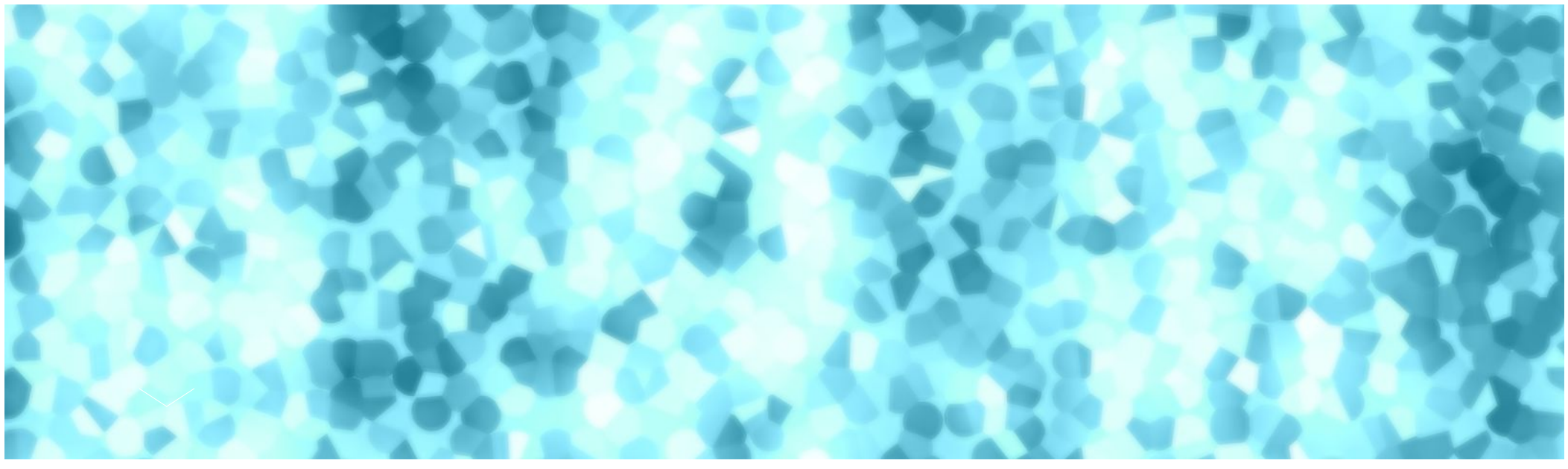
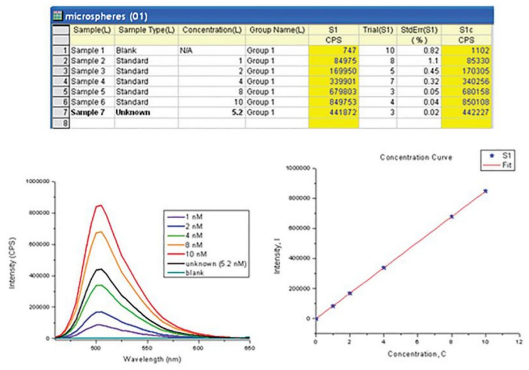


Fluorescence Spectroscopy

Steady State Fluorescence Techniques

What are the uses for single point fluorescence intensity?



	Sample(L)	Sample Type(L)	Concentration(L)	Group Name(L)	S1 CPS	Trial(S1)	StdErr(S1) (%)	S1c CPS
1	Sample 1	Blank	N/A	Group 1	747	10	0.82	1102
2	Sample 2	Standard	1	Group 1	84975	8	1.1	85330
3	Sample 3	Standard	2	Group 1	169950	5	0.45	170305
4	Sample 4	Standard	4	Group 1	339901	7	0.32	340256
5	Sample 5	Standard	8	Group 1	679803	3	0.05	680158
6	Sample 6	Standard	10	Group 1	849753	4	0.04	850108
7	Sample 7	Unknown	5.2	Group 1	441872	3	0.02	442227
8								

Fig. 7: Top: Table of single point intensities for a calibration curve of fluorescent microspheres in different concentrations in solution measured on a FluoroMax-4 spectrofluorometer. Bottom Left: Spectra for the same microspheres. Bottom Right: Intensity vs. Concentration with a linear fit to the calibration curve.

Because fluorescence intensity depends on the concentration of the fluorescent molecule, standard concentration curves can be generated easily and used to determine concentrations of the same molecule in unknown samples.

This is useful in quenching experiments, where additives decrease the intensity of the fluorophores in a systematic way. Concentration curves can also be created to study how other molecules interact with things like proteins, and can be used for tracking protein structural changes, folding, unfolding, association and dissociation systematically.

As an example of a single point fluorescence experiment, here is a calibration curve of a known set of fluorescent microspheres. Five known concentration solutions were used to create the standard curve. The curve was fit to a linear polynomial and the fit was used to calculate the concentration of beads in an unknown solution.

How does temperature affect fluorescence?

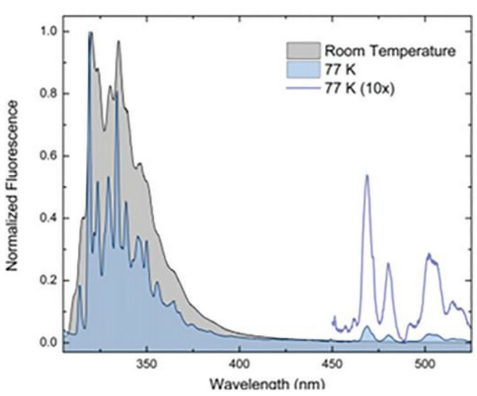


Fig. 8: Temperature can add thermal broadening to fluorescence spectra and increase phosphorescence quenching. Spectra of naphthalene dissolved in methanol measured at room temperature (298K) and in a liquid nitrogen dewar accessory (77K) on a HORIBA Fluorolog-3. The low temperature spectrum reveals rich vibrational structure and longer wavelength phosphorescence. Phosphorescence peaks are also shown magnified 10x for clarity. (HORIBA FluoroMax Series, n.d.)

Temperature does have an effect on fluorescence intensity and sometimes spectral wavelength and shape as well. A fluorescence emitter's molar extinction coefficient (or radiative rate constant) are usually weakly dependent on the temperature. However, the non-radiative rate constant, which is governed by vibrational coupling, is strongly affected and will increase with increasing temperature. This means that the fluorescence will decrease with increasing temperature, in general. Similarly, collisional quenching will also increase with temperature, also decreasing fluorescence intensity.

Fluorescence is greatly influenced by the molecules around the emitting molecule. Also, fluorophores measured at very low temperatures using liquid nitrogen or liquid helium show increased fluorescence and also increased amounts of vibrational structure in the excitation and emission spectra. Collisional quenching decreases and the non-radiative rate constant will decrease as well, increasing fluorescence emission intensity.

Molecules such as polyaromatic hydrocarbons (i.e. anthracene, naphthalene, pyrene, perylene, etc.) have vibrational peaks that show up in the spectrum at room temperature. At liquid nitrogen temperature of 77 K, these fluorescence peaks become more structured and phosphorescence peaks become measurable. These below were measured at both 298 K and at 77 K in a liquid nitrogen Dewar sampling accessory.

How do I remove solvent Raman peaks from a fluorescence spectrum?

Raman peaks will often show up in a fluorescence spectrum, especially if the fluorescence intensity is weak. To remove a Raman peak, the spectrum of a blank solvent can be measured under the same conditions as that of the sample and this "blank" spectrum subtracted from the fluorescent sample spectrum. Because a Raman peak is a vibrational phenomenon that is related to the energy of the excitation light, the position can be controlled somewhat. A Raman peak will move closer or further from the excitation wavelength if the excitation is moved more to the blue or to the red, respectively. So, if a Raman peak is interfering with a sample fluorescence spectrum, the excitation wavelength can be moved to put the Raman peak in a slightly different location if necessary.

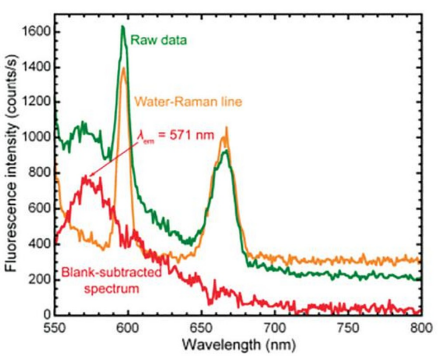


Fig. 9: Blank-subtracted emission spectrum (red) of red microspheres at 6.7x104 beads/mL (lexc=542 nm): The non-subtracted spectrum is green. Here, two water Raman peaks for – OH bending and stretching modes (corresponding to 3380 and 1700 cm-1) can be shown in the spectrum of the blank solvent (orange) and subtracted from the sample

fluorescence spectrum to better resolve the emission peak at 571 nm. All measurements were performed on a FluoroMax-4 spectrofluorometer.

How can fluorescence spectra be used to characterize proteins?

Fluorescence spectra can also be used to track changes in protein folding or unfolding. This is an example of how the fluorescence spectrum of tryptophan in a solution of 1 µM BSA looks with increasing temperature. These curves represent the temperature going from 5 to 70 °C. You can see the intensity decreasing as well as the spectrum shifting to shorter wavelengths as temperature is increased. Anisotropy or time-resolved anisotropy could be used to get information on the size, shape, and orientation motion of the protein as well.

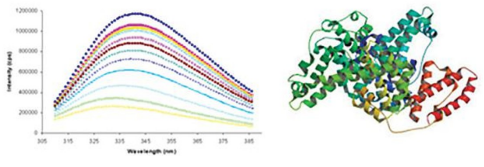


Fig. 10: Left: Spectral changes as a result of thermal unfolding of Bovine serum albumin (BSA) using a QuantaMaster fluorometer. Right: Basic structure of BSA (Bujacz, 2012)

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