The 8th LFD workshop on Advanced Fluorescence Imaging and Dynamics
October 21-25, 2013

A Nano-History of Fluorescence
(with some comments on the contributions of Gregorio Weber)

David Jameson
The discovery and characterization of Fluorescence

Quiz: When was the first report on “fluorescence”?

Contrary to popular belief it was not from Enrico’s kitchen!
Nor even the Old West!
The discovery and characterization of Fluorescence

Quiz: When was the first report on “fluorescence”?

Nicolás Monardes (1577), a Spanish physician and botanist who wrote on medicines of the New World, is usually credited as being the first to describe the bluish opalescence of the water infusion from the wood of a small Mexican tree. When made into cups and filled with water, a peculiar blue tinge was observed.

Actually, Bernardino de Sahagún, a Franciscan missionary, independently described the wood – called “coatli” by the Aztecs.

I am indebted to Ulises Acuna for this picture and for information about these early studies.

Coatli .....patli, yoan aqujxtiloni, matlatic iniayo axixpatli.. “it is a medicine, and makes the water of blue color, its juice is medicinal for the urine”

(7) Sahagún, B. Matritensis Codex; Spanish Royal Academy of History, ca. 1560–1564, f203v.
An early Latin translation (1574) by the influential Flemish botanist Charles de L’Écluse (1526-1609), in which the wood’s name is given as Lignum Nephriticum (kidney wood), helped to extend awareness of its strange optical properties in Europe. This wood was very popular in XVI - XVII Europe, because of its medicinal virtues for treating kidney ailments.

An Englishman, John Frampton, translated Mondares description as “.. white woodde which gives a blewe color” when placed in water that was good “for them that doeth not pisse liberally and for the pains of the Raines of the stone..”
The German Jesuit priest Athanasius Kircher, among his numerous achievements, wrote a book in 1646 called:

_Ars Magna Lucis et Umbrae_  
(The Great Art of Light and Shadow)

In this book he described his observation of the wood extract _Lignum nephriticum_. Light passing through an aqueous infusion of this wood appeared more yellow while light reflected from the solution appeared blue.
Robert Boyle (1670) was inspired by Monardes’ report and investigated this system more fully. He discovered that after many infusions the wood lost its power to give color to the water and concluded that there was some “essential salt” in the wood responsible for the effect. He also discovered that addition of acid abolished the color and that addition of alkali brought it back.

I thank Katherine Reinhart from Johns Hopkins Univ. for providing a copy of the original 1670 Boyle manuscript.

Hence Boyle was the first to use fluorescence as a pH indicator!
In the ensuing centuries the wood was no longer used and the botanic identity of the LN was lost in a confusion of several species. Safford, in 1915, succeeded in disentangling the botanic problem and identified the species which produced the Mexican LN as *Eynsenhardtia polystachia*. More recently, several highly fluorescent glucosyl-hydroxichalcones were isolated from this plant.
The identification of the fluorescing molecule from *Lignum Nephriticum* was finally made in 2009!

**Structure and Formation of the Fluorescent Compound of *Lignum nepheriticum***

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Received May 11, 2009

**ABSTRACT**

The intense blue fluorescence of the infusion of *Lignum nepheriticum* (Erythrina pseudolatifolia), first observed in the sixteenth century, is due to a novel four-ring tetrahydrobenzofuran-2,3-dione which is not present in the plant but is the end product of an unusual, very efficient iterative spontaneous oxidation of at least one of its tris’s flavonoids.

**Figure 3.** Spectral properties of matlaline (2) in water solution. Absorption (red) and corrected fluorescence (blue) spectra, emission yield ($\Phi_\text{F}$), and lifetime ($\tau_\text{F}$) of the dianion form at pH 9; absorption spectrum of the nonemitting form (black) at pH 4.

**Scheme 1.** Plausible Sequential Reaction That Transforms Coatline B (1) into Matlaline (2) at Room Temperature in Water Solution
Galileo Galilei (1612) described the emission of light (phosphorescence) from the famous Bolognian stone, discovered in 1603 by Vincenzo Casciarolo, a Bolognian shoemaker. Galileo wrote: "It must be explained how it happens that the light is conceived into the stone, and is given back after some time, as in childbirth."
Recently, Enrico Gratton pointed out to me an even greater discovery from Bologna

But that’s another story
David Brewster (1833) described that when a beam of white light passed through an alcohol solution of leaves a red beam could be observed from the side (which was of course chlorophyll fluorescence).

Chlorophyll fluorescence is now very important in studies on plant health and photosynthesis and portable fluorescence instruments are routinely taken out into the field.
David Brewster (1833) described that when a beam of white light passed through an alcohol solution of leaves a red beam could be observed from the side (which was of course chlorophyll fluorescence).

John Herschel (1845) made the first observation of fluorescence from quinine sulfate - he termed this phenomenon “epipolic dispersion”.


Received January 28, 1845,—Read February 13, 1845.

an extremely vivid and beautiful celestial blue colour.
Enrico was so impressed by this “beautiful celestial blue color” that he recently renovated his living room to allow him to appreciate it on a daily basis.
George Gabriel Stokes (1852) published his massive treatise “On the Change of Refrangibility of Light” – more than 100 pages.

In this work he initially using the term “dispersive reflection” to describe the phenomenon presented by quinine sulphate.

Fortunately for all of us today, however, he then wrote:

* I confess I do not like this term. I am almost inclined to coin a word, and call the appearance *fluorescence*, from fluor-spar, as the analogous term *opalescence* is derived from the name of a mineral.
Stokes used a prism to disperse the solar spectrum and illuminate a solution of quinine. He noted that there was no effect until the solution was placed in the ultraviolet region of the spectrum.

He wrote:

*It was certainly a curious sight to see the tube instantaneously lighted up when plunged into the invisible rays: it was literally *darkness visible*. Altogether the phenomenon had something of an unearthly appearance.*

This observations led Stokes to proclaim that fluorescence is of longer wavelength than the exciting light, which led to this displacement being called the **Stokes Shift**.
In 1856, at the age of 18, William Henry Perkin set out with the idea of making quinine by oxidizing allytoluidine – instead, he accidentally produced the synthetic dye, mauve, a derivative of coal tar with an aniline base.

Fortunately for him Queen Victoria loved it! Not long afterward Perkin produced a green and a violet, and soon the canal outside his factory was turning a different color every week.

Although others – including Friedlieb Runge and Robert Rumney – had synthesized synthetic dyes, Perkins was the first to recognize the potential for commercialization and really started the synthetic dye industry.

Histologists started using the dyes to stain samples within a decade of Perkin’s discovery.
Adolph Von Baeyer (1871) a German chemist, synthesized Spiro[isobenzofuran-1(3H),9'-[9H]xanthen]-3-one, 3',6'-dihydroxy.

**FLUORESCEIN!!!**

**Adolf Baeyer: Ueber eine neue Klasse von Farbstoffen.**
(Vorgetr. vom Verf.)


He apparently coined the name “fluorescein”, from “fluo” and “rescein,” (resorcinol) which he reacted with phthalic anhydride.

In 1905 he was awarded the Nobel Prize in Chemistry "in recognition of his services in the advancement of organic chemistry and the chemical industry, through his work on organic dyes and hydroaromatic compounds".
Adolph Von Beyer (1871) a German chemist, synthesized Spiro[isobenzofuran-1(3H),9'-[9H]xanthen]-3-one, 3',6'-dihydroxy.

**FLUORESCEIN!!!**

One of the first uses of fluorescein was in 1877 in a major ground-water tracing experiment in southern Germany.

The results of this experiment showed that the River Danube and Rhine are connected by underground streams. Fluorescein was placed in the Danube and about 60 hours later it appeared in an affluent of the Rhine.

10 Kilograms of fluorescein were used!

Enrico and I actually submitted a grant proposal to repeat this great experiment using Alexa 488... To our disappointment the funding agency balked at the cost of 10 kgm of Alexa 488 which was a mere $2,210,000,000
Adolph Von Beyer (1871) a German chemist, synthesized Spiro[isobenzofuran-1(3H),9'-[9H]xanthen]-3-one, 3',6'-dihydroxy.

FLUORESCIN!!!
Every year on St. Patrick’s Day, the Chicago river is dyed green with about 40 pounds of fluorescein.
The opposite of a single molecule experiment!
Adolph Von Beyer (1871) a German chemist, synthesized Spiro[isobenzofuran-1(3H),9'-[9H]xanthen]-3-one, 3',6'-dihydroxy.

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Paul Ehrlich (1882) used uranin (the sodium salt of fluorescein) to track secretion of the aqueous humor in the eye. First *in vivo* use of fluorescence.
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R. Meyer (1897) used the term “fluorophore” to describe chemical groups which tended to be associated with fluorescence; this word was analogous to “chromophore” which was first used in 1876 by O.N. Witt to describe groups associated with color.

Otto Heimstaedt and Heinrich Lehmann (1911-1913) developed the first fluorescence microscopes as an outgrowth of the UV microscope (1901-1904). The instrument was used to investigate the autofluorescence of bacteria, protozoa, plant and animal tissues, and bioorganic substances such as albumin, elastin, and keratin.
Stanislav Von Prowazek (1914) employed the fluorescence microscope to study dye binding to living cells.


Albert Coons (1941) labeled antibodies with Fluorescein Isocyanate, thus giving birth to the field of immunofluorescence.


Riggs et al. first reported FITC, in 1958, which they synthesized to circumvent problems inherent in the isocyanate derivative, including the difficulty of its synthesis and its instability.

Gregorio Weber (1952) synthesized dansyl chloride for attachment to proteins and used polarization to study protein hydrodynamics - these studies initiated the field of quantitative biological fluorescence.

Shimomura, Johnson and Saiga (1962) discovered Green Fluorescent Protein in the Aequorea victoria jellyfish

Osamu Shimomura in the lab in the basement of his home. He is holding a sample of GFP isolated from Aequorea victoria, not produced by bacteria.
“The jellyfish *Aequorea* and its light-emitting organs”
The outer ring of the jellyfish had to be isolated. Initially scissors were used but then a “ring-cutting” machine was built.
Figure 1. Top left: a male *U. vocans* from the Atlantic Forest under white light. Top right: the same bee pictured on the left under UV light. Bottom left: a male *U. anabrytes* from the Amazon Basin under white light. Bottom right: the same bee pictured on the left under UV light.

Muscle cell expression

Japanese eel

Molecular cloning

apoUnaG

Bilirubin

holoUnaG

New clinical assay for billirubin

Heterologous expression
Polarizers have been in use for a very long time - the Vikings used a “sunstone” (thought to have been composed either of the mineral cordierite or iceland spar – calcite – both of which are naturally polarizing materials) to observe the location of the sun on foggy or overcast days. Since scattered sunlight is highly polarized compared to light coming along the direction to the sun, the distribution of the sky’s brightness could be observed through the sunstone and hence the sun’s position could be localized and, if the time of day were known, the compass directions.
In 1808, Malus observed sunlight reflected from the windows of the Luxemburg Palace in Paris through an Iceland spar (Calcite) crystal that he rotated.

(Erasmus Bartholin (1625-1698) discovered the double refraction of light by Iceland spar in 1669)

Malus discovered that the intensity of the reflected light varied as he rotated the crystal and coined the term “polarized” to describe this property of light.


Malus also derived an expression for calculating the transmission of light as a function of the angle (θ) between two polarizers. This equation (Malus’ Law) is now written as: $I_\theta = I_0 (\cos^2 \theta)$
Sir David Brewster (1781-1868)

David Brewster studied the relationship between refractive index and angle of incidence on the polarization of the reflected light.

III. On the law of the partial polarization of light by reflexion. By David Brewster, LL.D. F.R.S. L. & E.

Read February 4, 1830.

He discovered that for normal glass and visible light, an incidence angle of \( \sim 56 \) degrees resulted in total reflection of one plane of polarization – this angle is now known as Brewster’s Angle.

\[
\theta_B = \tan^{-1} \left( \frac{n_2}{n_1} \right)
\]

This discovery allowed Brewster to construct a polarizer composed of a “pile of plates”:

- a. Linear stacked array polarizer
- b. Tent polarizer
William Nicol (1770-1851)

In 1828, Nicol joined two crystals of Iceland spar, cut at an angle of 68°, using Canada balsam.

Other important calcite polarizers developed around this time include: Glan-Foucault; Glan-Thompson; Glan-Taylor; Wollaston; Rochon

But the Henry Ford of polarizers was.....

Edwin Herbert Land (1909-1991)

In 1929 Edwin Land patented the sheet polarizer (the J-sheet), consisting of crystals of iodoquinine sulfate embedded in nitrocellulose film followed by alignment of the crystals by stretching which led to dichroism. In 1938 he invented the H-sheet which was comprised of polyvinyl alcohol sheets with embedded iodine.
The Time Interval between Absorption and Emission of Light in Fluorescence.


(Received June 12, 1921.)

Some experiments were then made at the University of Wisconsin, in collaboration with Prof. C. E. Mendenhall, during my visit to Madison in December. We used a high pressure, six-cylinder pump, and obtained a jet velocity of about 200 metres per second, with a fine glass nozzle about 0.2 mm. in diameter. More recently, Prof. Mendenhall has increased the velocity to 230 metres per second, and, by blackening one side of the jet tube, leaving a small clear space for the entrance of the sunlight, has assured himself that there is no displacement as great as 0.1 mm. (observing the fluorescent patch with a short-focus lens). This means that the duration of the fluorescence is less than 1/2,300,000 second.
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i.e. < 435ns

anthracene
Ein Fluorometer.
Apparat zur Messung von Fluoreszenzabklingungszeiten.

Von E. Gaviola in Berlin.

Mit 9 Abbildungen. (Eingegangen am 24. März 1927.)

Fig. 1. Original apparatus of Gaviola\(^1\) for the measurement of fluorescence lifetimes, described in text. \(B\), Source of exciting light; \(T\), cuvette containing the fluorescent solution; \(S\), mirror.
Ramón Enrique Gaviola was born in the city of Mendoza on August 31, 1900. In 1917, he was a student in La Plata University, when his professor, Richard Gans, advised him that if he really wanted to ‘learn physics’ he had to do it in Germany. Following the suggestion, Gaviola studied physics in the Georg August Universität, Göttingen, from 1922 to 1923, and in the Friedrich Wilhelms Universität, Berlín, from 1923 to 1926. The list of his professors is impressive: James Franck, David Hillbert, Richard Courant, Max Born, Richard Pohl, Hans Reichenbach, Max Plank, Max von Laue, Edler von Mises, Peter Pringsheim, Wolfgang Köhler, Albert Einstein, Walter Nernst and Lise Meitner. His Ph.D. thesis (1926) was co-directed by Walter Nernst and Max von Laue.

The eight papers (five of them before his graduation) on fluorescence and polarisation published by Gaviola in Zeitschrift für Physik and in Annalen der Physik are the basis of the scientific field that has relevance in today’s biology and biochemistry: Fluorescence Spectrometry. Gaviola constructed the first-phase—fluorometer in the 1920s and measured with great precision the lifetime of the excited state of fluoresceine.
<table>
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<tr>
<th>Farbstoff</th>
<th>Abklingungszeiten</th>
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<tr>
<td></td>
<td>in Wasser</td>
</tr>
<tr>
<td></td>
<td>Sekunden</td>
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<tr>
<td>Uranin</td>
<td>4,5 \cdot 10^{-9}</td>
</tr>
<tr>
<td>Fluorescein</td>
<td>—</td>
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<tr>
<td>Rhodamin B</td>
<td>2,0 \cdot 10^{-9}</td>
</tr>
<tr>
<td>Rhodulin Orange</td>
<td>2,7</td>
</tr>
<tr>
<td>Erythrosin</td>
<td>1,8</td>
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<tr>
<td>Tetrajodfluor. Na</td>
<td>1,0</td>
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<tr>
<td>Eosin 5 B</td>
<td>1,9</td>
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<tr>
<td>Uranylsulfat</td>
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<td>Uranylsulfat in Schwefelsäure</td>
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<td>Chinisarin in Pentan</td>
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<td>Uranglas</td>
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<td>Rubinkristall</td>
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The instrument was capable of dissecting the image into areas of interest, and can therefore be classified as an imaging fluorescence lifetime instrument. Lifetime measurements were carried out on "fluorphores bound to the nuclei of tumor cells, as well as autofluorescence of biological tissue samples."

\[ \tan \Delta \phi = \omega \tau. \]
Milestones in the Theory of Resonance Energy Transfer

1922 G. Cario and J. Franck demonstrate that excitation of a mixture of mercury and thallium atomic vapors with 254nm (the mercury resonance line) also displayed thallium (sensitized) emission at 535nm.

1924 E. Gaviola and P. Pringsham observed that an increase in the concentration of fluorescein in viscous solvent was accompanied by a progressive depolarization of the emission.
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1925  J. Perrin proposed the mechanism of resonance energy transfer

1928  H. Kallmann and F. London developed the quantum theory of resonance energy transfer between various atoms in the gas phase. The dipole-dipole interaction and the parameter $R_0$ are used for the first time

1932  F. Perrin published a quantum mechanical theory of energy transfer between molecules of the same specie in solution. Qualitative discussion of the effect of the spectral overlap between the emission spectrum of the donor and the absorption spectrum of the acceptor

1946-1949  T. Förster develop the first complete quantitative theory of molecular resonance energy transfer
From Förster’s famous book “Fluoreszenz Organischer Verbindungen”; which everyone references, but few read,

setzt, wobei \( n_{AB} \) die Häufigkeit des zwischenmolekularen Energieübergangs ist. Sie ist gleich der Zahl der Übergänge in der Zeiteinheit unter der Annahme, daß nach jedem Übergang stets wieder der ursprüngliche Zustand hergestellt wird. Aus Gl. (13.5) erhält man so, indem man gleichzeitig von der bisher benutzten Kreisfrequenz \( \omega \) zur Frequenz \( \nu \) selbst übergeht:

\[
(13.6) \quad n_{AB} = \pi^2 e^4 \frac{\kappa^2}{16 \pi^3 n^2 m^2 \hbar^2} \int_0^\infty f_n^{(A)}(v) \cdot f_n^{(B)}(v) \frac{dv}{v^2}.
\]

Statt der Oszillatorenstärkenfunktionen \( f_n^{(A)}(v) \) und \( f_n^{(B)}(v) \) führt man hier zweckmäßig die leichter zugänglichen Größen \( f_n^{(A)}(v) \) (Quantispektrum des energieabgebenden Moleküls \( A \)) und \( \epsilon_n^{(B)}(v) \) (molarer dekadischer Extinktionskoeffizient des energiaufnehmenden Moleküls \( B \)) nach Gln. (12.46) und (12.43) ein. Man erhält so für die Übergangshäufigkeit

\[
(13.7) \quad n_{AB} = \frac{\kappa^2}{128 \pi^3 n^2 N^* \hbar^2} \int_0^\infty f_n^{(A)}(v) \cdot \epsilon_n^{(B)}(v) \frac{dv}{v^2}.
\]

Diese Beziehung ist auch auf exakter quantenmechanischer Grundlage zu gewinnen [Förster (1947a)].

Die dimensionslose Konstante \( \kappa \) ist nach Gl. (13.1) durch die Feldstärkekomponente des Oszillators \( A \) am Ort und in der Richtung des Oszillators \( B \) bestimmt. Sie hängt daher von der Orientierung beider Oszillatoren zueinander und zu deren Verbindungsline ab. Seien \( \varphi_A \) und \( \varphi_B \) die Winkel zwischen den einzelnen Oszillationsrichtungen und dieser Verbindungslinie und \( \varphi_{AB} \) der Winkel zwischen den Oszillationseintrachtung, so ist nach einer elementaren Formel für die Wechselwirkungsenergie zweier Dipole:

\[
(13.8) \quad \kappa = \cos \varphi_{AB} - 3 \cos \varphi_A \cdot \cos \varphi_B.
\]

Wegen der Rotationsbewegung der Moleküle wechseln sämtliche Richtungen in den meisten Fällen so rasch, daß statt der Momentanwerte von \( \kappa^2 \) dessen statistischer Mittelwert über sämtliche Orientierungen einzusetzen ist. Dieser ergibt sich am einfachsten durch Mittelbildung über die vier in Fig. 16 angegebenen Hauptlagen unter Berücksichtigung deren statistischer Gewichte zu \( \kappa^2 = 2/3 \). Die exakte Berechnung durch Integration über sämtliche Richtungen liefert den gleichen Wert.
The 1st Theodor Förster International Lecture series at the University of Cambridge was inaugurated at the Li Ka Shing Centre for Cancer Research, on the 12th of November 2007 by Professor Enrico Gratton
Instrumentation

During World War II, the United States government issued a desperate call to scientists and doctors: find a treatment for malaria! Since Japan had taken over most of the world's supply of quinine—the best known treatment—Allied forces in the Pacific Theater needed a new drug, and fast.

With an instrument they called a fluorometer, Brodie and Udenfriend could measure how much of the drug was in a patient's plasma sample.
The earliest commercial instruments were essentially attachments for spectrophotometers such as the Beckman DU spectrophotometer; this attachment allowed the emitted light (excited by the mercury vapor source through a filter) to be reflected into the spectrophotometer’s monochromator. The first description of this type of apparatus was by R.A. Burdett and L.C. Jones in 1947 (J. Opt. Soc. Amer. 37:554).

**Fig. 20.** Attachment for measuring fluorescence spectra with the Beckman Model DU and DK spectrophotometers.
The first commercial spectrofluorimeters with monochromators for both excitation and emission were inspired by the work of Bowman at the NIH and were produced by Aminco-Bowman and Farrand. These early instruments allowed biologists to use fluorescence to develop clinically relevant assays for a wide variety of biological molecules.
1956

Cost: over $8,000
– which is about $100,000 in 2013 dollars
Fluorescence in the 20th Century

Most of the basic principles of fluorescence were developed during the 1920's and 1930's.

- Excited state lifetime (Gaviola)
- Quantum yield (Wavilov)
- Polarization of fluorescence (Weigert, F. Perrin)
- Fluorescence resonance energy transfer (J. and F. Perrin; T. Förster)

Until the second half of the 20th century, however, the use of fluorescence in biology and biochemistry was, descriptive in nature and primarily limited to a role in the isolation, purification and quantification of fluorescent substances such as riboflavin and porphyrins. True “quantitative” biological fluorescence began with the pioneering work of Gregorio Weber.
During the last few decades, fluorescence spectroscopy has evolved from a narrow, highly specialized technique into an important discipline widely utilized in the biological, chemical and physical sciences.

As in all scientific disciplines, the development of modern fluorescence spectroscopy has benefited from the contributions of many individuals from many countries.

However, one individual, Gregorio Weber, can be singled out for his outstanding and far-reaching contributions to this field.
Biographical Sketch of Gregorio Weber

1916  Born in Buenos Aires, Argentina (July 4)

1943  M.D. degree from the University of Buenos Aires (teaching assistant with Bernardo Houssay)

1943/47  Attended Cambridge University supported by a British Council Fellowship. Thesis Advisor - Malcolm Dixon

1947  Awarded Ph.D. - Thesis title "Fluorescence of Riboflavin, Diaphorase and Related Substances"

1948/52  Independent investigator at the Sir William Dunn Laboratory for Clinical Research, Cambridge University

1953  Joined Biochemistry Department of Sheffield University

1962  Joined the Biochemistry Division of the Chemistry Department at the University of Illinois at Urbana-Champaign

Bernardo Houssay was awarded the 1947 Nobel Prize in Physiology and Medicine for his discovery of the role of pituitary hormones in the regulation of glucose in the blood. He was also the first Argentine and Latin American to be awarded with a Nobel Prize in some field of the Sciences.
At the University of Buenos Aires, Bernardo Houssay suggested that his young protégé apply for a prestigious British Council Fellowship to support Ph.D. studies at Cambridge University, UK.

Travel to England in 1943 was an adventure - Weber’s voyage took 44 days in a convoy.
At Cambridge, Weber entered St. John's College where he met Malcolm Dixon, the well-known enzymologist, and talked with him about applying techniques of Physical Chemistry to the study of proteins.

At that time, Dixon was already acknowledged as one of the world’s preeminent physical biochemists and the leading authority on enzymes. He had recorded the first absorption spectrum of cytochrome c.
Gregorio Weber was thus given the task of “sorting out” this area.

At that time, Weber knew little about fluorescence but soon learned that there were a number of low molecular weight flavin compounds, such as riboflavin and FAD, that differed greatly in fluorescence intensity. A few flavoproteins had been purified but only one of them showed fluorescence comparable to the free prosthetic group.

Dixon suggested that Weber consider applying fluorescence techniques to the study of the naturally fluorescent flavin and flavoprotein systems.

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Cambridge

The Chapel Tower -St. John’s College

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Microviscosity
versus
Macroviscosity

The final chapter of Gregorio Weber’s thesis is devoted to the application of polarization measurements to determine the viscosity of gels
the low microscopic viscosity necessary for the rapid diffusion of metabolites within it.

**Note on the determination of protoplasm viscosity.**

The polarization method appears very convenient for the determination of the viscosity of protoplasm.

Both the microscopic and macroscopic viscosity of the protoplasm are of importance; the first in relation to diffusion processes and the second in relation to the organization of the cell. The fluorescent method would allow the determination of the microscopic viscosity only.
This prescient observation anticipated the work he would publish 24 years later which first delineated the application of fluorescence probes to studies of the physical state of lipid systems.
Microviscosity and Order in the Hydrocarbon Region of Micelles and Membranes Determined with Fluorescent Probes. I. Synthetic Micelles

M. Shinitzky,† A.-C. Dianoux,‡ C. Gitler,§ and G. Weber||

ABSTRACT: The viscosity in micelle interiors, termed here as microviscosity, was derived from an adequate determination of the degree of fluorescence depolarization of perylene or 2-methylnaphthalene when dissolved in the tested micelles and in American white oil U. S. P. 35. The latter was used as a reference system of known viscosities. In the series studied, lauryltrimethylammonium bromide, myristyltrimethylammonium bromide, cetyltrimethylammonium bromide (CTABr), and stearyltrimethylbenzylammonium bromide, the determined microviscosities at 27° were all in the range of 17-50 cP. The change in microviscosity with temperature in this series was found to follow a simple exponential form with an activation energy in the range of 6.1-9.6 kcal mole⁻¹. Added salts affected only slightly the microviscosity values. Mixed micelles of perylene-labeled CTABr with cetyl alcohol or cholesterol and with sodium 1-hexadecanesulfonate, were used to test the effect of charge isolation and charge neutralization on the fluidity of the micelle interior. The microviscosity of these mixed micelles was found to increase rapidly with concentration of the admixed component, and at a molar ratio close to 1:1 microviscosities of several poises were obtained. The changes in apparent rotational diffusion with wavelength of excitation indicate that the depolarizing rotations are strongly anisotropic. In-plane rotations in perylene are ten times faster than out-of-plane rotations, independently of the medium (micelles, propylene glycol at -14°C, propylene glycol-glycerol at 4°C). This indicates that the resistance to the motion in the micelles must be close to isotropic. A summary of the findings presented leads to the conclusion that micelle interiors are similar in nature to aliphatic hydrocarbon solvents.

In general, the fluorescence emitted from molecules which are dispersed in a viscous medium is partially polarized. This is customarily expressed in terms of molecular anisotropy, $r$, or degree of polarization, $\rho$, which are measured at right angle to a polarized excitation beam and are defined as

$$ r = \frac{I_{||} - I_{\perp}}{I_{||} + 2I_{\perp}} \quad \rho = \frac{I_{||} - I_{\perp}}{I_{||} + I_{\perp}} $$

(1)

When $I_{||}$ and $I_{\perp}$ are the fluorescence intensities observed through a polarizer oriented parallel and perpendicular to the plane of polarization of the excitation beam. For a rotating fluorescent sphere the observed $r$ or $\rho$ values obey the well-known Perrin (1926) equation in which $r_0$ and $\rho_0$ are the values

$$ r = \frac{1}{\rho} - 3 \left(\frac{1}{\rho} - 1\right) = 1 + 6R_s \frac{1}{\lambda} $$

(2)

of $r$ and $\rho$ when the emitting molecules maintain their orientation excitation and emission (e.g., in a very viscous solvent), $R_s$ is the rate of rotation of the sphere and $\lambda$ is the rate of fluorescence emission. The term $r_0/\rho_0$ is defined here as the degree of depolarization.
The use of fluorescence probes in model and biological membrane systems has become an extremely important and wide-spread technique which, in recent years, has been extended to fluorescence microscopy.
From 1948 to 1952 Weber carried out independent investigations at the Sir William Dunn Institute of Biochemistry at Cambridge. He began to delve more deeply into the theory of fluorescence polarization and also to develop methods which would allow him to study proteins which did not contain an intrinsic fluorophore.

He invested considerable time and effort in synthesizing a fluorescent probe which could be covalently attached to proteins and which possessed absorption and emission characteristics appropriate for the instrumentation available in post-war England.

The result of two years of effort was dimethylaminonaphthalene sulfonyl chloride or dansyl chloride - a probe which is still utilized today.
Designing fluorescence probes - The beginning

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Designing fluorescence probes - continued...

Bis-ANS

LAURDAN \( R = -(CH_2)_{10}CH_3 \)

PRODAN \( R = CH_2CH_3 \)

DANCA \( R = - \)

-80°C

+60°C
Fay Ferris and Gregorio Weber
With Dansyl chloride and with new instrumentation Weber began to investigate several protein systems, publishing his theory and experimental results in two classic papers published in Biochemical Journal in 1952.

“The Polarization of the fluorescence of macromolecules. I. Theory and experimental method.”

“The Polarization of the fluorescence of macromolecules. II. Fluorescent conjugates of ovalbumin and bovine serum albumin.”

The theory paper - which interestingly contains an acknowledgment to F. Perrin for his suggestions - includes an extension of Perrin’s theory of depolarization to the case of ellipsoidal molecules carrying randomly oriented oscillators of absorption and emission.
Polarization theory continued...

In subsequent years, Weber continued to advance the theory of fluorescence polarization.

This second paper (1972) is a re-examination of previous treatments by several groups (including Weber) and presents the generally accepted equation for the time-dependence of fluorescence polarization owing to rotational diffusion of fluorophores attached to rigid macromolecules.

\[ r(t) = \sum_{i=1}^{3} c_i \exp(-t/\tau_i) + \left[ (F + G)/4 \right] \exp(-|6D - 2\Delta|t) + \left[ (F - G)/4 \right] \exp(-|6D + 2\Delta|t), \]

where

- \( \tau(t) \) is the polarization anisotropy (2) at time \( t \).
- \( D = (D_1 + D_2 + D_3)/3 \), the mean rotational diffusion constant.
- \( \Delta = (D_1^2 + D_2^2 + D_3^2 - D_1 D_2 - D_2 D_3 - D_3 D_1)/4 \).
- \( c_i \) are the direction cosines of the absorbing dipole with respect to the principal rotation axes.
- \( \alpha_i, \alpha_j, \alpha_k \) are the corresponding direction cosines of the emitting dipole.

\( \tau_1 = t(3D + 3D_1) \).
\( F = \sum_{i=1}^{3} \alpha_i^2 \).
In 1953, Hans Krebs recruited Weber for the new Biochemistry Department at Sheffield University.

Krebs received the Nobel Prize in 1953 for his elucidation of the metabolic reactions which produce energy in cells – the Tricarboxylic Acid or Krebs Cycle.

During his years at Sheffield Weber continued to lay the foundations of modern fluorescence spectroscopy developing both fluorescence theory and instrumentation.
Gregorio Weber’s pioneering contributions during these early years included his report, with Laurence (in 1954) of the fluorescence properties of anilino-naphthalene sulfonate (ANS).

Fluorescent Indicators of Adsorption in Aqueous Solution and on the Solid Phase. By G. Weber and D. J. R. Laurence. (Department of Biochemistry, University of Sheffield and Postgraduate Medical School, London, W. 12)

It is interesting to note that even today, more than 50 years after that first report, ANS is still being used in protein studies, quite often as an indicator of the “molten globular” state.
Intrinsic Protein Fluorescence

During his years at Sheffield, Weber and his postdoctoral fellow, John Teale, began their studies on the fluorescence of the aromatic amino acids and proteins.

In the late 1950’s and early 1960’s, Weber and Teale published a series of important papers including the first description of the excitation and fluorescence spectra of the aromatic amino acids - tryptophan, tyrosine and phenylalanine.

Ultraviolet Fluorescence of the Aromatic Amino Acids

By F. W. J. TEALE AND G. WEBER
Department of Biochemistry, The University, Sheffield 10
(Received 25 June 1956)

Figure 7 from this paper has been reproduced many times in review articles and books.
Ultraviolet Fluorescence of the Aromatic Amino Acids

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(Received 25 June 1958)

Fig. 4. Excitation spectrum of phenylalanine fluorescence in water. Abscissa: wavelength (m.μ). Ordinate: molecular extinction coefficient. The continuous line is the optical density spectrum; the dots are the values of log Ω in equation (5).

Fig. 5. Excitation spectrum of tyrosine fluorescence in water. Co-ordinates are as in Fig. 4.

Fig. 6. Excitation spectrum of tryptophan fluorescence in water. Co-ordinates are as in Fig. 4.

Fig. 7. Fluorescence spectra of the aromatic amino acids in water. Abscissa: wavelength (m.μ). Ordinate: relative number of quanta.
Phase Fluorometry

During the 1950’s, Gregorio Weber started to think about constructing a fluorescence lifetime instrument. Influenced perhaps by the work of fellow Argentinean Enrique Gaviola, Weber worked on designing a phase fluorometer. At that time Birks and others had also built several types of phase fluorometers.

At the University of Illinois in the mid-1960’s Weber, together with his graduate student Richard Spencer, constructed a highly versatile phase and modulation fluorometer utilizing the principle of cross-correlation (Annals New York Acad. Sci. 158, 361)

MEASUREMENTS OF SUBNANOSECOND FLUORESCENCE LIFETIMES WITH A CROSS-CORRELATION PHASE FLUOROMETER

Richard D. Spencer and Gregorio Weber

The cross-correlation approach proved to be the key to modern phase fluorometry and is still used universally today.
When Enrico Gratton joined Weber’s laboratory as a postdoctoral fellow from 1975-1976, he worked, at the suggestion of Weber, on the development of a phase and modulation fluorometer with continuously variable light modulation frequencies.

Enrico returned to Urbana in 1978 as an Assistant Professor in the Physics Department. By this time he had finished the first true multifrequency phase and modulation instrument, utilizing a Pockels cell as the light modulator, thus completing Weber’s vision.
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Weber’s work with phase and modulation fluorometry also led indirectly to the development of the Phasor approach to lifetime imaging by Enrico Gratton
Awards

American Academy of Arts and Sciences - 1968

1st National Lecturer of the Biophysical Society - 1969

Guggenheim Foundation Fellow - 1970

Corresponding Member of the National Academy of Exact Sciences of Argentina - 1971

National Academy of Sciences (U.S.) - 1975

Rumford Premium of American Academy of Arts and Science - 1979

ISCO Award for excellence in Biochemical Instrumentation - 1983

First recipient of Repligen Award for the Chemistry of Biological Processes: Awarded by the American Chemical Society - 1986

First recipient of the International Jablonski Award for Fluorescence - 1996
Established in 1839, the Rumford Premium is one of the oldest scientific prizes in the US. and recognizes contributions to the fields of heat and light, broadly interpreted. The endowment was created by a bequest to the Academy from Benjamin Thompson, Count Rumford, in 1796.

Previous winners include:


American Academy of Arts and Sciences.
Boston, Massachusetts.

Rumford Award Ceremony, February 13, 1980
Robert L. Mills, Chen Ning Yang, Milton Katz and Gregorio Weber
Repligen Corporation Award in Chemistry of Biological Processes

The Repligen Award

The Repligen Award for Chemistry of Biological Processes was established in 1985 and consists of a silver medal and honorarium. Its purpose is to acknowledge and encourage outstanding contributions to the understanding of the chemistry of biological processes, with particular emphasis on structure, function, and mechanism. The Award is administered by the Division of Biological Chemistry of the American Chemical Society.

1986  Gregorio Weber
1987  Thomas C. Bruice
1988  Robert H. Abeles
1989  Stephen J. Benkovic
1990  Harold A. Scheraga
1991  William W. Parson
1992  Frank H. Westheimer
1993  Jeremy R. Knowles
1994  Judith P. Klinman
1995  W. Wallace Cleland
1996  William P. Jencks
1997  James A. Spudich
1998  David S. Eisenberg
1999  Christopher T. Walsh
2000  Perry A. Frey
2001  Rowena G. Matthews
2002  C. Dale Poulter
2003  John A. Gerlt
2004  JoAnne Stubbe
2005  David E. Cane
2006  Vern L. Schramm
2007  Michael Marletta
2008  Hung-Wen (Ben) Liu
2009  Frank Raushel
2010  Ronald T. Raines
Welcome to the 6th International Weber Symposium on Innovative Fluorescence Methodologies in Biochemistry and Medicine and 8th Zeiss Workshop on Fluorescence Correlation Spectroscopy and Related Methods

Kauai - 2005
Kauai - 2008
Come join us at the 8th Weber Symposium on Innovative Fluorescence Methodologies in Biochemistry and Medicine

To be held on Kauai, Hawaii
June 12-17, 2011
(updates to be posted on www.lfd.uci.edu)
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Kauai, Hawaii
June 15-20, 2014

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Deadline: March 1, 2014

For info: www.lfd.uci.edu
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David M. Jameson and Enrico Gratton

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11. Francisco Cardarelli – Italy
12. Claus Seidel – Germany
13. Debora Foguel - Brazil
14. Richard Day – USA
15. Taekjip Ha – USA
16. Qiaoqiao Ruan – USA

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Paul French – England
Joachim Muller - USA

First committee selects top three candidates
Second committee select winner
Laboratory for Fluorescence Dynamics
A NIH research resource center for biomedical fluorescence spectroscopy at the University of California, Irvine

Gregorio Weber

In tribute to the outstanding contributions of Gregorio Weber (1916-1997) to the field of fluorescence, the Laboratory for Fluorescence Dynamics (LFD) organizes and sponsors the

- International Weber Symposium on Innovative Fluorescence Methodologies in Biochemistry & Medicine
- Gregorio Weber International Prize in Biological Fluorescence

See also:

- List of Publications by Gregorio Weber, maintained by LFD.
- Gregorio Weber Award for Excellence in Fluorescence Theory and Applications, sponsored by ISS, Inc.
- Gregorio Weber Homepage, maintained by David Jameson.
- Tributes to Gregorio Weber, collected by David Lloyd.


Excerpt from the article published by David M. Jameson in *Biophysical Journal* 75(1), 419-421, 1998.

"Early on the morning of July 18, 1997, at home and surrounded by family and friends, Gregorio Weber died of leukemia at the age of 81. His death ended a remarkable scientific career, which began in Buenos Aires, took form in England at Cambridge and Sheffield, and flourished at the University of Illinois at Urbana-Champaign.

Gregorio Weber's research career, spanning more than half a century, was characterized by an unbroken chain of highly original and important contributions to fluorescence spectroscopy and protein chemistry. Born in Buenos Aires, Argentina, in 1916, Weber completed his M.D. degree at the University of Buenos Aires in 1942. While attending medical school, he worked as a teaching assistant for Bernardo Houssay, who was to receive the Nobel Prize for Physiology and Medicine in 1947. Houssay nominated Gregorio Weber for a British Council Fellowship, which would support his graduate studies at Cambridge University. Travel to England during the war years was an adventure, and his voyage took 44 days in a convoy that endured occasional U-boat attacks. At Cambridge, Malcolm Dixon, the well-known enzymologist, became his thesis advisor and suggested that the young Argentinean investigate the fluorescence of flavins and flavoproteins. Weber soon learned that, during the 1920s and 1930s, fluorescence had already greatly impressed the physicists and to some extent the biologists, but had not drawn much attention from the chemists. For example, a fellow Argentinean, the physicist Guinov, had already constructed a phase fluorometer in the 1920s and had measured the excited state lifetime of fluorescein with good precision. Weber soon came upon the writings of Francis Perrin (the son of Jean Perrin, who had worked on the translational diffusion of macroscopic particles), on the depolarization of fluorescence by Brownian rotation and on energy transfer. Perrin's beautifully crafted theories and clarity of thought and expression inspired Weber to apply these methods to biochemistry."

Read more on the Gregorio Weber Homepage, maintained by David M Jameson.
Final observations...

You know David, when I was much younger an older colleague said to me “Gregorio, when you pass the age of 60 you will begin to notice that your students have more ideas than you and better ideas than you”

Huh?

Gee, really Professor?

...I have not found this to be the case...

How about you Enrico?

And David?

In their dreams!