectra. The use of multichannel recording enabled the dynamic range to be broadened and the accuracy and sensitivity of measurement of the absorption coefficients to be increased. Absorption coefficients in the range 3×10^{-9} to 2×10^{-8} cm^{-1} were measured with an accuracy of 30% and those in the range 3×10^{-8} to 10^{-6} cm^{-1} with an accuracy of 10%. The detection threshold of the absorption lines was 10^{-9} cm^{-1}.

PACS numbers: 07.65.Eh, 42.60.Kg

Intracavity laser spectroscopy,[1,2] which has substantially increased the sensitivity of absorption spectrum analysis, is being used to investigate the kinetics of chemical reactions and to detect small amounts of gas-

weak absorption lines $K_L(\omega)$, i.e., $K_A(\omega)=K_0+K_L(\omega)$, where $K_L(\omega)\ll K_0$, the variation in the spectral distribution will occur with a dependence similar to the Lambert–Beer law,

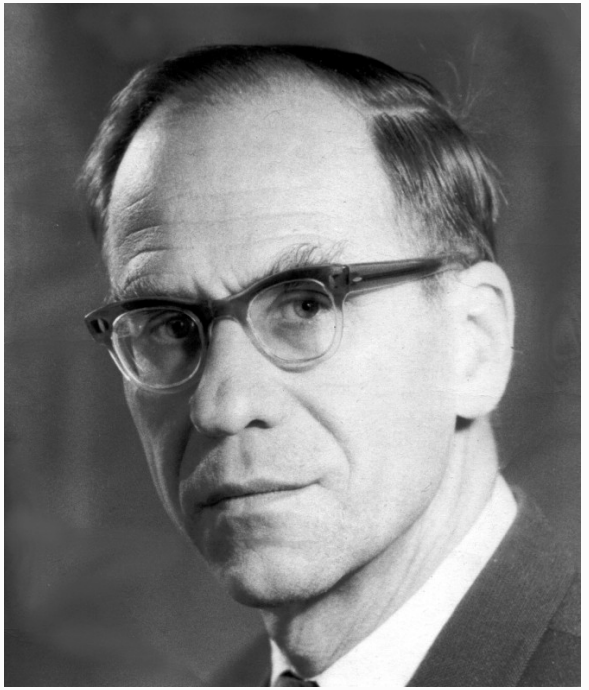
My first project: Intracavity Laser Spectroscopy (1977-1982)

What I did:

1. Built an electronic interface that allowed to output laser spectra directly to a mainframe computer,
2. Wrote software for the data analysis,
3. Analyzed intracavity laser spectra.

This drew the attention of Professor Michael D. Galanin

Professor Michael D. Galanin



A pioneer of Time-Correlated Single Photon Counting (TCSPC),

The author of the Förster-Galanin equation.

Förster equation for the donor decay:

$$I(t) = I_0 \exp\left(- \frac{t}{\tau_0} - \frac{R_0^6}{R^6} \frac{t}{\tau_0} \right)$$

Förster-Galanin equation for the donor decay:

$$I(t) = I_0 \exp\left(- \frac{t}{\tau_0} - \frac{4}{3} \pi^{\frac{3}{2}} R_0^3 C_A \sqrt{\frac{t}{\tau_0}} \right)$$

My second project: Time-Correlated Single Photon Counting & Data Analysis (1982-1990)

I was asked to:

1. Build an electronic interface that would allow to output TCSPC data directly to a mainframe computer,
2. Write software for the data analysis,
3. Fit TCSPC data using different fluorescence decay models, including the Förster-Galanin equation.

To help me start, Dr. M. D. Galanin handed to me seven reprints.

To help me start, Dr. M. D. Galanin handed to me seven reprints:

I. Isenberg, R. D. Dyson, R. Hanson, *Biophys. J.* **13,** 1090 (1973).

W. R. Ware, L. J. Doemeny, T. L. Nemzek, *J. Phys. Chem.* **77,** 2038 (1973).

A. Grinvald, I. Z. Steinberg, *Anal. Biochem.* **59,** 583 (1974).

A. Gafni, R. L. Modlin, L. Brand, *Biophys. J.* **15,** 263 (1975).

A. E. McKinnon, A. G. Szabo, D. R. Miller, *J. Phys. Chem.* **81,** 1564 (1977).

D. V. O'Connor, W. R. Ware, J. C. Andre, *J. Phys. Chem.* **83,** 1333 (1979).

M. G. Badea, L. Brand, *Meth. Enzymol.* **61,** 378 (1979).

The last three reprints compared different methods of data analysis:

1. Method of Moments
2. Method of Modulating Functions
3. Laplace Transform
4. Fourier Transforms (two variants)
5. Exponential Series A. K. A. Linear Least Squares
6. Iterative Convolution A. K. A. Nonlinear Least Squares

All three comparisons were in favor of the Iterative Convolution procedure, however, only Badea and Brand (1979) explicitly stated this conclusion:

Comparison of Deconvolution Techniques. McKinnon *et al.*[44] have carried out a detailed evaluation of the various procedures for deconvolution of fluorescence decay data. They found that the iterative convolution procedure gave good recovery of decay parameters under a variety of conditions. The reader is referred to the original paper which includes an excellent discussion of the problems associated with the analysis of fluorescence decay data.

```
      SUBROUTINE TOPFIT(MODEL,PAR,STD,FLO,NP,DOMAIN,ARGM,
     &DATA,FIT,WGHT,ND,CHI2,OK,SWRITE,DWRITE)
C
C   Nonlinear Weighted Least Squares Estimator by Dmitri Toptygin,
C   based on the classical Gauss-Newton algorithm.
C   Calls external subroutine MODEL & external logical function DOMAIN.
C
      IMPLICIT REAL*8 (A-H,O-Z), INTEGER*4 (I-N)
      EXTERNAL MODEL, DOMAIN
      DIMENSION PAR(NP), STD(NP), FLO(NP)
      DIMENSION ARGM(*)
      DIMENSION DATA(ND), FIT(ND), WGHT(ND)
      LOGICAL FLO, DOMAIN, OK, SWRITE, DWRITE
      PARAMETER(MAXNP=64, MAXLTRAIN=2, NABORT=256)
      PARAMETER(ACCUR=1.D-8, ALPHA=1.D-8)
      DIMENSION TRIALPAR(MAXNP), DELTA(MAXNP), DCOPY(MAXNP)
      DIMENSION DERIV(MAXNP,MAXLTRAIN)
      DIMENSION HESSIAN(MAXNP,MAXNP)
 4    FORMAT(1X,/,1X,A,' ABORT RESULTS.')
 5    FORMAT(1X,/,1X,A,' THE REGION OF TOLERATED VALUES IN PARAMETER SPA
     &CE. ABORT RESULTS.')
 6    FORMAT(1X,/,1X,A,' IS CURRENTLY SET EQUAL TO ',I4)
C
      CHI2=0.D0
      IF(NP.LE.0)THEN
      WRITE(*,4)'ZERO OR NEGATIVE NUMBER OF PARAMETERS.'
      GOTO 801
      ENDIF
      IF(NP.GT.MAXNP)THEN
      WRITE(*,6)'MAX. NO. OF PARAMETERS',MAXNP
```

The phone call at 1:30 AM that changed my life completely

1. Förster-Galanin equation is used to fit donor decay in liquid crystals.
2. The donor decay is non-exponential even without the acceptor.
3. In anisotropic media the decay rate depends on the orientation.
4. There is no simple mathematical model to describe this dependence in liquid crystals.
5. Collaboration with the Charles University in Prague, Czechoslovakia, where they study fluorescence of DPH in lipid membranes.
6. I introduce a simple mathematical model for the orientational dependence of the decay rate in lipid membranes.
7. Unable to test the theoretical model due to inferior experimental equipment in Prague at that time (1989).
8. Dr. Jaroslava Svobodova sends my science proposal to Dr. Michael Edidin.
9. Dr. Michael Edidin hands my science proposal to Dr. Ludwig Brand.

The phone call at 1:30 AM that changed my life completely

Biochemistry **1986**, *25*, 1811–1816

Anisotropy Decay Associated Fluorescence Spectra and Analysis of Rotational Heterogeneity. 2. 1,6-Diphenyl-1,3,5-hexatriene in Lipid Bilayers†

Lesley Davenport,‡ Jay R. Knutson,§ and Ludwig Brand*

Biology Department and McCollum-Pratt Institute, The Johns Hopkins University, Baltimore, Maryland 21218

Received August 22, 1985

10. Dr. Ludwig Brand studied fluorescence decay of DPH in membranes since about 1981.
11. In about 1987 Dr. R. E. Dale finds a paper where the orientational dependence of decay rate in thin layers was obtained from classical electrodynamics and brings it to the attention of Dr. Brand:

    W. Lukosz, *Phys. Rev. B* **22,** 3030 (1980).

12. Dr. Ludwig Brand writes about the decay rate dependence in his NIH grant proposal, but he finds nobody who would like to take on this project... until Dr. Michael Edidin hands my science proposal to him.

The phone call at 1:30 AM that changed my life completely

Baltimore to Moscow time difference = 8 hours.

9:30 AM + 8 hours = 5:30 PM

The phone call at 1:30 AM that changed my life completely

Baltimore to Moscow time difference = 8 hours.

9:30 AM + 8 hours = 5:30 PM

5:30 PM + 8 hours = 1:30 AM

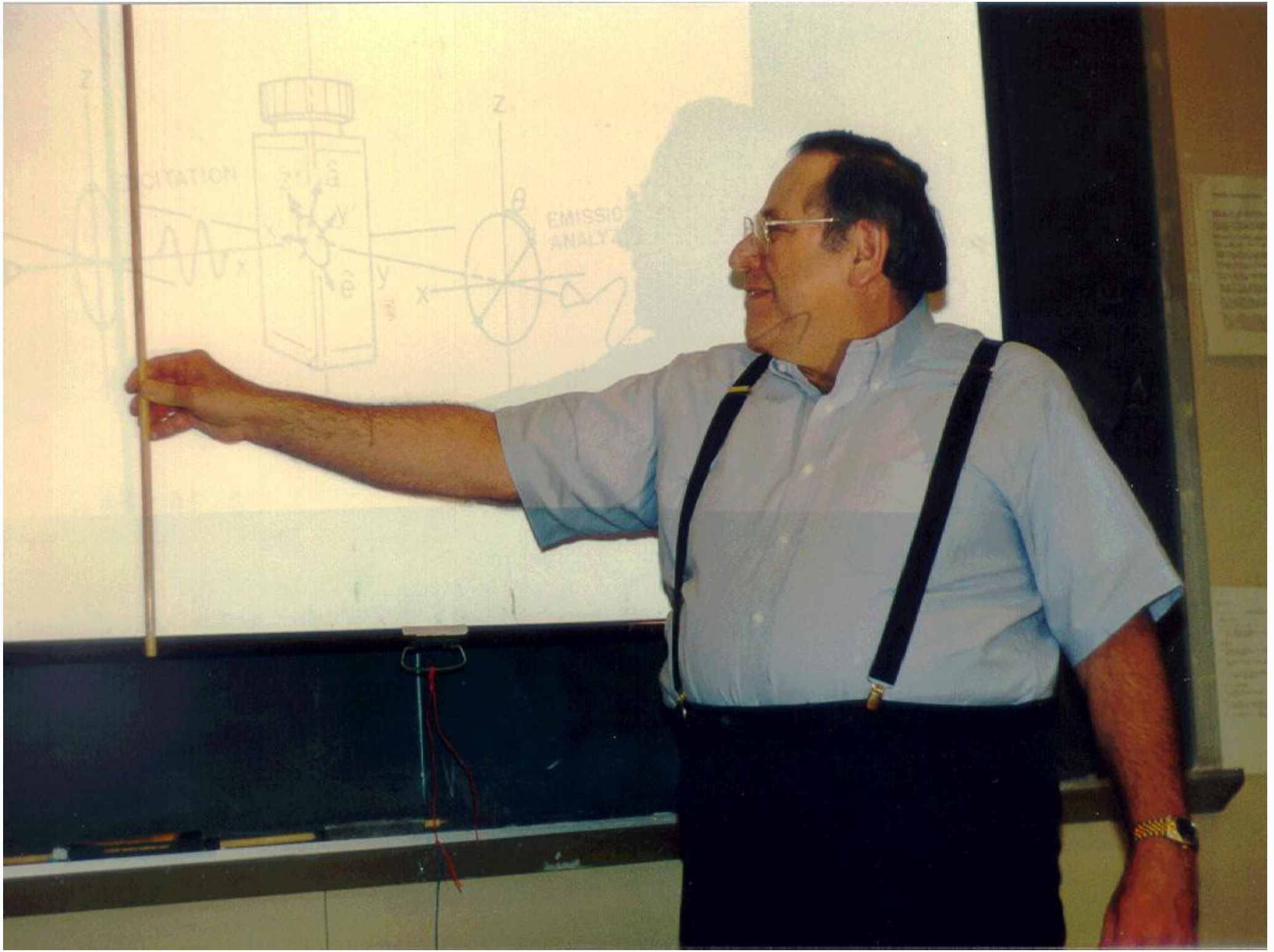
1. Toptygin, D.; Svobodova, J.; Konopasek, I.; Brand, L. *“Fluorescence intensity and emission anisotropy decay of diphenylhexatriene in single bylayer phospholipid membranes.”* Abstract at the 35th Annual Meeting of the Biophysical Society. *Biophys. J.,* **59,** 360A (1991).

2. Toptygin, D.; Svobodova, J.; Konopasek, I.; Brand, L. *“Fluorescence decay and depolarization in membranes.”* Time-Resolved Laser Spectroscopy in Biochemistry III, Los Angeles, CA, Jan. 20-22, 1992. *Proceedings of SPIE,* **1640,** 739-751 (1992).

3. Toptygin, D.; Svobodova, J.; Konopasek, I.; Brand, L. *“Fluorescence decay and depolarization in membranes.” J. Chem. Phys.,* **96,** 7919-7930 (1992).

4. Toptygin, D.; Brand, L. *“Fluorescence decay of DPH in lipid membranes: influence of the external refractive index.” Biophys. Chem.,* **48,** 205-220 (1993).

5. Toptygin, D.; Brand, L. *“Determination of DPH order parameters in unoriented vesicles.” Journal of Fluorescence,* **5,** 39-50 (1995).


z
θ
x
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EMISSIO
ANALY


CASAD

Area of Commercial Ship Traffic in Maryland and Virginia

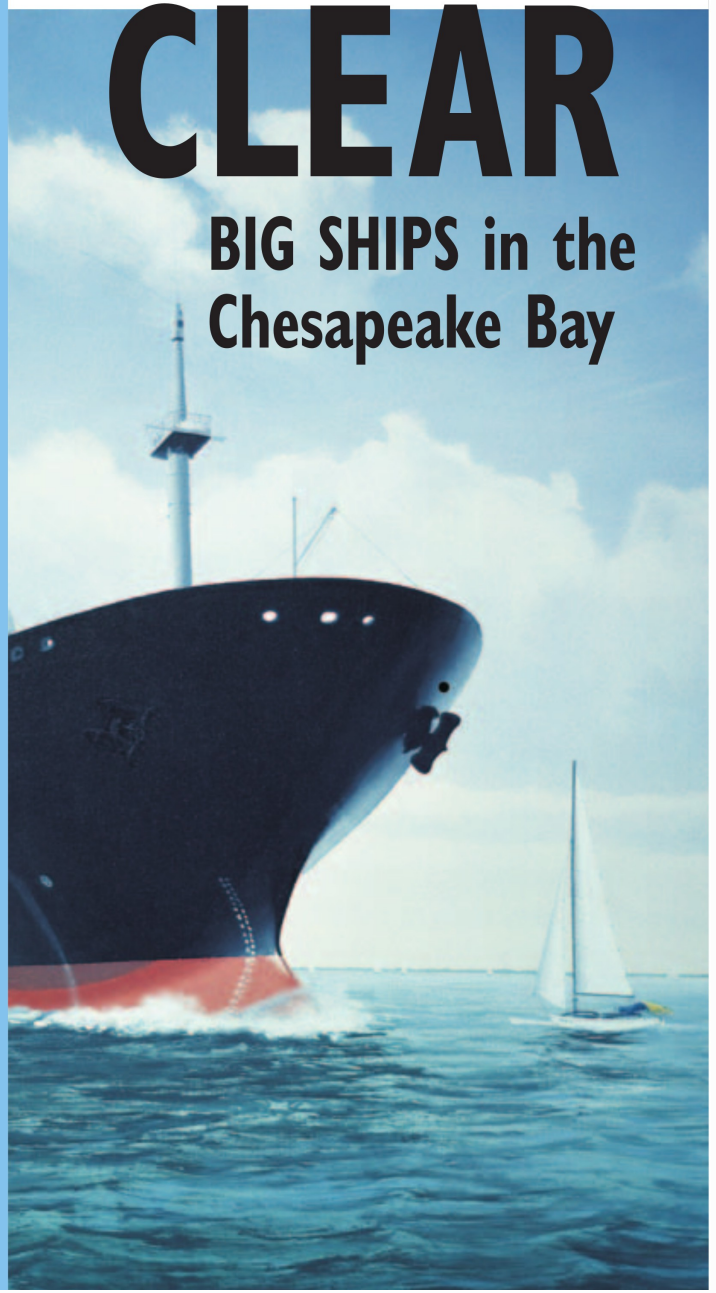
Following the drowned river valley of the ancient Susquehanna, the Chesapeake's main ship channel brings large vessels to ports like Baltimore and Norfolk, but leaves them little room to maneuver. When crossing the ship channel, small boats should take great care, especially at night or in poor visibility.

Sea Grant
Maryland Sea Grant
University System of Maryland
College Park, Maryland 20742
www.mdsg.umd.edu

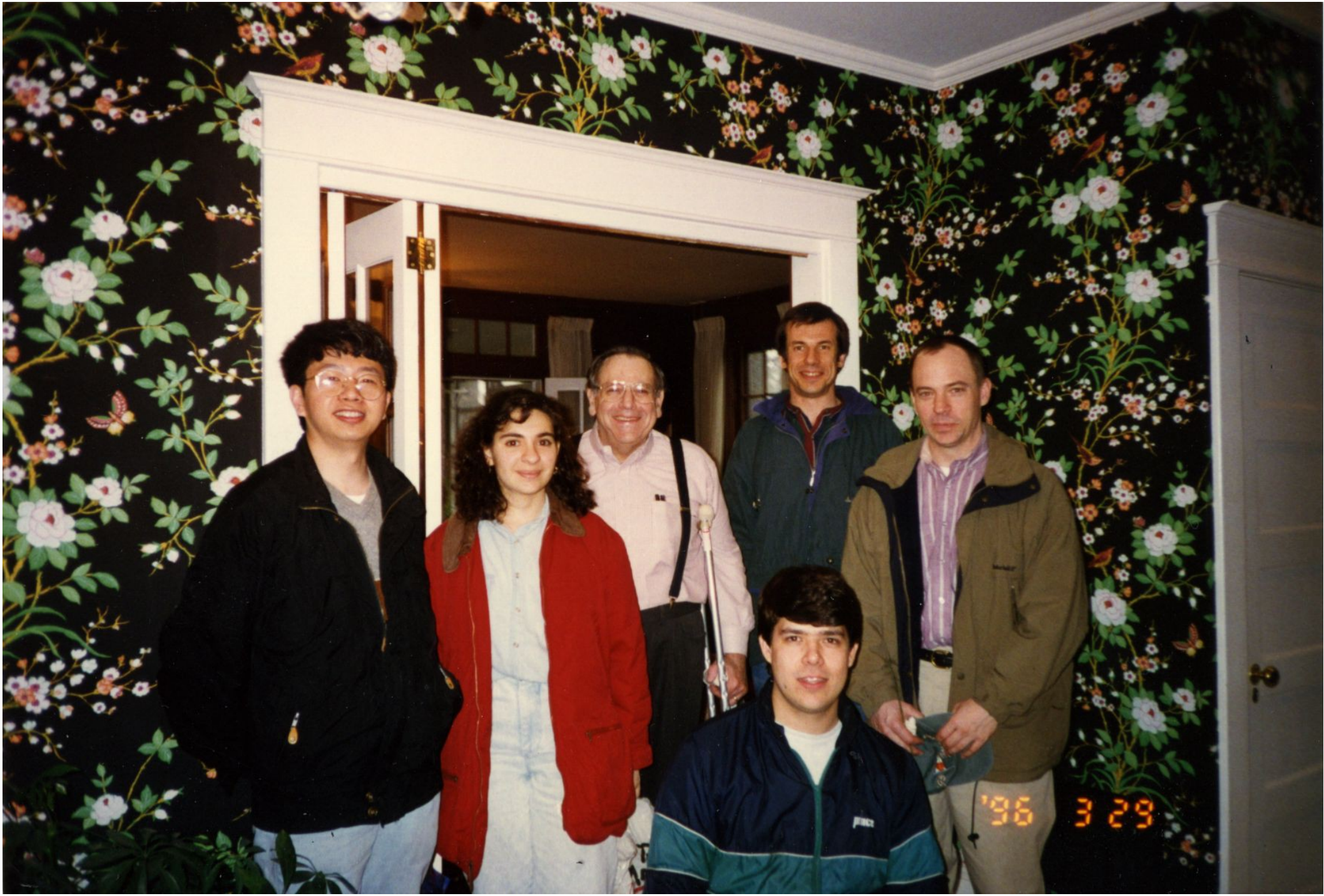
Cover art from an original oil painting by Maryland Pilot Brian Hope.

KEEP CLEAR

BIG SHIPS in the Chesapeake Bay




SIGNAL TRANSDUCTION PATHWAYS

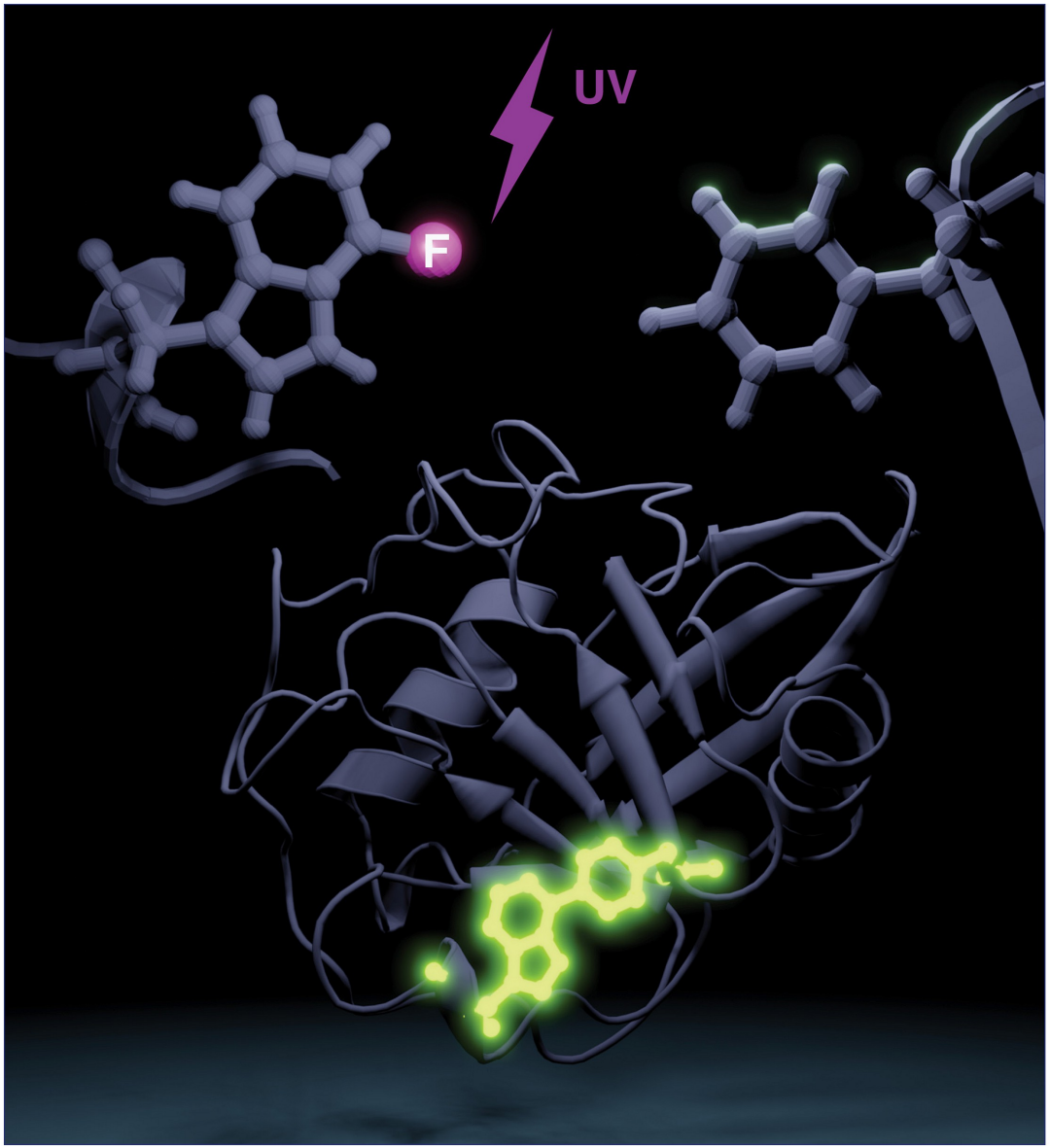

'96 3 29


echo $PATH
NADP
NAADP
NAD
Nicotinamide

June 22, 2022
Volume 144
Number 24
pubs.acs.org/JACS

JACS

JOURNAL OF THE AMERICAN CHEMICAL SOCIETY



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Article

The Magic of Linking Rings: Discovery of a Unique Photoinduced Fluorescent Protein Crosslink

Manman Lu, Dmitri Toptygin, Yufei Xiang, Yi Shi, Charles D. Schwieters, Emma C. Lipinski, Jinwoo Ahn, In-Ja L. Byeon, and Angela M. Gronenborn*

Cite This: J. Am. Chem. Soc. 2022, 144, 10809−10816

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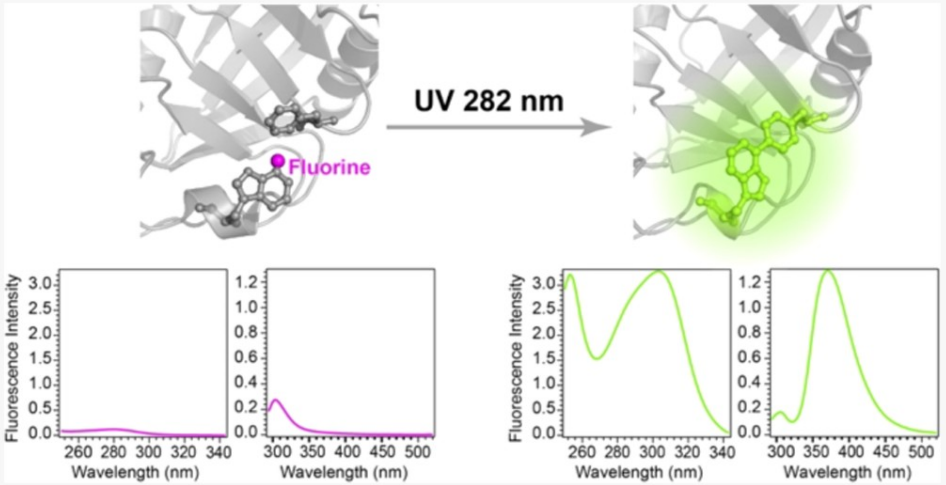
ABSTRACT: Fluorosubstituted tryptophans serve as valuable probes for fluorescence and nuclear magnetic resonance (NMR) studies of proteins. Here, we describe an unusual photoreactivity introduced by replacing the single tryptophan in cyclophilin A with 7-fluoro-tryptophan. UV exposure at 282 nm defluorinates 7-fluoro-tryptophan and crosslinks it to a nearby phenylalanine, generating a bright fluorophore. The crosslink-containing fluorescent protein possesses a large quantum yield of ∼0.40 with a fluorescence lifetime of 2.38 ns. The chemical nature of the crosslink and the three-dimensional protein structure were determined by mass spectrometry and NMR spectroscopy. To the best of our knowledge, this is the first report of a Phe−Trp crosslink in a protein. Our finding may break new ground for developing novel fluorescence probes and for devising new strategies to exploit aromatic crosslinks in proteins.



7-fluoro-tryptophan (7F-Trp) is completely non-fluorescent.

7F-Trp variant of the cyclophilin A (CypA) protein was created at the University of Pittsburgh for NMR studies.

7F-Trp-CypA displayed weak fluorescence of Tyr residues, peak at 302 nm.

Dr. Manman Lu discovers that after photoactivation by UV radiation, 7F-Trp-CypA becomes highly fluorescent, with the peak near 370 nm.

Dr. Angela M. Gronenborn sends a sample of 7F-Trp-CypA to me. Why did she send it to me?

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Nanosecond Relaxation Dynamics of Protein GB1 Identified by the Time-Dependent Red Shift in the Fluorescence of Tryptophan and 5-Fluorotryptophan

Dmitri Toptygin,*,† Angela M. Gronenborn,‡,§ and Ludwig Brand†

Department of Biology, Johns Hopkins University, Baltimore, Maryland 21218, and Laboratory of Chemical Physics, NIDDK, National Institutes of Health, Bethesda, Maryland

Received: July 17, 2006; In Final Form: October 19, 2006

The quantum yield (η) of new fluorophore at λ_{ex} = 305 nm equals 0.395±0.003

The main lifetime (τ) is 2.38 ± 0.01 ns

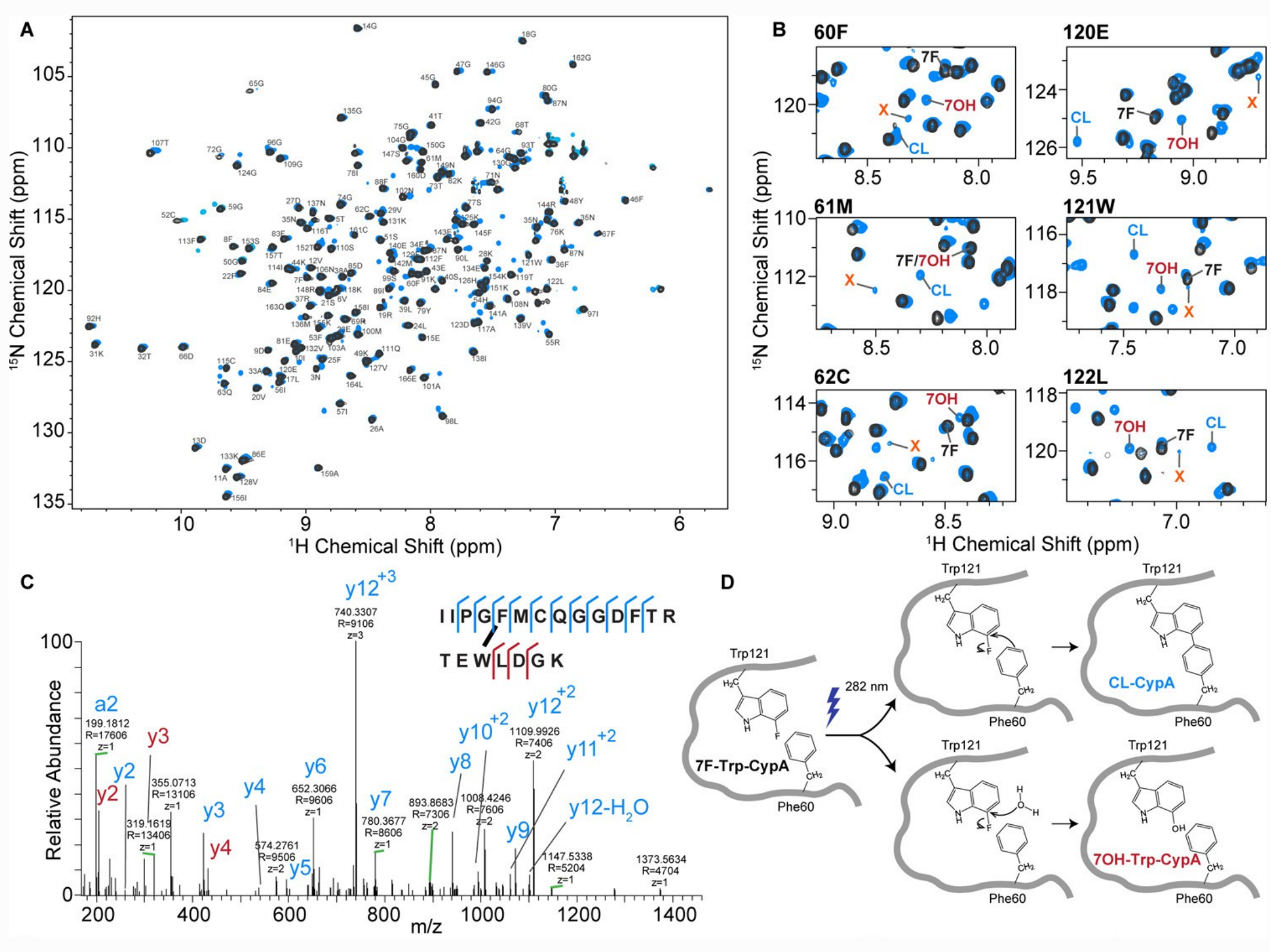
The natural lifetime: $\tau_0 = \tau/\eta$ = 6.02 ns

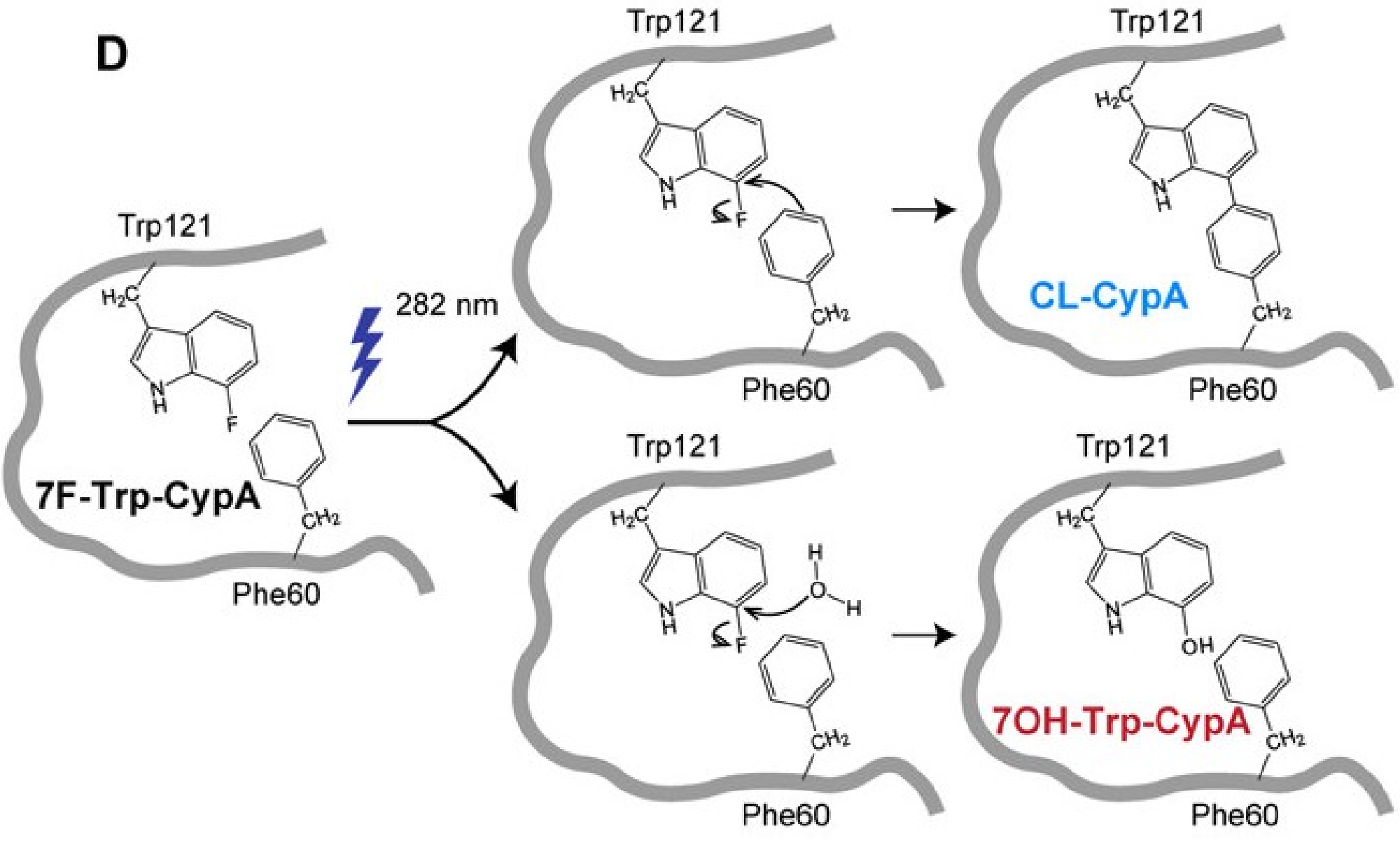
The radiative decay rate: $k_r = \eta/\tau = 1.66\times10^8\ s^{-1}$

This high radiative decay rate cannot be achieved if the fluorophore is the size of the Trp side chain (indole).

CONCLUSION:

The fluorophore must consist of more than just two fused aromatic rings.


A
^{15}N Chemical Shift (ppm)
^{1}H Chemical Shift (ppm)
B
60F
120E
61M
121W
62C
122L
7F
7OH
CL
X
7F/7OH
^{15}N Chemical Shift (ppm)
^{1}H Chemical Shift (ppm)
C
Relative Abundance
m/z
y12^{+3}
740.3307
R=9106
z=3
I I P G F M C Q G G D F T R
T E W L D G K
a2
199.1812
R=17606
z=1
y3
y2
355.0713
R=13106
z=1
319.1619
R=13406
z=1
y4
574.2761
R=9506
z=2
y5
y6
652.3066
R=9606
z=1
y7
780.3677
R=8606
z=1
y8
893.8683
R=7306
z=2
y10^{+2}
1008.4246
R=7606
z=2
y12^{+2}
1109.9926
R=7406
z=2
y11^{+2}
y9
y12-H$_2$O
1147.5338
R=5204
z=1
1373.5634
R=4704
z=1
D
Trp121
Phe60
7F-Trp-CypA
282 nm
CL-CypA
7OH-Trp-CypA


D
Trp121
7F-Trp-CypA
Phe60
282 nm
Trp121
Phe60
Trp121
CL-CypA
Phe60
Trp121
Phe60
Trp121
7OH-Trp-CypA
Phe60

Fluorescence decay of photoactivated 7F-Trp-CypA was not exponential. This could indicate the presence of multiple fluorophores and/or relaxation. One-dimensional data, like:

- an emission spectrum $F(\lambda_{em})$ obtained at one excitation wavelength
- an excitation spectrum $F(\lambda_{ex})$ obtained at one emission wavelength
- a nanosecond decay curve $F(t)$ obtained at one emission wavelength

are not sufficient to separate the contributions of multiple fluorophores.

We collected and analyzed two-dimensional data of two kinds:

1. $F(t, \lambda_{em})$ - Decay Associated Spectra (DAS),
2. $F(\lambda_{ex}, \lambda_{em})$ - Singular Value Decomposition of the Excitation-Emission Matrix (SVD ExEm).

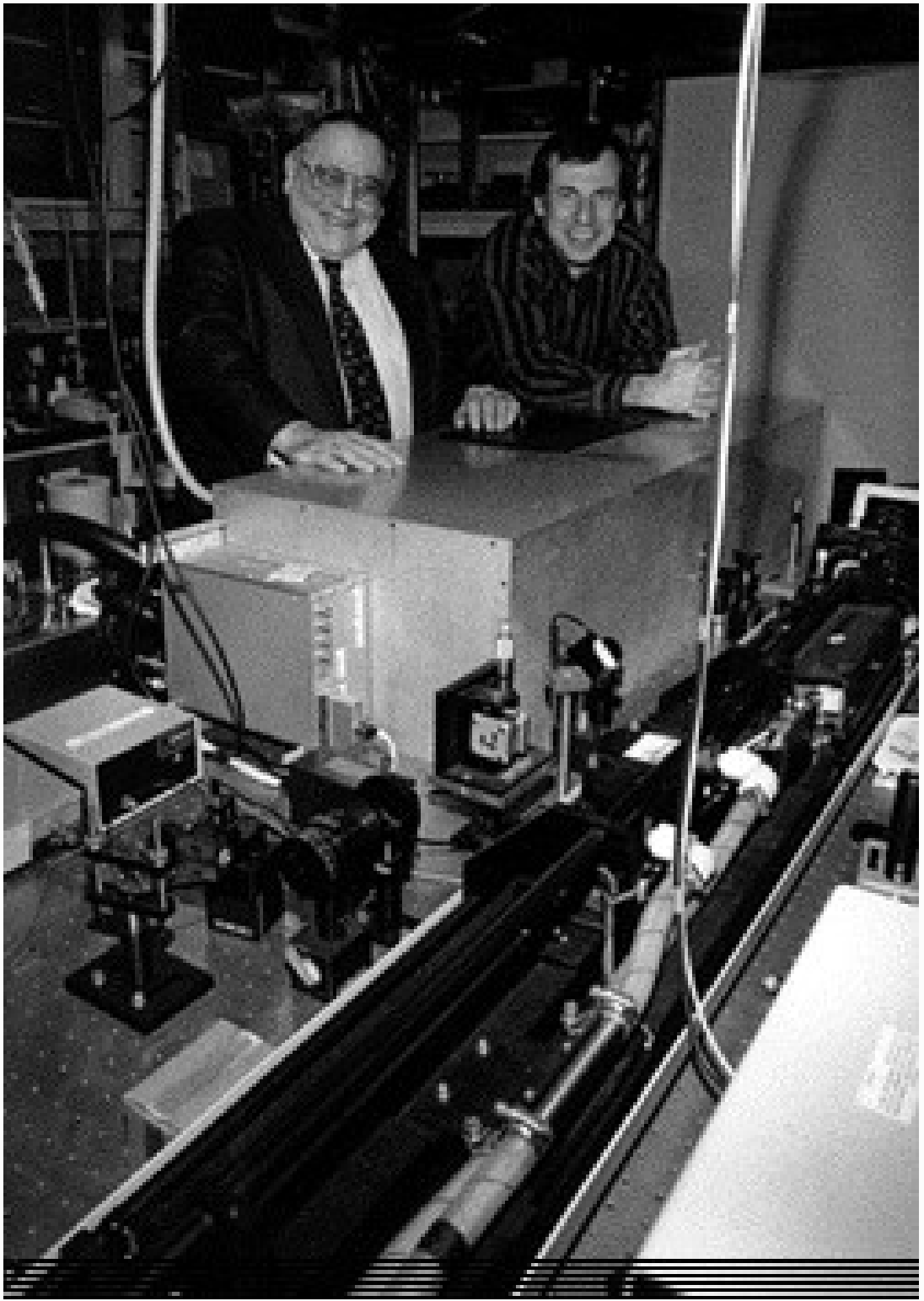
Decay Associated Spectra (DAS):

$$F(t,\lambda_{em}) = \sum_{n=1}^{N_{\exp}} \alpha_n(\lambda_{em}) \exp(-t/\tau_n) = \sum_{n=1}^{N_{\exp}} f_n(\lambda_{em}) \frac{1}{\tau_n} \exp(-t/\tau_n)$$

Singular Value Decomposition of the Excitation-Emission Matrix

$$F(\lambda_{ex},\lambda_{em}) = \sum_{n=1}^{N_{SV}} u_n(\lambda_{em})\ s_n\ v_n(\lambda_{ex})$$

$$F(\lambda_{ex},\lambda_{em}) = \sum_{n=1}^{N_{SV}} f_n(\lambda_{em})\ g_n(\lambda_{ex})$$



The instrument for collecting time-resolved fluorescence was built in the 1980's by Dr. Ludwig Brand and his colleagues.

After 1990 it was substantially improved: the productivity went up tenfold; the time resolution improved twofold.

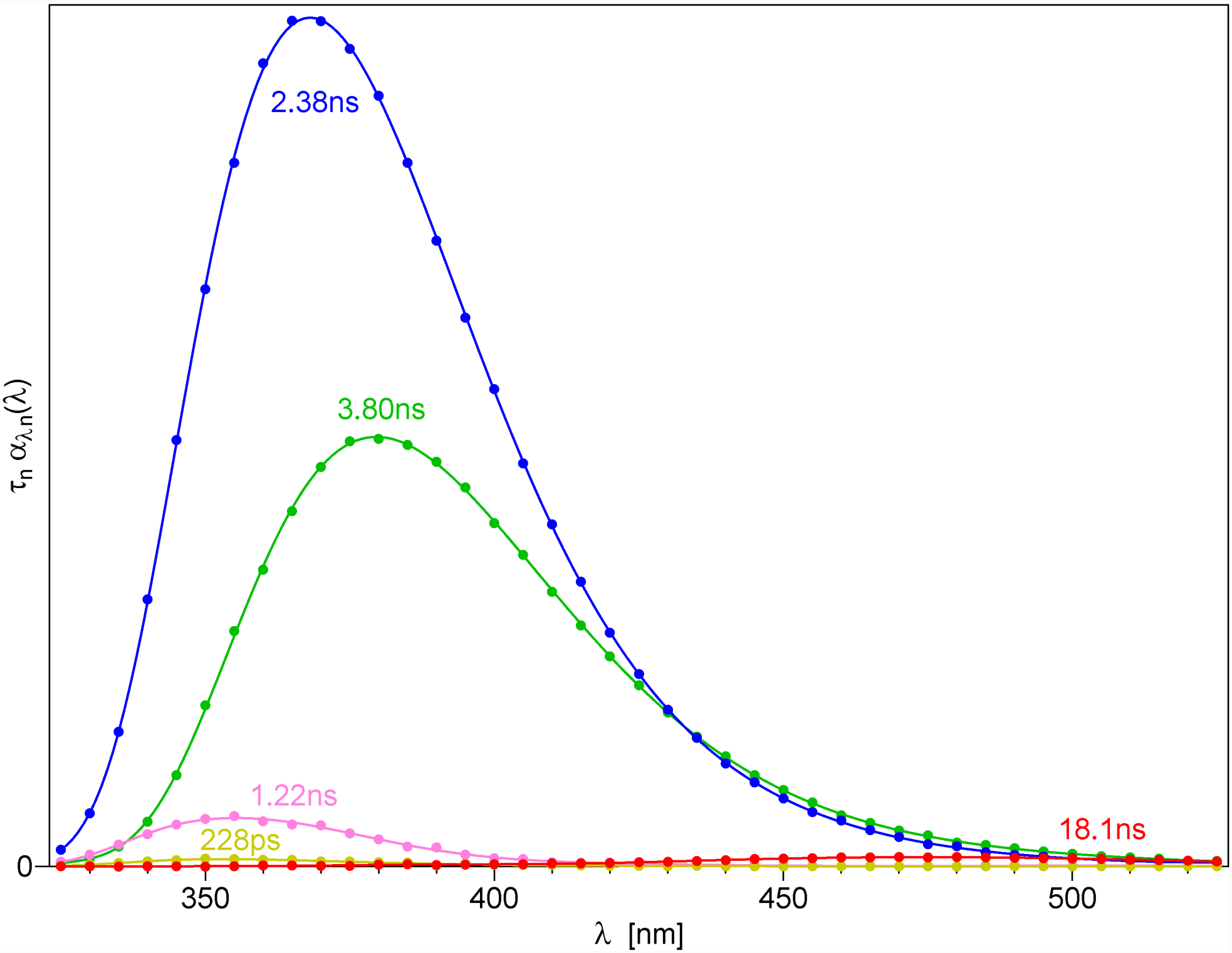
The concept of Decay Associated Spectra (DAS) was first introduced by Jay R. Knutson, Dana G. Walbridge, and Ludwig Brand:

Biochemistry **1982**, *21*, 4671–4679

Decay-Associated Fluorescence Spectra and the Heterogeneous Emission of Alcohol Dehydrogenase†

Jay R. Knutson, Dana G. Walbridge, and Ludwig Brand*

ABSTRACT: A procedure is described for using nanosecond time resolved fluorescence decay data to obtain decay-associated fluorescence spectra. It is demonstrated that the individual fluorescence spectra of two or more components in a mixture can be extracted without prior knowledge of their spectral shapes or degree of overlap. The procedure is also of value for eliminating scattered light artifacts in the fluorescence spectra of turbid samples. The method was used to separate the overlapping emission spectra of the two tryptophan residues in horse liver alcohol dehydrogenase. Formation of a ternary complex between the enzyme, NAD^+, and pyrazole leads to a decrease in the total tryptophan fluorescence. It is shown that the emission of *both* tryptophan residues decreases. The buried tryptophan (residue 314) undergoes dynamic quenching with no change in the spectral distribution. Under the same conditions, the fluorescence intensity of tryptophan (residue 15) decreases without a change in decay time but with a red shift of the emission spectrum. There is also a decrease in tryptophan fluorescence intensity when the free enzyme is acid denatured (succinate buffer, pH 4.1). The denatured enzyme retains sufficient structure to provide different microenvironments for different tryptophan residues as reflected by biexponential decay and spectrally shifted emission spectra (revealed by decay association). The value of this technique for studies of microheterogeneity in biological macromolecules is discussed.


2.38ns
3.80ns
1.22ns
228ps
18.1ns
0
350
400
450
500
λ [nm]
τn αλn(λ)

Singular Value Decomposition (SVD) of the Excitation-Emission Matrix History of the Method

- Early 1990's. Dr. Beverly Packard attached two different rhodamine dyes to a polypeptide. The excitation and emission spectra of the two dyes need to be separated. I try to separate them using Nonlinear Least Squares, with little success.

Singular Value Decomposition (SVD) of the Excitation-Emission Matrix History of the Method

- Early 1990's. Dr. Beverly Packard attached two different rhodamine dyes to a polypeptide. The excitation and emission spectra of the two dyes need to be separated. I try to separate them using Nonlinear Least Squares, with little success.
- 1992. Dr. Ludwig Brand and Dr. Michael Johnson are editors of the Methods in Ezymology vol. 210 "Numerical Computer Methods".

Methods in ENZYMOLOGY

Volume 210

Numerical Computer Methods

Edited by

Ludwig Brand

Michael L. Johnson

Singular Value Decomposition (SVD) of the Excitation-Emission Matrix History of the Method

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- 1992. Dr. Ludwig Brand and Dr. Michael Johnson are editors of the Methods in Ezymology vol. 210 "Numerical Computer Methods".
- Dr. Ludwig Brand learns about my failed attempt to separate the excitation and emission spectra of two dyes. He brings Vol. 210 to me and shows this article:

  Henry, E. R.; Hofrichter, J. Methods Enzymol. 210, 129 (1992).

[8] Singular Value Decomposition: Application to Analysis of Experimental Data

By E. R. HENRY and J. HOFRICHTER

I. Introduction

The proliferation of one- and two-dimensional array detectors and rapid scanning monochromators during the 1980s has made it relatively straightforward to characterize chemical and biochemical systems by measuring large numbers of spectra (e.g., absorption or emission spectra) as a function of various condition parameters (e.g., time, voltage, or ligand concentration). An example of such a data set is shown in Fig. 1. These data were obtained by measuring absorption difference spectra as a function of time after photodissociation of bound carbon monoxide from a modified hemoglobin. The difference spectra are calculated with respect to the CO-liganded equilibrium state. We will use this data set as an illustrative example at several points in the following discussion. As such

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- Dr. Ludwig Brand learns about my failed attempt to separate the excitation and emission spectra of two dyes. He brings Vol. 210 to me and shows this article:

  Henry, E. R.; Hofrichter, J. Methods Enzymol. 210, 129 (1992).

- I write the first program to perform SVD of the Excitation-Emission Matrix, but it does not work at all because of scattered light peaks (when the excitation and emission wavelengths are equal, scattered light intensity is enormous).

Singular Value Decomposition (SVD) of the Excitation-Emission Matrix History of the Method

- By 2002 I develop "Iterative SVD" algorithm that ignores the scattered light peaks.

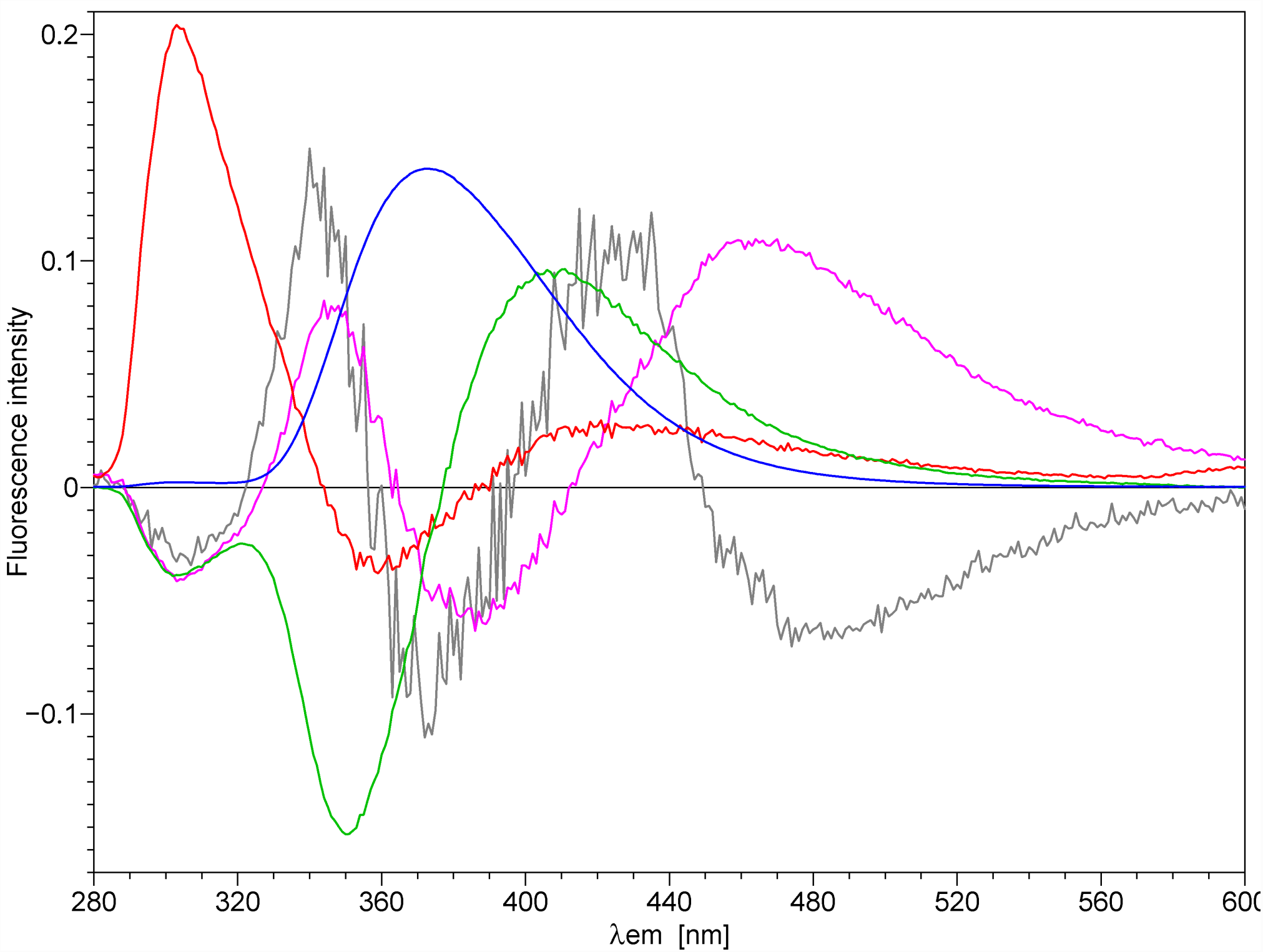
Iterative SVD algorithm. SVD of an excitation-emission matrix requires an iterative algorithm because of the presence of scattered light in the experimental spectra. Scattered excitation light passes through emission monochromator if $|\lambda_{ex}-\lambda_{em}|\leq w_{ex}+w_{em}$, where λ_{ex} and λ_{em} are the excitation and emission wavelengths, respectively; w_{ex} and w_{em} are the full widths at half maximum of the excitation and emission monochromators, respectively. The matrix elements for which the condition $|\lambda_{ex}-\lambda_{em}|<w_{ex}+w_{em}$ is satisfied are called the affected elements; those elements cannot be used in SVD, since their use would result in a very large effective rank k, defeating the purpose of SVD. In general, for a $m\times n$ data matrix $\mathbf{M}$, a corresponding $m\times n$ Boolean array is defined, the elements of which are true for the matrix elements not affected by the scattered light and false for the affected elements that should not be used in SVD. The Boolean array can be also used to exclude inelastic Raman scattered light and elastic scattered light that passes through both monochromators due to the second-order diffraction in one of them. The iterative SVD algorithm uses the Boolean array in the following way: For the zeroth approximation the algorithm uses the matrix $\mathbf{M}^{(0)}$ instead of the experimental data matrix $\mathbf{M}$. The non-affected elements in $\mathbf{M}^{(0)}$ are the

Singular Value Decomposition (SVD) of the Excitation-Emission Matrix History of the Method

- By 2002 I develop "Iterative SVD" algorithm that ignores the scattered light peaks.
- Then the next problem: how to transition from the orthogonal SVD "basis vectors" to the excitation and emission spectra of the actual fluorophores.

$$F(\lambda_{ex}, \lambda_{em}) = \sum_{n=1}^{N_{SV}} u_n(\lambda_{em})\ s_n\ v_n(\lambda_{ex})$$

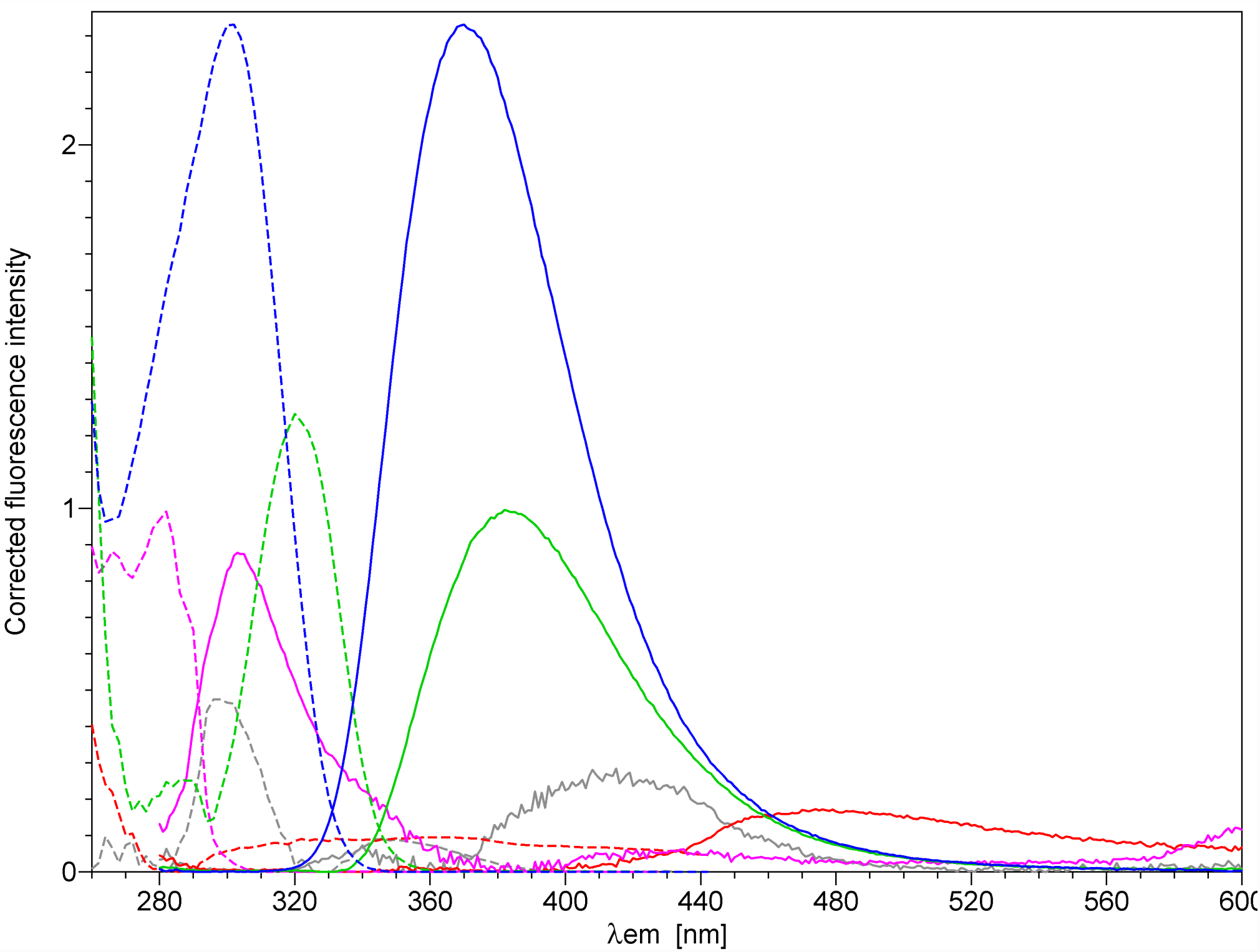
$$F(\lambda_{ex}, \lambda_{em}) = \sum_{n=1}^{N_{SV}} f_n(\lambda_{em})\ g_n(\lambda_{ex})$$

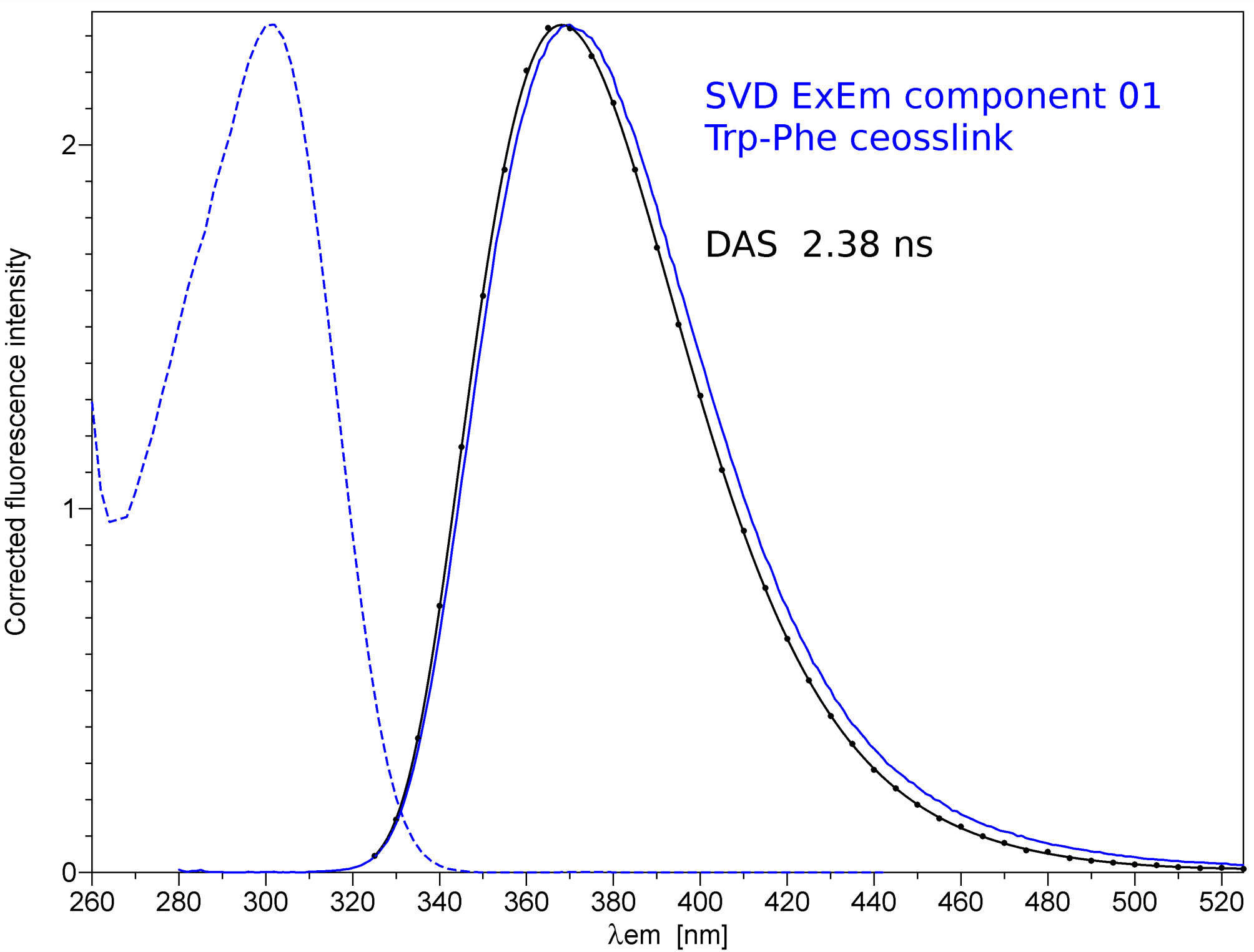

0.2
0.1
0
−0.1
Fluorescence intensity
280
320
360
400
440
480
520
560
600
λem [nm]

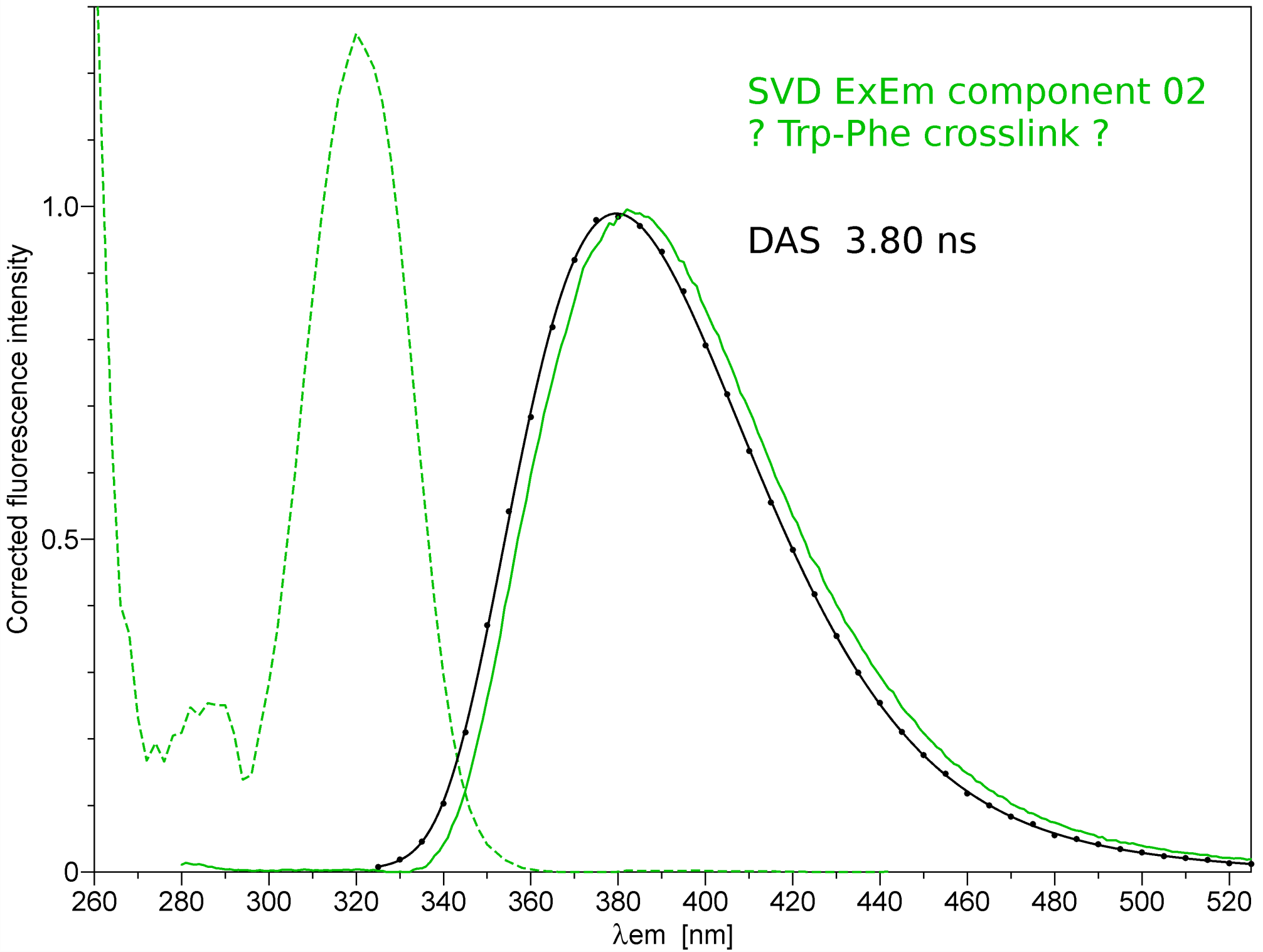
Singular Value Decomposition (SVD) of the Excitation-Emission Matrix History of the Method

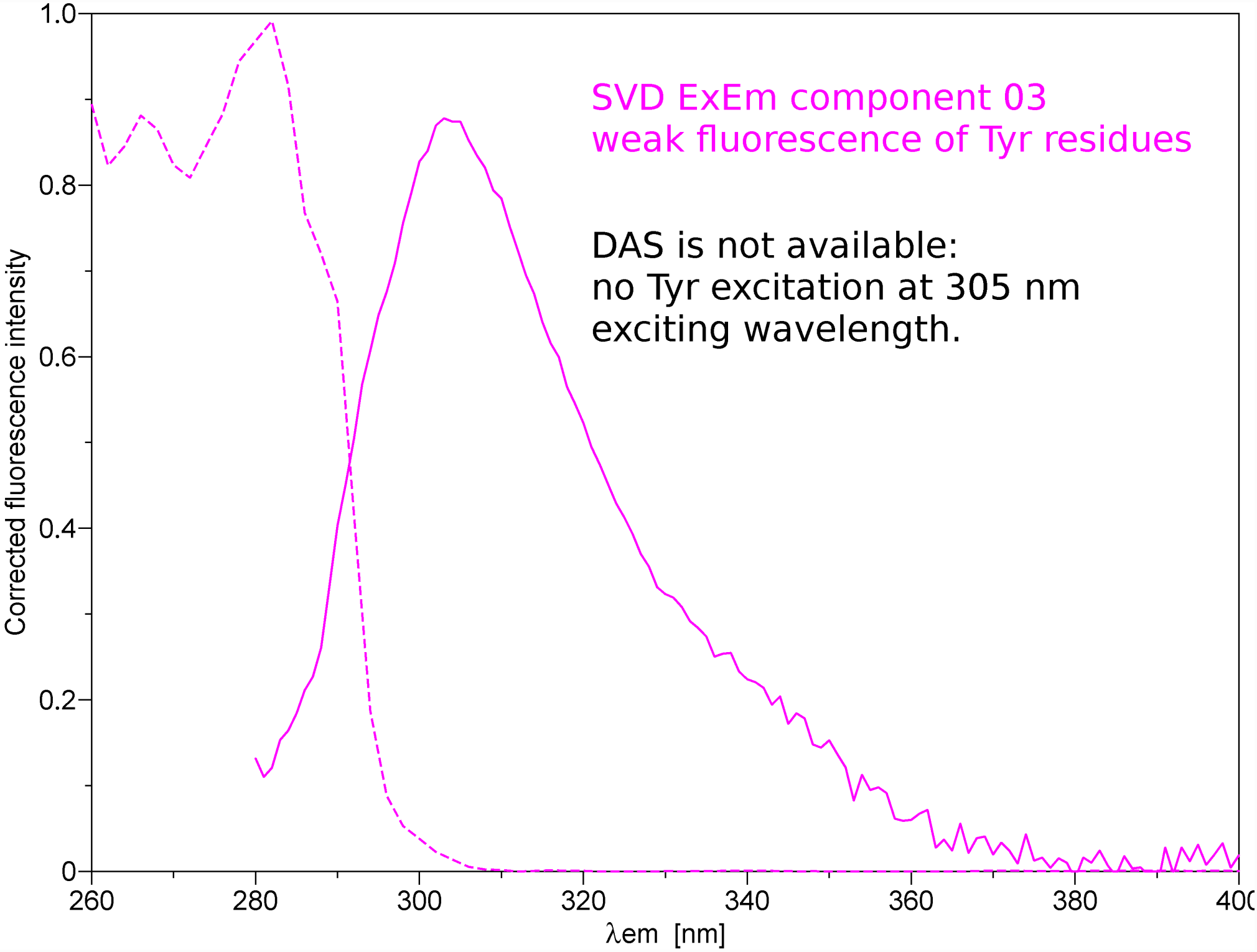
- By 2002 I develop "Iterative SVD" algorithm that ignores the scattered light peaks.
- Then the next problem: how to transition from the orthogonal SVD "basis vectors" to the excitation and emission spectra of the actual fluorophores.
- By 2004 I develop a transition algorithm that requires some *a priori* knowledge, e.g., a fluorophore does not absorb at wavelengths longer than λ_{red_edge} .
- In 2021 I develop a method that does not require *a priori* knowledge except for the fact that fluorescence intensity cannot be negative.

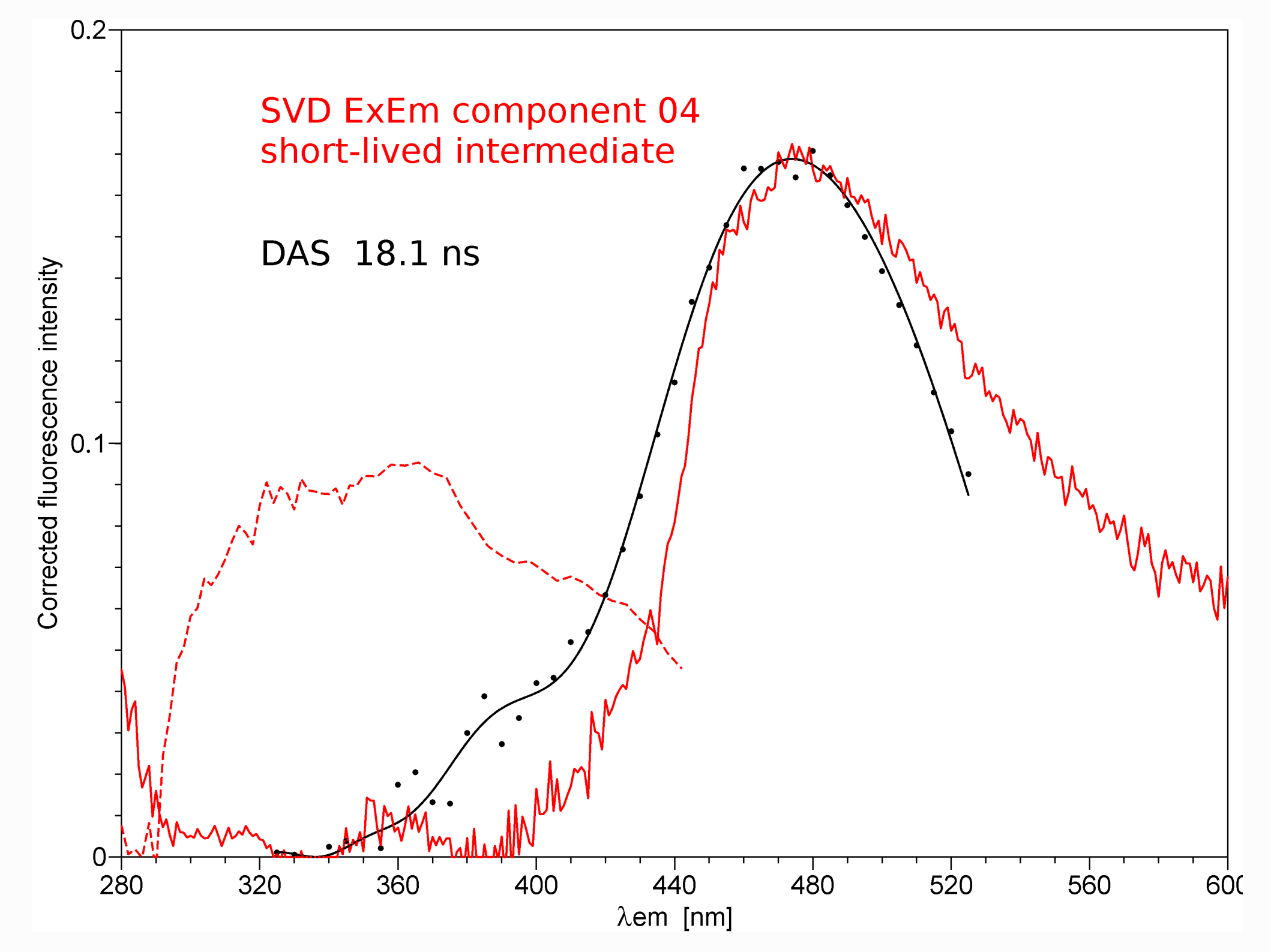
Transition from SVD basis vectors to excitation and emission spectra. Vibrational relaxation of fluorophores at room temperature takes picoseconds. The lifetimes of the singlet electronic excited states responsible for fluorescence are in the nanosecond range. This demonstrates that practically all fluorescence emission originates from the relaxed excited state. Therefore, for a single fluorophore, the fluorescence emission spectrum is independent of the excitation wavelength, and the fluorescence excitation spectrum is independent of the emission wavelength. In accordance with this physical model, a reconstructed excitation-emission matrix $\mathbf{M}_k$ of reduced rank k can be represented as a product $\mathbf{FG}$, where the columns of an $m \times q$ matrix $\mathbf{F}$ represent fluorescence emission spectra of q fluorophores, and the rows of an $q \times n$ matrix $\mathbf{G}$ represent fluorescence excitation spectra of the same q fluorophores. Note, that if $q<k$, then $\mathbf{M}_k$ cannot be equal to $\mathbf{FG}$, because matrix $\mathbf{M}_k$ is of rank k, while the rank of the product $\mathbf{FG}$ cannot exceed q. On the other hand, if $q>k$, then the matrix equation $\mathbf{M}_k=\mathbf{FG}$ will always have an infinite number of solutions, rendering it of little practical use. Therefore, one has to postulate that the number of detectable fluorophores is equal to the effective rank of the excitation-emission matrix,


Corrected fluorescence intensity
0
1
2
280
320
360
400
440
480
520
560
600
λem [nm]


SVD ExEm component 01
Trp-Phe ceosslink
DAS 2.38 ns
Corrected fluorescence intensity
0
1
2
260
280
300
320
340
360
380
400
420
440
460
480
500
520
λem [nm]


SVD ExEm component 02
? Trp-Phe crosslink ?
DAS 3.80 ns
Corrected fluorescence intensity
1.0
0.5
0
260
280
300
320
340
360
380
400
420
440
460
480
500
520
λem [nm]


SVD ExEm component 03
weak fluorescence of Tyr residues
DAS is not available:
no Tyr excitation at 305 nm
exciting wavelength.
Corrected fluorescence intensity
1.0
0.8
0.6
0.4
0.2
0
260
280
300
320
340
360
380
400
λem [nm]


SVD ExEm component 04
short-lived intermediate
DAS 18.1 ns
Corrected fluorescence intensity
0.2
0.1
0
280
320
360
400
440
480
520
560
600
λem [nm]

CONCLUSIONS

- Both DAS and SVD of ExEm matrix can separate the contributions of fluorophores in a mixture.
- DAS produces more reliable results, but it requires expensive picosecond instrumentation.
- SVD of ExEm matrix requires only steady-state equipment, but results are less reliable.
- DAS was invented by Dr. Brand & colleagues.
- SVD of ExEm matrix was inspired by Dr. Brand.

I deeply appreciate everything Lenny did for me and my family.

I will always miss him.