

CONJUGATE BRIGHTNESS

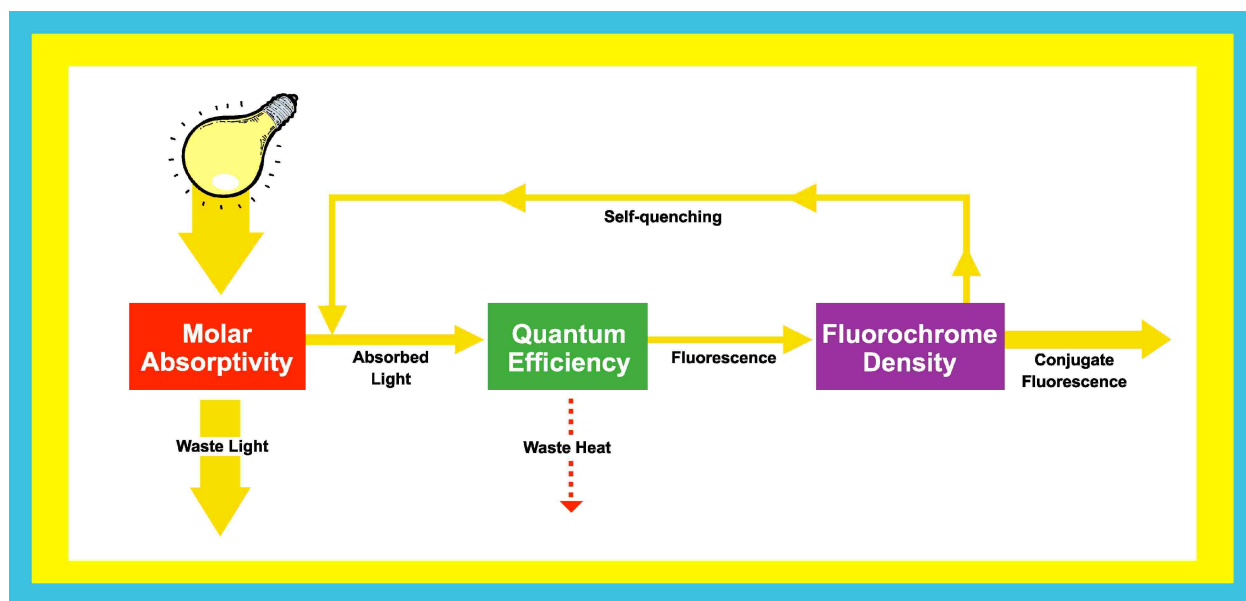


Figure 1 - Factors That Determine Conjugate Brightness.

When preparing fluorescent conjugates for various applications, investigators are confronted by a wide selection of suitable fluorescent molecules (fluorochromes). Many are offered in conjugation kits, where prepared reagents and prescribed protocols relieve the investigator of the task of chemistry development.

Fluorochromes are selected for optimal performance in each application based on such factors as photostability, solubility, spectral overlap, nonspecific binding, and the physical size of the finished conjugate. But often, the foremost concern is conjugate brightness. High brightness not only provides high sensitivity, it also contributes to a robust assay, which allows greater latitude in the manipulation of other assay parameters. Therefore, it is important to understand the factors that make one conjugate brighter than another.

Three characteristics of the fluorochrome-ligand complex combine to determine brightness:

- Molar absorptivity
- Quantum efficiency
- Fluorochrome density

The first two factors are properties of the fluorochrome itself; the conjugation chemistry should not significantly alter their values if performed properly. Fluorochrome density (the number of fluorochromes incorporated per ligand molecule) is a result of the conjugation process.

Figure 1 illustrates the interaction of these factors to produce conjugate fluorescence. A portion of the photons from the light source are absorbed by the fluorochrome depending on its absorptivity spectrum (red box). Some of the absorbed energy is

emitted as fluorescence depending on the fluorochrome's quantum efficiency; the balance is dissipated as heat (green box). The amount of fluorescence per binding event may be enhanced by increasing the number of fluorochromes present in each conjugate (purple box), although self-quenching due to the physical proximity of multiple fluorochromes can limit the efficacy of this strategy.

For this discussion, we assume that the light source and detection system can be optimized for the chosen fluorochrome. The choice among instruments, with very different light sources and detection modes, is outside the scope of this discussion and will not be included here.

Molar Absorptivity

Often absorbance spectra are the first characteristics that a user evaluates when choosing a fluorochrome for a specific application. However, it is also instructive to consider how well a molecule absorbs light in an absolute sense.

A molecule in the light path will absorb a portion of the light as a function of the magnitude and spectral distribution of its absorptivity. Absorptivity at a chosen wavelength (normally, the wavelength of maximum absorbance, λ_{Max} , or other useful wavelength) is called the molar extinction coefficient and is reported in units of $\text{cm}^{-1}\cdot\text{M}^{-1}$ or $\text{cm}^2\cdot\text{mol}^{-1}$. Table 1 shows molar extinction coefficients measured at the λ_{Max} for several commonly available natural and synthetic fluorochromes, along with IgG and streptavidin for comparison. The values demonstrate just how variable this characteristic can be: *the most absorptive fluorochromes are more than two orders of magnitude more absorptive than the least.*

Most absorbance spectra are presented with the maximum normalized to a value of one; other points on the spectrum are shown as relative values. Only the shape, not the magnitude, of the spectrum is apparent.

Table 1 - Molar Extinction Coefficients

Molecule	($\times 10^3$)	λ_{Max} (nm)
IgG ¹	210	280
Streptavidin ¹	176	280
Oyster [®] 645	25	645
Cascade Blue [®]	28	400
Alexa Fluor [®] 488	71	495
BODIPY [®] FL	75	505
Oregon Green [®]	77	488
Alexa Fluor 532	80	532
Rhodamine B	80	570
Fluorescein	85	498
Texas Red [®]	85	595
Alexa Fluor 555	130	555
Cy3 [™]	140	550
Oyster 556	155	556
Alexa Fluor 633	165	632
Oyster 656	222	656
Alexa Fluor 647	239	650
Alexa Fluor 750	240	749
Cy5 [™]	250	649
Cy7 [™]	250	743
PerCP	380	482
APC	700	652
RPE	1,960	566
BPE	2,410	545

¹280 nm absorptivity of non-fluorescent proteins

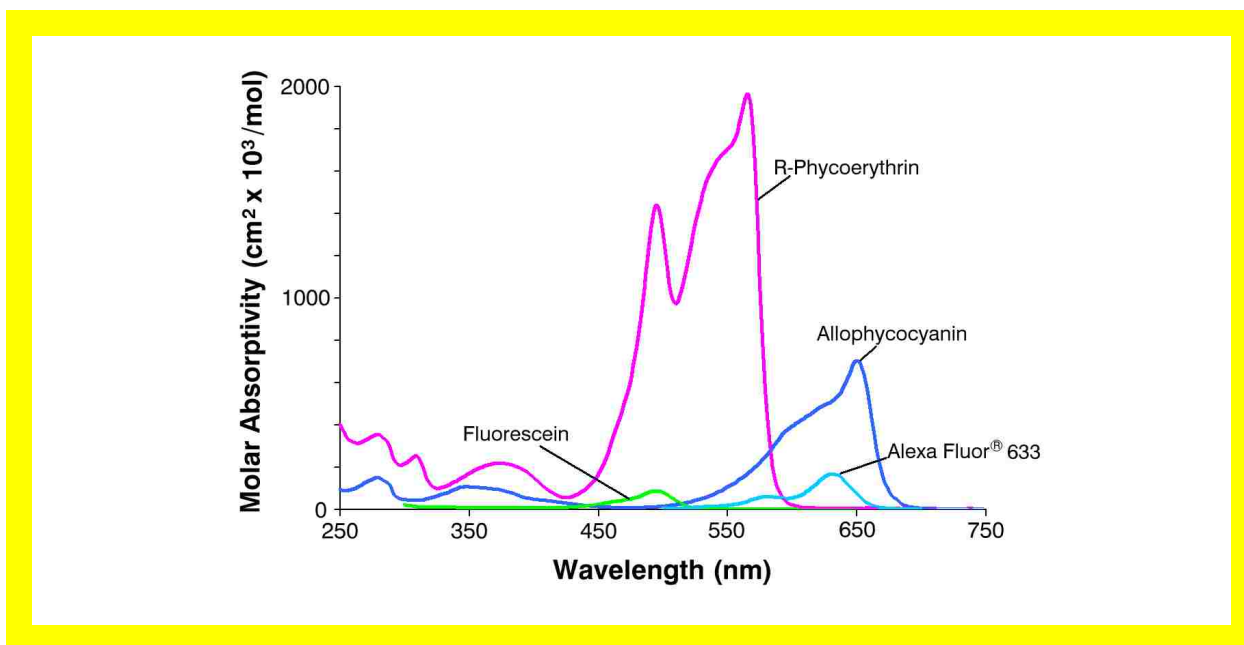


Figure 2 - Molar Absorptivity Spectra for Selected Fluorochromes

The ability of molecules to gather light at different wavelengths is better illustrated by their absorptivity spectra (see examples in Figure 2). This representation of the data makes it clear, for example, that highly absorptive fluorochromes, excited far from their absorbance maximum, may still be brighter than less absorptive ones excited at their λ_{Max} .

Quantum Efficiency

Quantum efficiency is the proportion of absorbed photons released as fluorescence, expressed as a percent, and measures how well the fluorochrome converts absorbed light into fluorescence. Quantum efficiency is a difficult parameter to measure, and independent determinations of this value for various fluorochromes—employing carefully engineered apparatus—have not been highly reproducible.

Regardless of the difficulties inherent in its measurement, quantum efficiency is measured on a closed-end scale, since it cannot exceed one. Useful fluorochromes fall into an even narrower range, say about 0.25 – 0.9+, so they will differ from one another by no more than a factor of 3 – 4. As a result, quantum efficiency will be of

secondary importance in determining brightness, relative to absorptivity, which ranges over orders of magnitude.

Fluorochrome Density

The manner in which conjugations are performed will determine the precise composition of the conjugates obtained. Conceptually, it is easy to think of conjugates as composed of a single fluorochrome bound to a single ligand. In reality this is seldom the case: the presence of multiple potential conjugation sites on one or both molecules leads to the formation of complexes comprising several molecules.

For many applications, the exact composition of the complex is of little concern, provided that the binding site of the ligand is not obscured. For more exacting uses, the composition of the conjugate can be manipulated through control of conjugation conditions and/or downstream purification of the conjugate. (For example, ProZyme offers a series of compositionally defined conjugates of R-phycoerythrin and streptavidin (product codes PJ31S, PJ35S and PJ37S), each of which has proved useful in specific applications.)

When determining conjugate brightness, the number of fluorochromes per conjugate molecule (or possibly in some applications the ratio of fluorochrome molecules to ligand molecules) determines the fluorescence measured for each ligand binding event.

With low-molecular-weight, low-absorptivity fluorochromes such as fluorescein, improved signal can be achieved if a number of molecules can be conjugated to each ligand molecule. In practice, several factors limit the utility of this approach, including the number of conjugation sites per ligand molecule, potential reduction in solubility of the complex, and potential self-quenching by fluorochromes placed in close proximity (see below).

See TechNote TNPJ200 *PhycoLink® Conjugate Evaluations* for a discussion of the methods for determination of molar ratios of phycoprotein conjugate components and estimation of conjugate molecular weight. From this, the fluorochrome density may be inferred. Conjugates of other fluorochromes may be assessed by similar methods.

Self-quenching

(See also TechNote TNPJ100.20 *Self-quenching in FRET Assays*.)

Fluorescent molecules emit photons over a probability-based range of wavelengths called their emission spectrum. In practice, this distribution of photon energies can overlap the absorbance spectrum (see Figure 3). Photons with energies in the overlapping range can be absorbed by other nearby fluorochrome molecules before arriving at the detector, reducing the apparent fluorescence signal (Figure 4).

This may appear to be of little concern, because a reabsorbed photon is in fact eligible to participate again in the fluorescence process. In practice, however, this reabsorbed photon has a lower probability of being re-emitted, since quantum efficiency never equals 100%.

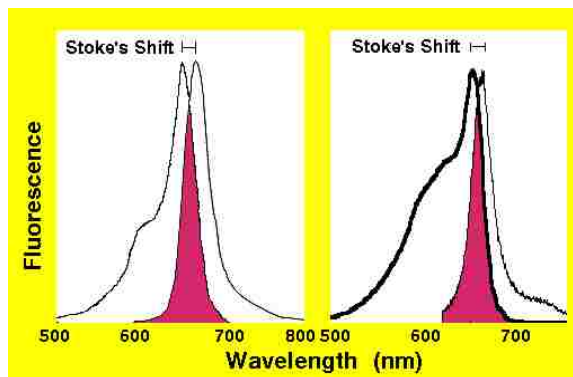


Figure 3 - Overlap (shaded area) between Absorbance and Emission Spectra of Cy 5™ (left) and Allophycocyanin (right).
NOTE: fluorescence scale is relative.

When molecules are randomly dispersed in solution (e.g. in Figure 4), concentration must be sufficiently high to impart significant absorbance to the bulk solution before self-quenching becomes a concern. Fluorochrome concentrations are seldom this high in fluorescent applications. When the molecules are non-randomly dispersed due to aggregation, however, self-quenching can become significant. This type of aggregation is effectively achieved when many small fluorochromes are incorporated into the same conjugate: the local concentration of the fluorochrome is high enough for self-quenching to be a real concern.

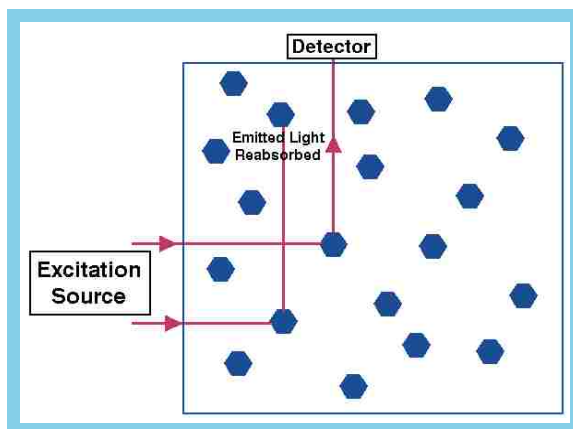


Figure 4 - Reabsorption of Fluorescence in a Fluorochrome Solution

Benefits of Brighter Conjugates

Like many measurements, the photo-multiplier counts obtained in fluorometric measurements are subject to less overall variability as measurement level increases. Relative standard deviations (RSD = (standard deviation/mean)) decline steadily with increasing counting levels: Figure 5 provides an example of RSD *vs.* counting level. While there is a great deal of scatter in this relationship, both inherent to the statistical variability of the counts and also owing to other factors such as pipetting error that can contribute to variability, it is nevertheless clear that the overall trend is the expected reduction in RSD as counts increase. Thus, higher counts are inherently less noisy, and any steps taken to increase counts will, all other factors remaining equal, lead to improvements in the signal-to-noise ratio.

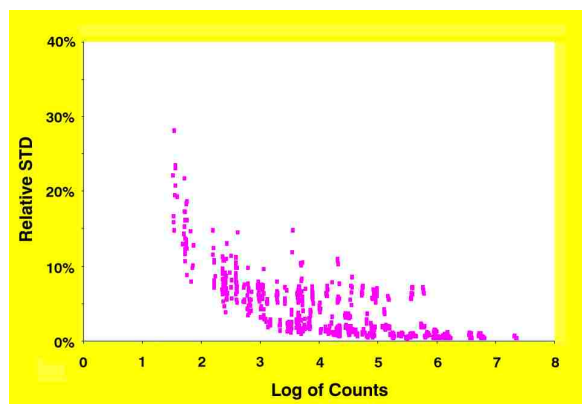


Figure 5 - Relative Standard Deviation vs. Counting Level

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REFERENCES

This and other TechNotes are available on ProZyme's webpage:

<http://www.prozyme.com/technical/index.html#technotes>

Absorbance and emission spectra for Phycopigments and other fluorochromes offered by ProZyme are available on the website:

<http://www.prozyme.com/technical/spectra/index.html>



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