New Long-wavelength Fluorescent Protein Labels with Advanced Characteristics

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Introduction

In this presentation we investigate the newly developed water-soluble, long-wavelength labels **Seta-646** and **Seta-660**. These squaraine-based labels, commercially available as free carboxylic acids, amine-reactive NHS esters (K8-1672, K8-1682) and thiol reactive maleimides (K8-1673, K8-1683) from SETA BioMedicals, show advanced spectral characteristics as labels compared to **Cy5** and **Alexa 660**.

Spectral Characteristics

Seta-646 absorbs and emit at the same spectral range as **Cy5** while **Seta-660** is a better alternative for **Alexa-660** (Fig. 1, Table 1) as a protein label. The extinction coefficients (ε) for these new labels are also in the same order as for **Cy5** and **Alexa 660**.

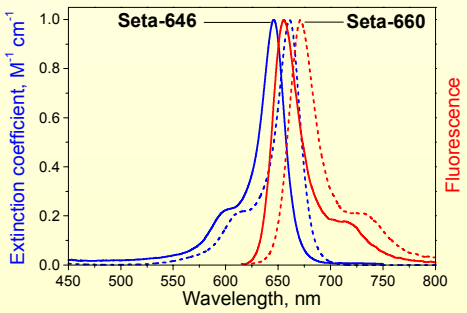

Fig. 1. Absorption and emission spectrum of **Seta-646** in phosphate buffer (pH 7.4)

The initial quantum yields (Q.Y.s) of **Seta-646** and **Seta-660** in aqueous solutions are lower compared to **Cy5** or **Alexa 660** but they substantially increase after covalent binding to proteins.

Typically an increase of the dye-to-protein ratio for any dye-conjugate is associated with a decrease in Q.Y. Thus, the **Cy5** protein conjugates show a gradual decrease of the Q.Y. (Fig. 2). Importantly the decrease of the quantum yields of **Seta-646** and **Seta-660** is less pronounced as compared to **Cy5**, which results in an over-all higher quantum yield (Fig. 2) and total brightness for Seta-dye bioconjugates (Fig. 3).

Table 1. Spectral properties of the **Seta-646**, **Seta-660**, and **Cy5** free in solution and after covalent binding to IgG

Dye	Free dye in phosphate buffer pH 7.4					IgG conjugate, D/P = 1			
	λ_{max} (Abs) [nm]	ε [$M^{-1}cm^{-1}$]	λ_{max} (Em) [nm]	Q.Y. [%]	τ_{mean} [ns]	λ_{max} (Abs) [nm]	λ_{max} (Em) [nm]	Q.Y. [%]	τ_{mean} [ns]
Seta-646	646	207,000	656	10	0.39	650	661	33	1.50
Seta-660	661	151,000	672	11	1.06	665	675	49	2.10
Cy5	647	240,000	664	27	1.00	651	670	29	1.15
Alexa-660	663	132,000	690	37	1.2	663	690	37	-

In Figure 3 the total fluorescence of the dye-IgG conjugates was calculated as the Q.Y. multiplied by the dye-to protein ratio (D/P) and plotted against the D/P ratio. The total fluorescence of **Cy5** remains almost constant over the D/P range between 0.5 and 7 and decreases thereafter, while those for **Seta-646** and **Seta-660** are increasing up to a D/P of 10.

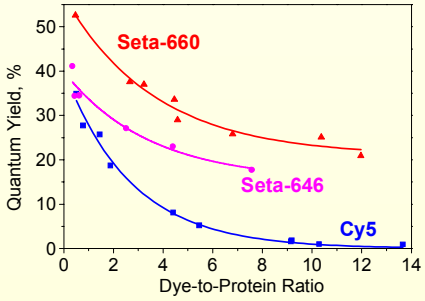

Fig. 2. Quantum yield vs. dye-to-protein ratio of Dye — IgG conjugates in phosphate buffer (pH 7.4)

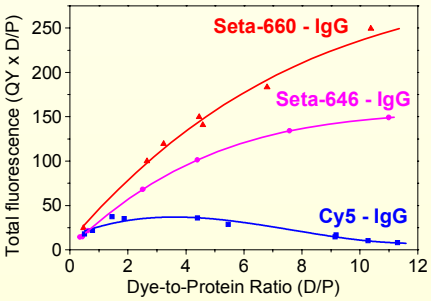

Fig. 3. Total fluorescence vs. dye-to-protein ratio of Dye — IgG conjugates in phosphate buffer (pH 7.4)

Fluorescence lifetime

The mean fluorescence lifetime (τ_{mean}) of **Seta-660** in buffer is about the same as for **Cy5** or **Alexa 660**, but for **Seta-646** the τ_{mean} is about 2.5-fold lower (Table 1). Remarkably, the τ_{mean} increases by factor of 2 for **Seta-660** and by factor of 3.8 for **Seta-646** after binding to IgG (D/P = 1) while for **Cy5** it is almost unchanged. This large lifetime change upon binding to proteins suggests that **Seta-646** and **Seta-660** could be useful in the fluorescence lifetime (FLT) based applications.

Photostability

The relative photostability was determined *via* measurement of the relative change in absorption (Fig. 4) and fluorescence intensity (Fig. 4) upon exposure to light from a 500 W halogen lamp. The data reveals that the photostability of **Seta-646** and **Seta-660** is about 5-fold higher compared to **Cy5**, which is of importance for their use in biomedical assay, single molecule and fluorescence imaging applications.

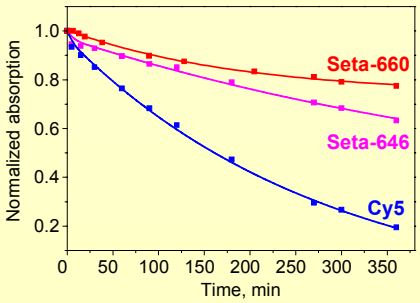

Fig. 4. Relative decrease of the long-wavelength absorption maxima of **Seta** dyes as compared to **Cy5** upon irradiation with a halogen lamp

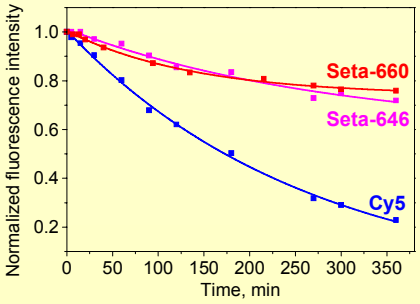

Fig. 5. Relative decrease of the emission maxima of **Seta** dyes as compared to **Cy5** upon irradiation with a halogen lamp

Conclusion

Due to their favourable properties **Seta-646** and **Seta-660** are superior replacements for **Cy5** and **Alexa 660** for many biomedical applications.

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