Internet-based education on the structure, function, and imaging of fluorescent proteins

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ABSTRACT

Despite the explosive growth of the Internet (in terms of the World Wide Web) as an informational resource for the original scientific literature pertaining to fluorescent protein investigations, there remains an obvious void in educational Websites targeted at beginning students and novices in the field. To address this issue, educational sites dedicated to optical microscopy and digital imaging being constructed and hosted at The Florida State University are turning their attention to the increasing application of fluorescent proteins for live-cell imaging studies. The primary focus of this effort is to create new sections of the sites that address the structure and properties of fluorescent proteins as well as optimizing their utility in imaging experiments.

Keywords: fluorescent proteins, optical highlighters, biosensors, photoconversion, confocal microscopy, FRET, FRAP, EGFP, DsRed, Internet, Web, Java, Flash

1. INTRODUCTION

Shortly after the primary structure of green fluorescent protein (GFP) was elucidated[1] and the protein was demonstrated to be a useful marker for gene expression[2], the Internet became a practical vehicle for the international dissemination of information with the establishment of hypertext transfer protocol[3-5] (HTTP) as the underlying foundation for electronically linking digitally-encoded documents through a virtual World Wide Web of interconnected computer systems. The first edition of HTTP was functional for the transfer of hypertext documents, but was unable to support graphics, multimedia (audio and video), or proprietary file formats, such as Microsoft Word or Adobe PostScript. During the mid-1990s, the protocol started to rapidly evolve from a trivial request/response application into a true messaging vehicle capable of transferring a wide spectrum of media formats between localized servers and remote clients.

By 1997, the Web was burgeoning in popularity due, in no small part, to the introduction of novel software applications, such as Java[6] and Flash[7], and the deployment of standards for transmission of audio and video multimedia files. During this period, client software (termed "Web browsers") also became increasingly more sophisticated as the competing packages from various manufacturers were incrementally being equipped with the ability to display a larger array of image formats, interface with audio sound systems, and embed specialized "plug-in" modules to enable the control capabilities of emerging interactive (Java and Flash) software. The rapid advances in Internet software development were paralleled by equally dramatic steps in the capability of personal computers to multi-task and handle the continuously increasing hardware demands of video, audio, and memory-hungry applications. At the turn of the century, a third critical factor in the utility of the Internet, search engine technology, had advanced to the stage where the millions of Websites being cataloged on a daily basis could be logically categorized and sequentially hyperlinked (ranked) according to a variety of criteria for fast and efficient searches[8].

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As the Web grew in popularity and became a viable alternative to libraries, a majority of the scientific publications began to make their content available on-line in the form of abstracts, HTML documents, and downloadable portable document format[9] (PDF) files that could be viewed in a Web browser and printed locally. These journals are formally indexed by such services as PubMed[10] (National Center for Biotechnology Information at the National Institutes of Health), Chemical Abstracts[11] (American Chemical Society), and PhysicsWeb[12] (Institute of Physics), as well as being informally cataloged by the search engines (Google[13], Yahoo![14], etc.) and numerous Websites of researchers in the field.

Today, virtually every laboratory involved with fluorescent proteins has the potential ability to maintain a site that assists users, the general public, and interested scientists with information relating to equipment, specimens, scheduling, as well as providing image galleries, and educational tutorials. Several Websites[15-18] have been developed by researchers, students, and the microscope manufacturers to specifically address current educational topics in disciplines related to the structure, function, and imaging of fluorescent proteins, however, most of these sites are limited in scope. Unfortunately, exhaustive Web searches have revealed scant educational information other than hyperlinks directly from the search engine return pages to PubMed for the original research articles, which are often difficult to understand for beginning students. To address this issue, the microscopy educational Websites, described below, are being populated with information on fluorescent proteins in the form of well-illustrated review articles, interactive Java tutorials and, image galleries. Also featured are links to related Websites and the original research reports on PubMed or similar indexing services.

2. MICROSCOPY EDUCATIONAL WEBSITES

The Molecular Expressions Website[19], which has been built over the past 10 years and is hosted in the National High Magnetic Field Laboratory at The Florida State University (FSU), currently is 9500+ HTML pages in size and contains almost 75 image galleries featuring a variety of contrast-enhancing techniques, such as phase contrast, differential interference contrast (DIC), darkfield, fluorescence, confocal, Hoffman modulation contrast, and brightfield. The site also offers hundreds of review articles on various topics in optical microscopy[20], which are accompanied by over 500 interactive Java tutorials that enable the visitor to experiment with variables in a manner similar to operating an actual microscope. Included in the many features on this site are an introduction to the physics of light and color, basic principles of the optical microscope, advanced and specialized contrast-enhancing techniques, fluorescence, photomicrography, digital image acquisition and processing, stereomicroscopy, laser scanning confocal microscopy, and a virtual museum of microscopes developed through the ages[21]. Similar Websites are offered at no charge to the general public by Nikon (MicroscopyU)[22] and Olympus (Microscopy Resource Center)[23]. Recently, Olympus has introduced an educational Website (FluoView Resource Center[24]) dealing specifically with confocal microscopy.

Among the most useful features of the microscopy educational Websites is that, unlike printed material, the dynamic nature of HTML enables the ability to continuously modify electronic documents in a timely manner in order to maintain current information. As an example, review articles and interactive tutorials dealing with continuously evolving fields, such as optical highlighter fluorescent proteins[25-27], can be easily modified to incorporate new information on these valuable probes as it becomes available. In addition, Web-based tables of fluorescent protein properties (see Table 1), which are useful in comparing fluorophores during the complex task of choosing the ideal protein for a particular experiment, can be readily modified and maintained to ensure that the latest information is available. Finally, in contrast to the static reference sections of printed scientific publications, HTML listings of literature references[16] can be hyperlinked to the originating journal Websites or to indexing service databases, such as PubMed[10], which enable researchers and students to conveniently view abstracts and/or download PDF files of the original research reports, news commentaries, and review articles.

The compilation of optical highlighter fluorescent protein data presented in Table 1 is an excellent example of a rapidly changing research frontier that can greatly benefit from the dynamic nature of the Internet. New highlighter proteins have been reported at the rate of one or two every several months over

the past couple of years, necessitating periodic updates to this table in order to preserve the timeliness and accuracy of information presented to visitors. Other educational Website features that require frequent monitoring are listings of Internet resources with hyperlinks to external Websites that may be of interest to visitors. Typically about 10-15% of the external links on the combined FSU microscopy educational Websites require editing on a semi-annual basis due to changes in the URLs.

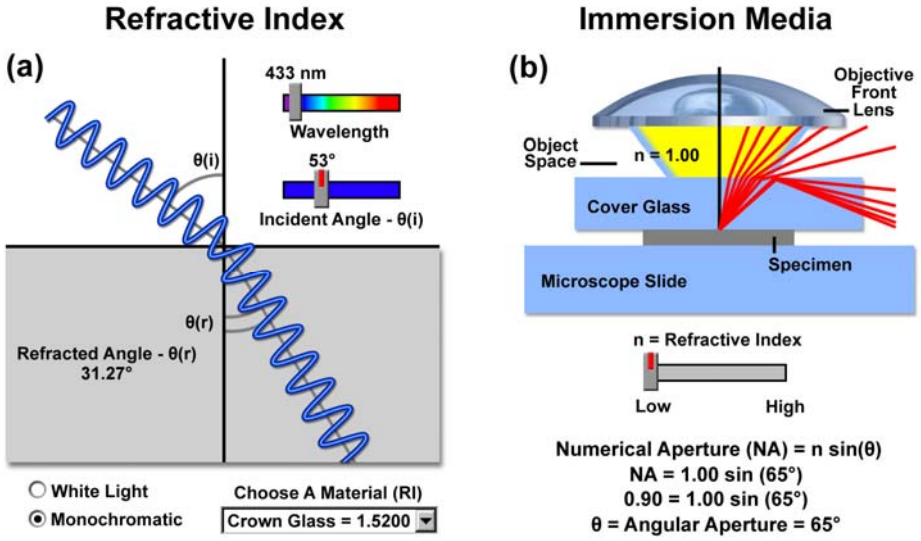

Figure 1. Interactive Java tutorials on the refraction of light and the concept of refractive index **(a)**, and the effect of immersion media refractive index on resolution **(b)**. Visitors to the Website are able to operate the tutorials using sliders (wavelength and refractive index), pull-down menus (material selection), and radio buttons (white or monochromatic light). The wavefront trajectories are dynamically controlled by the sliders as the wavelength and refractive index are altered. Changes to the numerical aperture of the objective-specimen system illustrated in **(b)** are continuously updated as the slider position is altered.

Addressing topics of interest to science education in the K-12 arena, the Web programming team at FSU has developed a site entitled Science, Optics and You[28], a microscopy curriculum package being targeted at middle and high school teachers, students, and parents. These activities are designed to promote the asking and answering by students of fundamental questions related to light, color, and optics. The program begins with basic information about lenses, shadows, prisms, light and color, and related topics in optics, leading up to the application of sophisticated instruments, such as microscopes, binoculars, digital cameras, and telescopes. The goal of Science, Optics and You is for students to acquire the skills with which they can conduct optical and microscopic analysis on a variety of specimens. This program is supported by Web-based student and teacher activities, interactive tutorials, a timeline in optics, and biographies of pioneering scientists in the field of optics. In addition, two low-cost microscopes designed for students, the Digital Blue QX3 (and QX5) and the Olympus MIC-D, are thoroughly reviewed.

Illustrated in Figure 1 are several interactive Java tutorials that serve as typical examples of the material found on the FSU microscopy educational Websites. The tutorial in Figure 1(a) demonstrates the basic properties of refraction in a single wavefront of light as it passes between two media of dissimilar refractive index[29]. Students learn from this tutorial that the effects of refraction are responsible for a variety of familiar phenomena, such as the apparent bending of an object that is partially submerged in water and the mirages observed on a dry, sandy desert. More importantly, they also discover that the refraction of ultraviolet, visible, and near-infrared light is also a fundamental characteristic of lenses that enables them to focus a beam of light onto a single point. The importance of refractive index to imaging with the microscope is presented in Figure 1(b), which is a critical factor in determining the working numerical aperture of a microscope objective[30]. A dramatic increase in numerical aperture (and, thus, resolution) is observed when the objective design is modified to operate with an immersion medium such as oil, glycerin, or water between the front lens and the specimen cover glass. By using an immersion medium with a refractive index similar to that of the glass coverslip, image degradation due to thickness variations of the

cover glass are practically eliminated whereby rays of wide obliquity no longer undergo refraction and are more readily grasped by the objective.

More advanced tutorials[31] on the microscopy educational Websites are designed to explore complex techniques, such as creation and optical displacement of the wavefront field in differential interference contrast, the origin of specimen contrast using a condenser annulus and phase ring in phase contrast microscopy, the interaction of birefringent materials with linearly and circularly polarized light in optical microscopy, and the effect of condenser numerical aperture in darkfield microscopy. In the fast-paced arena of digital imaging in optical microscopy, a large collection of tutorials[32] is available to teach students how the fundamental tools of digital image processing can be utilized to manipulate, rehabilitate, edit, resize, rotate, and store images captured with an optical microscope (or other digital image recording device). These interactive tutorials each consider a specific algorithm or related series of algorithms that are useful for processing digital images. In addition, review articles on each topic are included for in-depth study in far greater detail.

Optical Highlighter Fluorescent Protein Properties

Fluorescent Protein (Acronym-Color)	Ex (nm)	Em (nm)	Molar Extinction Coefficient	QY	*in vivo* Molecular Structure	Relative Brightness (% EGFP)	Literature Reference(s)
PA-GFP (G/NA)	400	515	20,700	0.13	Monomer	8	33, 34, 35
PA-GFP (G)	504	517	17,400	0.79	Monomer	41	33, 34, 35
PS-CFP (C)	402	468	34,000	0.16	Monomer	16	36
PS-CFP (G)	490	511	27,000	0.19	Monomer	15	36
PS-CFP2 (C)	400	468	43,000	0.20	Monomer	26	36
PS-CFP2 (G)	490	511	47,000	0.23	Monomer	32	36
PA-mRFP1 (R)	578	605	10,000	0.08	Monomer	3	37
Kaede (G)	508	518	98,800	0.88	Tetramer	259	38, 39
Kaede (R)	572	580	60,400	0.33	Tetramer	59	38, 39
KikGR (G)	507	517	53,700	0.70	Tetramer	112	40
KikGR (R)	583	593	35,100	0.65	Tetramer	68	40
mEosFP (G)	505	516	67,200	0.64	Monomer	128	41, 42
mEosFP (R)	569	581	37,000	0.62	Monomer	68	41, 42
Kindling (R)	580	600	59,000	0.07	Tetramer	12	43, 44, 45
Dronpa (G)	503	518	95,000	0.85	Monomer	240	46, 47

Table 1. A compilation of important data for optical highlighter fluorescent proteins that display significant potential in applications as *in vivo* probes targeting cellular structure and function. Included are the excitation (or absorption) and fluorescence emission maximum wavelengths for both the activated and non-activated species, as well as the molar extinction coefficients, quantum yields, *in vivo* molecular structure (monomer, dimer, tetramer, etc.), and relative brightness level compared to enhanced green fluorescent protein (EGFP). Also listed are references to the original literature sources and review articles. This table should serve as a convenient guide for comparing the properties of optical highlighters, but will probably be obsolete by the time this article is published. For a current version of the table see http://www.olympusconfocal.com/applications/opticalhighlighters.html.

The Olympus FluoView Resource Center Website[24] is focused on delivering educational materials targeting laser scanning confocal microscopy. Included on this site are review articles and interactive Java tutorials on a variety of topics relating to confocal techniques, including basic fluorescence excitation and emission fundamentals, resolution and contrast, spectral bleed-through artifacts, acousto-optic tunable filters, objectives, lasers, photomultipliers, scanning systems, and signal-to-noise considerations. The Website also contains an exhaustive listing of useful fluorochromes, which is periodically updated as new synthetic probes are reported, as well as an extended glossary of common terms that attempts to simplify the complex nomenclature of fluorescence microscopy and related techniques. A literature section on the

site describes the most current books available on confocal microscopy and provides links to the appropriate pages from booksellers on the Internet.

The broad range of applications available to laser scanning confocal microscopy, and discussed on the FluoView Website, includes a wide variety of studies in neuroanatomy and neurophysiology, as well as morphological studies of a wide spectrum of cells and tissues. In addition, the growing use of new fluorescent proteins is rapidly expanding the number of original research reports coupling these useful tools to modern microscopic investigations. Other applications include resonance energy transfer, stem cell research, photobleaching studies, lifetime imaging, multiphoton microscopy, total internal reflection, DNA hybridization, membrane and ion probes, bioluminescent proteins, and epitope tagging. Many of these powerful techniques are described in detail on the Website while others are the subject of interactive tutorials. Specific review articles[48] address colocalization of fluorophores, fluorescent proteins, optical highlighters, epitope tagging, stem cells, resonance energy transfer and photobleaching techniques (FRET and FRAP), multiphoton microscopy, total internal reflection (TIRFM), and *in situ* hybridization (FISH). The Website also features numerous image galleries and links to related resources on the Internet. The hyperlinked resources include manufacturers and distributors of fluorescent filters, digital cameras, antibodies, fluorescent protein vector sources, lasers, specimen chambers, photometric detectors, live-cell imaging supplies, courses and workshops on microscopy, and software for three-dimensional volume rendering.

Laser Scanning Confocal Microscope Simulator

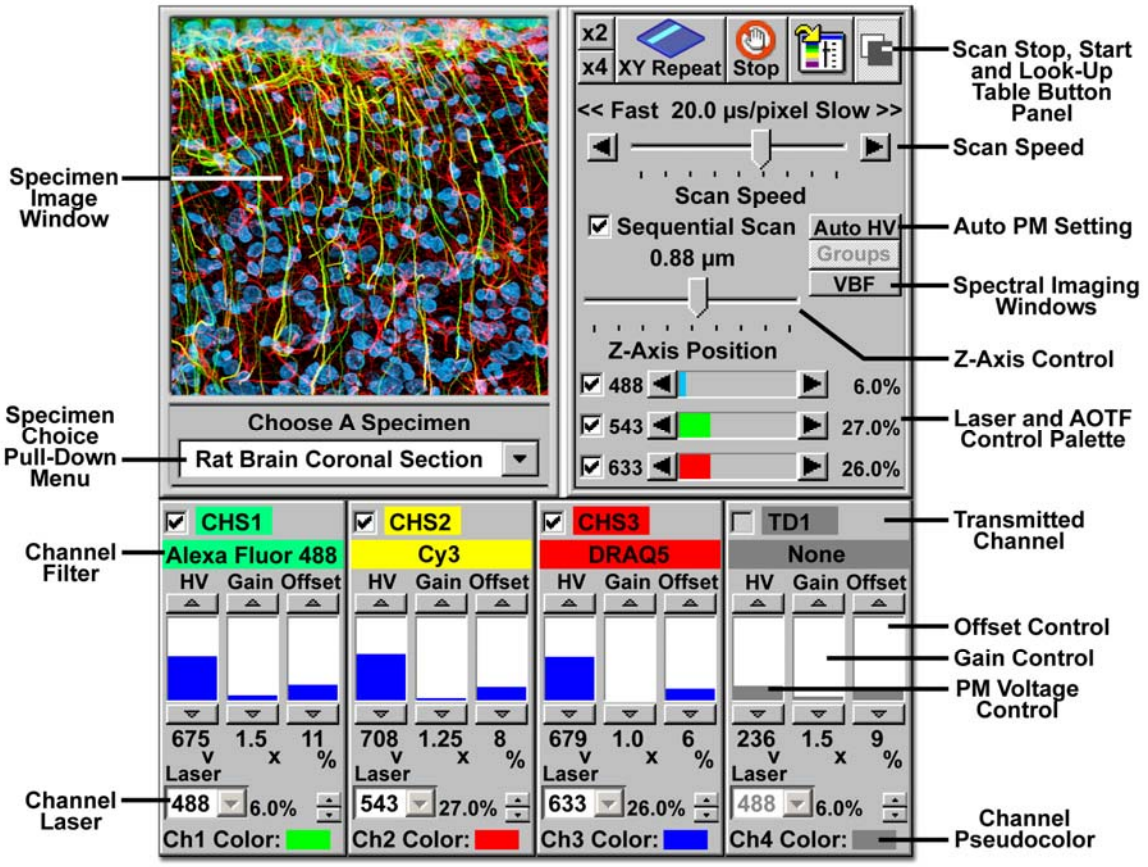

Figure 2. Laser Scanning Confocal Microscope Simulator interactive tutorial based on the software interface of the Olympus FluoView FV1000 microscope. Specimens in the tutorial were acquired as optical stacks with the FV1000, and image parameters, including intensity, bleed-through, and noise levels are based on the fluorophore emission spectral profiles.

One of the hallmark interactive tutorials on the FluoView Website, a Java-powered laser scanning confocal microscope simulator[49], is illustrated in Figure 2. This tutorial explores multi-laser fluorescence and differential interference contrast (DIC) scanning confocal imaging with the Olympus FluoView FV1000 confocal microscope software interface as a model. Using a series of selectable pre-recorded image stacks for specimens, the tutorial initializes with a randomly selected stack being scanned along the lateral x-y plane at a modest speed of 20 microseconds per pixel, and the detector channels being set very close to the optimum values with simultaneous scanning in all three channels. Imaging depth in the axial (z) direction is adjusted with the Z-Axis Position slider (see Figure 2) according to a range determined by the thickness of each specimen. Upon initialization, each channel corresponding to a fluorophore present in the specimen is activated and labeled with the specific probe being imaged. The transmitted light channel (TD) is turned off by default. Lasers are pre-assigned (and fixed) to the channels according to the following order: argon-ion (488 nanometers) - Channel 1; green helium-neon (543 nanometers) - Channel 2; and red helium-neon (633 nanometers) - Channel 3. The TD channel produces transmitted light differential interference contrast images using the argon-ion laser set at 488 nanometers.

Visitors are able to interact with the confocal microscope simulator tutorial by adjusting the voltage, gain, and offset values for each channel and immediately see the effect on the image. In addition, similar to the actual software, the tutorial is equipped with an automatic photomultiplier high voltage (Auto HV) control that maintains a constant signal level while increasing or decreasing the microscope scan speed. The individual laser intensities are also controllable (through a range of 0 to 100 percent) with sliders and arrow buttons adjacent to each laser activation checkbox (identical with the actual software). Additional features that simulate the microscope software and add a realistic "feel" to the tutorial are Auto Contrast for a "best-guess" initial setting of the detector channel levels (typically utilized for pre-scanning a specimen), a look-up table for adjustment of the gamma, intensity, contrast, and pseudocolor for each channel, and simulation of rapid (2x and 4x) specimen scanning.

In order to simulate image acquisition of specimens having strongly overlapping fluorophore emission spectra using the spectral imaging detector channels, the confocal simulator tutorial is equipped with a dialog box illustrating the emission spectra of the selected fluorophores superimposed over the detector slit settings for channels 1 and 2 (the spectral imaging channels). In a manner similar to the microscope software, the tutorial allows users to observe the effects of altering slit size and wavelength range while the simulator is scanning a specimen in order to demonstrate in real-time how increasing or decreasing these variables affects specimen contrast, intensity, and spectral bleed-through. A unique feature of this tutorial is that simulation of bleed-through in the specimen image is based on the degree of overlap between actual fluorophore emission spectral profiles. In our laboratory, students and entry-level technicians are first trained using the confocal microscope simulator software, and must display a minimum level of understanding and expertise before they are deemed qualified to operate the actual microscope.

Additional interactive tutorials on the Olympus FluoView Website relating to confocal imaging of fluorescent proteins include an analysis of fluorescent probe excitation efficiency as a function of laser wavelength (tutorial not illustrated) and a discussion of choosing fluorophore combinations for confocal and widefield fluorescence microscopy[50] (see Figure 3). The fluorophore excitation efficiency tutorial examines why the absorption and fluorescence emission spectral profiles of a fluorophore are two of the most important criteria that must be scrutinized when selecting probes. In addition to the wavelength range of the absorption and emission bands, the molar extinction coefficient for absorption and the quantum yield for fluorescence emission should be considered. At laser excitation levels that do not saturate the fluorophore, fluorescence intensity is directly proportional to the product of the extinction coefficient and the quantum yield. This interactive tutorial examines how these relationships can be utilized to match fluorophores with specific lasers for confocal microscopy. The tutorial addressing requirements for choosing fluorescent protein fluorophore combinations (Figure 3) addresses the fact that the judicious choice of probes is paramount in obtaining the best target signal while simultaneously minimizing bleed-through artifacts. This interactive tutorial is designed to explore the matching of dual fluorophores with efficient laser excitation lines or arc-discharge lamps, calculation of emission spectral overlap values, and determination of the approximate bleed-through level that can be expected as a function of the detection window wavelength profiles.

Matching Fluorescent Proteins for Dual Imaging Investigations

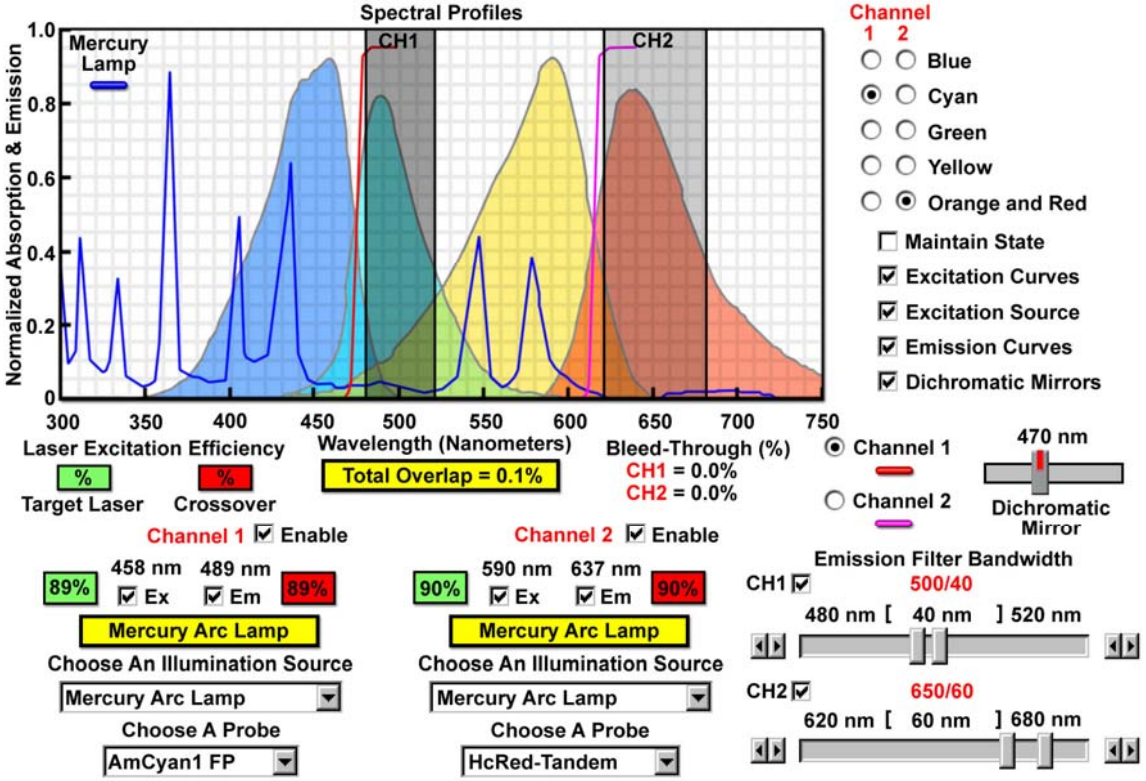

Figure 3. Interactive Java tutorial designed to aid in determining spectral parameters for imaging two fluorescent proteins simultaneously. The tutorial initializes with the absorption and fluorescence emission spectral profiles from a useful combination of fluorescent proteins (CFP AmCyan1 and HcRed-Tandem) appearing in the Spectral Profiles window superimposed over the emission spectrum of a mercury arc-discharge lamp. Also included in the window upon initialization are the profiles of dichromatic mirrors appropriate for the fluorophores, as well as the suggested starting points for the Emission Filter Bandwidth profiles. The mouse cursor can be used to vary parameters, such as the width of emission filters, cut-on wavelength of the dichromatic mirrors, or to disable one of the image channels. Radio buttons (upper right) are available to choose fluorescent protein emission color classes (blue, cyan, green, yellow, orange, and red). The illumination source can be toggled between arc-discharge lamps and lasers.

The Nikon MicroscopyU Website[22], also being constructed and hosted at FSU, is built around the Molecular Expressions model to act as a sister site, but contains a wealth of exclusive information relating to the microscopy instrumentation, digital cameras, and software being developed by Nikon. In addition, MicroscopyU hosts the annual Small World Photomicrography competition[23] and features interactive tutorials and review articles on basic and advanced techniques in optical microscopy. Covered in depth are the principles of resolution, numerical aperture, depth of field, image brightness, objective working distance, field of view, conjugate planes, and the useful magnification range, as well as comprehensive discussions of the microscope optical train, infinity optical systems, aberrations, and microscope objectives. As is the case with Molecular Expressions, each review article on MicroscopyU is accompanied by one or more interactive tutorials to assist students in assimilating difficult concepts. Several reviews and tutorials featured on MicroscopyU target the basic properties and imaging parameters for fluorescent proteins. In addition, new sections on live-cell imaging are being constructed that discuss how to keep cells alive on the microscope, and cell motility streaming videos with and without fluorescent proteins are being staged for the site.

3. FLUORESCENT PROTEINS ON THE MICROSCOPY WEBSITES

Over the past decade, fluorescent proteins have heralded a new era in cell biology by enabling investigators to apply molecular cloning methods, fusing the fluorophore moiety to a wide variety of protein and enzyme targets, in order to monitor cellular processes in living systems using optical microscopy and related methodology[52-54]. When coupled to recent technical advances in widefield fluorescence and confocal microscopy, including ultra-fast low light level digital cameras and multitracking laser control systems, the green fluorescent protein and its color-shifted genetic derivatives have demonstrated invaluable service in many thousands of live-cell imaging experiments. Current efforts on the microscopy Websites discussed above center on developing review articles, image galleries, literature source links, and interactive tutorials targeted at undergraduate-level education in fluorescent protein technology.

Among the most remarkable attributes of the original green fluorescent protein derived from the *Aequorea victoria* jellyfish, as well as the more recently developed palette of color-shifted genetic variants, is that the entire 27 kiloDalton polypeptide structure is essential for the development and maintenance of fluorescence in this very remarkable family of proteins[26]. The principle fluorophore (often termed a chromophore; see the interactive tutorial on EGFP fluorophore maturation in Figure 4) is a tripeptide consisting of the residues serine, tyrosine, and glycine at positions 65-67 in the sequence. Although this simple amino acid motif is commonly found throughout nature, it does not generally result in fluorescence.

Maturation of the Enhanced Green Fluorescent Protein Fluorophore

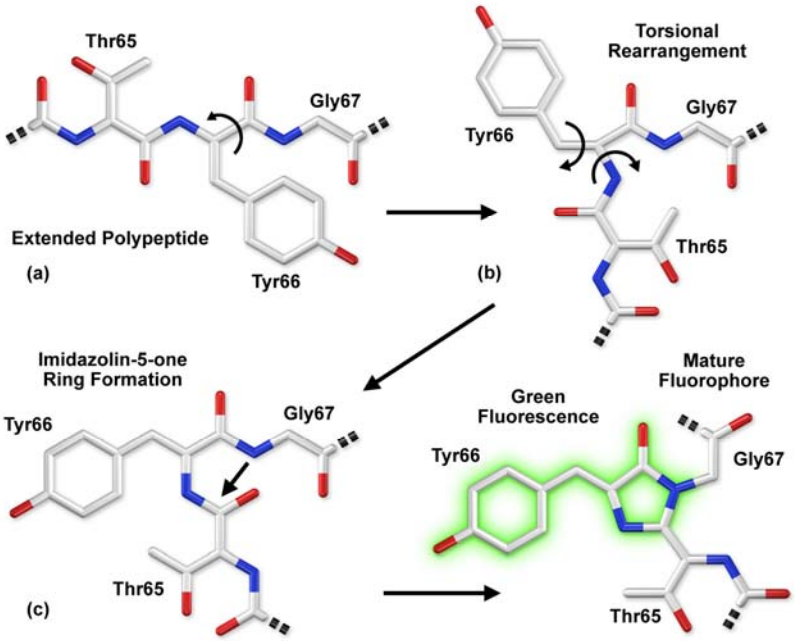

Figure 4. Interactive Java tutorial describing fluorophore formation of enhanced green fluorescent protein (EGFP). The tutorial initializes with an image of the pre-maturation EGFP fluorophore tripeptide amino acid sequence (Thr65-Tyr66-Gly67) stretched into a linear configuration so that the threonine residue is positioned at the extreme left end of the window (a). In the on-line tutorial, oxygen atoms are colored red, nitrogen atoms blue, carbon atoms white, and the black dashes at the peptide termini indicate continuation of the backbone beyond the portion illustrated. A slider (not illustrated) is used to transition through the self-catalyzed intramolecular re-arrangement of the tripeptide sequence that occurs during fluorophore maturation. The first step is a series of torsional adjustments (b) and (c) that relocate the carboxyl carbon of Thr65 in close proximity to the amino nitrogen of Gly67. Nucleophilic attack by this carbon atom on the amide nitrogen of glycine, followed by dehydration, results in formation of an imidazolin-5-one heterocyclic ring system (d). Fluorescence (indicated by a green glow surrounding the affected structural elements in the tutorial) occurs when oxidation of the tyrosine *alpha-beta* carbon bond by molecular oxygen extends electron conjugation of the imidazoline ring system to include the tyrosine phenyl ring and its para-oxygen substituent.

A broad range of fluorescent protein genetic variants have been developed[55] over the past several years that feature fluorescence emission spectral profiles spanning almost the entire visible light spectrum. Mutagenesis efforts in the original jellyfish green fluorescent protein have resulted in new fluorescent probes that range in color from blue to yellow[56], and are some of the most widely used *in vivo* reporter molecules in biological research. Longer wavelength fluorescent proteins, emitting in the orange and red spectral regions, have been developed from the marine anemone, *Discosoma striata*, and reef corals belonging to the class Anthozoa[57]. Still other species have been mined to produce similar proteins having cyan, green, yellow, orange, and deep red fluorescence emission[52]. Developmental research efforts are ongoing to improve the brightness and stability of fluorescent proteins, thus improving their overall usefulness.

Essential to the understanding of spectral diversity in the wide range of fluorescent proteins discovered thus far are structural investigations of the stereochemical nature of the fluorophore and the effects of its surrounding environment on fluorescent properties. Aside from the jellyfish proteins, there appears to be a high degree of variation in the fluorophores of red-shifted fluorescent proteins[58]. Even through the DsRed fluorophore configuration, termed planar *cis*, appears to be the predominant structure in most proteins that emit in the orange and red regions, there are at least two additional motifs, planar *trans* and non-planar *trans*, which have been elucidated through x-ray diffraction studies. A planar *trans* motif is found in the red fluorescent protein, eqFP611, isolated from the sea anemone *Entacmaea quadricolor*[59], which displays one of the largest Stoke's shifts and red-shifted emission wavelength profiles of any naturally occurring Anthozoan fluorescent protein. In contrast, the non-planar *trans* conformation is characteristic of the non-fluorescent chromoprotein Rtms5 from *Montipora efflorescens*[60]. Interactive tutorials on the Olympus FluoView Website examine conformational changes that occur during maturation of the DsRed, eqFP611, and HcRed[61] fluorophores, three proteins that exhibit orange-red to deep-red emission maxima. In addition, tutorials are available for studying the maturation process of the optical highlighters Kaede[39] (having an identical fluorophore with Eos and KikGR) and the Kindling fluorescent protein[62]. Also featured on the FluoView Website is a tutorial on the fluorophore from a yellow fluorescent protein, ZsYellow (originally referred to as zFP538[63]), which was discovered in the button polyp *Zoanthus.* Maturation of the ZsYellow fluorophore results in the formation of a novel three-ring system and peptide backbone cleavage, possibly due to the substitution of lysine for serine (in GFP) as the first amino acid residue in the chromophore tripeptide sequence.

Photobleaching and Photoactivation Kinetics with Fluorescent Proteins

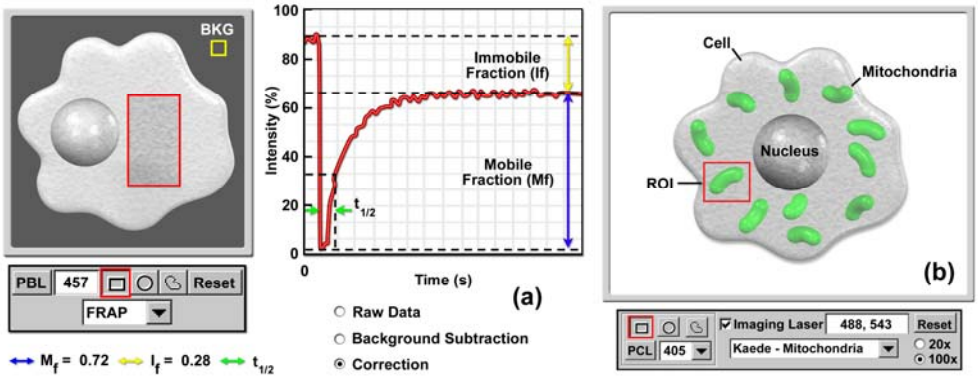

Figure 5. Interactive Java tutorials exploring the dynamics of photobleaching **(a)** and photoactivation/photoconversion kinetics **(b)** in living cells with fluorescent proteins. In fluorescence recovery after photobleaching (FRAP) studies **(a)**, fluorescent molecules in a selected region of interest (ROI) are photobleached and the recovery of fluorescence into the area is monitored as a function of time. Visitors can use the ROI toolbox to select rectangular, oval, or freehand areas, and then click on the photobleach button (PBL) to evoke a simulation of bleaching throughout the region. Recovery of fluorescence and the kinetic parameters are shown in real-time on the accompanying graph. The tutorial demonstrates inverse FRAP, photoactivation, fluorescence loss in photobleaching (FLIP), and spatial techniques. Similar tools are employed for the photoconversion tutorial **(b)**, which details individual organelles (i.e. mitochondria) as candidates for photoconversion or photoactivation. Among the proteins featured in this tutorial are Kaede, Eos, Dronpa, KFP, PA-GFP, and PS-CFP.

Compared to many traditional synthetic fluorophores, which are often toxic or photoreactive, the use of fluorescent proteins is minimally invasive for living cells, enabling visualization and recording of time-lapse image sequences for extended periods of time. Furthermore, continued advances genetically in fine-tuning the properties of fluorescent protein variants have led to increased brightness levels, improved photostability, and significantly better expression in mammalian cells[64]. These factors have stimulated a wide variety of investigations of protein dynamics and function using fluorescent protein chimeras imaged at low light intensities for many hours to extract valuable information about changes in the steady-state distribution. Unfortunately, time-lapse imaging alone is unable to reveal the kinetic properties of a protein to determine, for example, whether it is undergoing association with other components, freely diffusing, or bound to an immobile scaffold. The collective photobleaching techniques, including fluorescence recovery after photobleaching (FRAP and the inverse, iFRAP), fluorescence loss in photobleaching (FLIP), and fluorescence localization after photobleaching (FLAP), have been extremely useful when combined with fluorescent proteins to examine protein dynamics in living cells[65-68], as illustrated in Figure 5. These techniques have enjoyed increasing popularity in the past few years due to the development of laser scanning confocal microscopes equipped with acousto-optic tunable filters (AOTFs) capable of selecting discrete regions of interest that can be selectively bleached with short bursts of high laser power[66]. The various photobleaching techniques are differentiated by the size and location of the bleached region, the required number of bleaching events, and the manner in which recovery of fluorescence is analyzed. The FSU microscopy educational Websites examine photobleaching techniques with review articles, interactive Java tutorials (see Figure 5), and streaming videos.

Selecting Fluorescent Protein Pairs for Resonance Energy Transfer Experiments

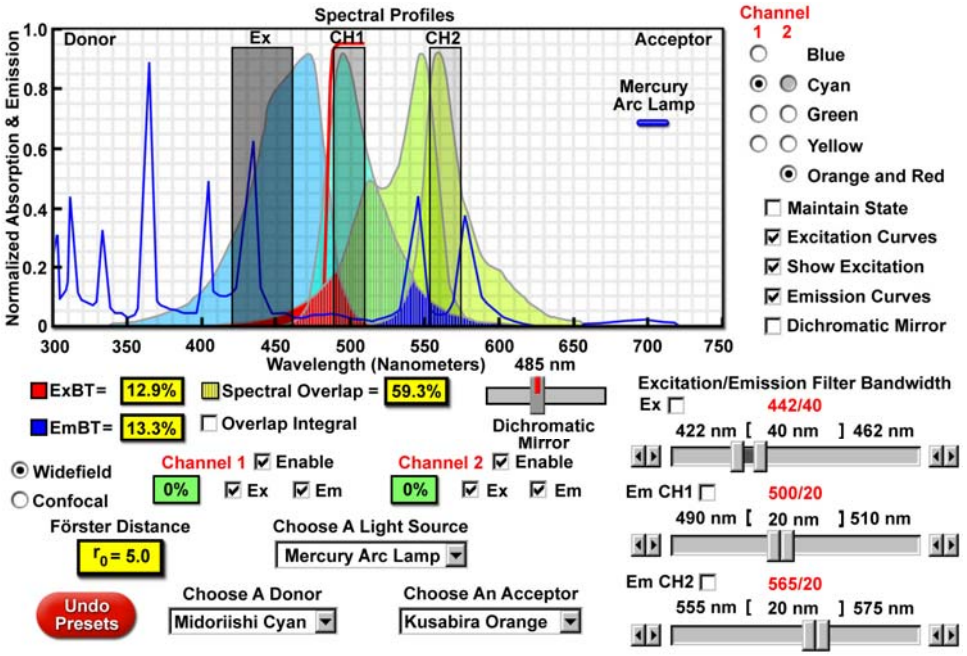

Figure 6. Interactive Java tutorial examining useful fluorescent pairs for resonance energy transfer (FRET) studies. The tutorial initializes with the normalized absorption and emission spectral profiles of the fluorescent proteins, Midoriishi-Cyan and Kusabira-Orange, appearing in the window superimposed over the emission spectrum of a mercury arc-discharge lamp. Visitors can alter the light source (lasers and arc lamps are available) and use pull-down menus to access the numerous fluorescent protein spectral profiles available in the tutorial. As new parameters are chosen by the visitor, several variables, including the excitation and emission bleed-through, spectral overlap, and Förster distance are automatically calculated by the tutorial and displayed in the window. In order to determine the optimum filter combination for a specific pair of proteins, the visitor can choose a dichromatic mirror and adjust the excitation filter profile (width and location), as well as the emission filter profiles for the donor and acceptor. To aid in developing usable filter sets, an Apply Presets button (not illustrated) can be used to provide suggestions for the initial settings.

The precise location and nature of the interactions between specific molecular species in living cells is of major interest in many areas of biological research, but investigations are often hampered by the limited resolution of the instruments employed to examine these phenomena. Conventional widefield fluorescence microscopy enables localization of fluorescently labeled molecules within the optical spatial resolution limits defined by the Rayleigh criterion, approximately 200 nanometers (0.2 micrometers). However, in order to understand the physical interactions between protein partners involved in a typical biomolecular process, the relative proximity of the molecules must be determined more precisely than diffraction-limited traditional optical imaging methods permit. The technique of fluorescence resonance energy transfer (FRET), when applied to optical microscopy[69-71], permits determination of the approach between two molecules within several nanometers, a distance sufficiently close for molecular interactions to occur. One of the major obstacles to the widespread implementation of FRET investigations in living cells has been the lack of suitable methods for labeling specific intracellular proteins with the appropriate fluorophores. The recent development of fluorescent proteins possessing a wide array of spectral profiles (from 450 to 650 nanometers) and the increasing sophistication of protein chimeras (fusions as well as biosensors) has resulted in a number of potential fluorescent protein pairs that are useful in FRET experiments. Several interactive tutorials on the FSU microscopy education Websites (see Figure 6) have been designed to address this issue and should be useful in enabling investigators to choose fluorescent protein combinations that will properly exhibit FRET characteristics and allow for the maximum dynamic range in order to maximize sensitivity when planning experiments.

Examining the FRET Mechanism in Biosensor Fluorescent Proteins

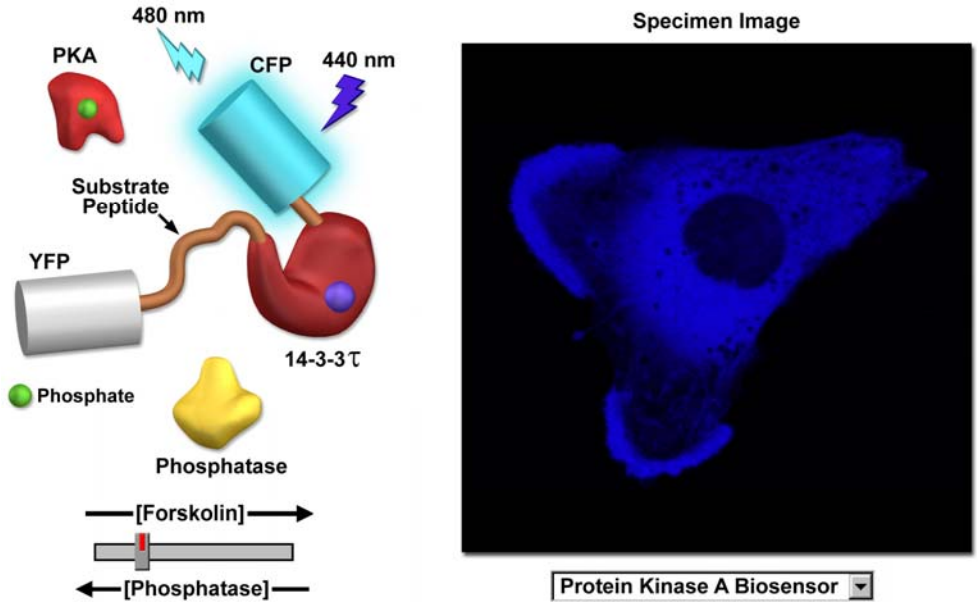

Figure 7. Typical fluorescent protein biosensors are constructed with a sandwich of two dissimilar fluorescent proteins (for example, CFP and YFP) surrounding a central polypeptide region that undergoes conformational changes upon binding a ligand. This interactive tutorial explores the alterations in several popular biosensor fluorescent proteins that occur as a result of ligand (such as calcium, cAMP, chloride, etc.) binding to the interior peptide. In addition to the animated cartoon sequence illustrating conformational changes (left), the tutorial also demonstrates real-time changes in fluorescence profiles as observed in the microscope (right). New biosensors can be selected using the pull-down menu.

An increasing number of biosensor fluorescent proteins[72-75], useful for monitoring a wide spectrum of intracellular processes and critical signaling events, are being reported on a monthly basis. Biosensors are collectively becoming one of the most important tools in cell biology and physiology. Several strategies have been employed in the construction of these chimeric molecules. The most widely utilized approach takes advantage of the sensitivity of resonance energy transfer to changes in the distance and

orientation between donor and acceptor molecules, in particular, fluorescent protein FRET partners. In the general approach, two dissimilar fluorescent proteins (typically CFP and YFP) are attached to the opposing ends of a sensor peptide to monitor changes in the conformation of the sensor through an increased or decreased level of FRET. A single excitation wavelength can be utilized to observe a ratiometric change in the fluorescence output of the biosensor. Another popular second strategy involves altered emission levels induced by modifications to the structure of a circularly permutated fused chimeric fluorescent protein upon binding small ligands. As mentioned above, the emission properties of fluorescent proteins are dictated by the interaction between the tripeptide chromophore and the surrounding amino acid structure of the *beta*-barrel protein. Alterations to the packaging of the fluorophore, either physically or through mutations, usually exhibit dramatic effects on the fluorescence emission properties. Tutorials being developed on the FSU microscopy Websites (see Figure 7) explore the mechanism underlying FRET signals in fluorescent protein biosensors and provide review articles that explain the technology.

4. CONCLUSIONS

Taking advantage of the dynamic nature of the World Wide Web, the microscopy educational Website initiative at The Florida State University has resulted in the production of several interactive sites targeted at entry-level and advanced students in biology, chemistry, and physics. In addition to hundreds of review articles on all aspects of optical microscopy, the Websites contain image galleries, streaming videos, interactive Java tutorials, listings of Internet resources, and reference sections with links to the original research documents. As the attention of the development team is re-focused on topics related to fluorescent proteins, new sections will emerge on the FSU microscopy Websites that are targeted at educational tutorials and review articles on this fascinating arena in cell and molecular biology.

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