

Spectral Imaging Microscopy Web Sites and Data

George McNamara,^{1,2*} Amit Gupta,³ James Reynaert,³ Thomas D. Coates,² and Carl Boswell^{3*}

¹Division of Cancer Immunotherapeutics and Tumor Immunology, Beckman Research Institute, City of Hope National Medical Center, Duarte, California

²Childrens Hospital Los Angeles Saban Research Institute, Congressman Julian Dixon Cellular Imaging Facility, Los Angeles, California

³Department of Molecular and Cellular Biology and The Arizona Cancer Center, University of Arizona, Tuscon, Arizona

Received 24 October 2005; Revision Received 8 April 2006; Accepted 14 April 2006

The Internet is enabling greater access to spectral imaging publications, spectral graphs, and data than that was available a generation ago. The spectral imaging systems discussed in this issue of Cytometry work because reagent and hardware spectra are reproducible, reusable, and provide input to spectral unmixing and spectral components recognition algorithms. These spectra need to be readily available in order to determine what to purchase, how to use it, and what the output means. We refer to several commercially sponsored and academic spectral web sites and discuss our spectral graphing and data sites. Sites include fluorescent dye graph servers from Invitrogen/Molecular Probes, BD Biosciences, Zeiss/Bio-Rad Cell Sciences, and filter set servers from Chroma Technology and Omega Optical. Several of these sites include data download capabilities. Recently, two microscope manufacturers have published on their web sites transmission curves for select objective lenses—crucial data for anyone doing multiphoton excitation microscopy.

The Internet has revolutionized science communication by making information available at a keystroke or mouse click. However, information tends to be spread over many sites and is available in different, and often limited, formats. Scientists using spectral imaging systems need to acquire reference spectra and optical performance data from their own specimens and optics. Even so, the acquired spectra should be compared to reference data to which all scientists have access. Optimizing performance of reagents and instruments is important, as is clear from the 0.0002–0.003 overall fluorescence detection efficiency for a typical mid-1990's confocal microscope, at different pinhole settings (at 600 nm), calculated by Sandison et al. (1). That this can be improved is evident by switching a multiphoton excitation fluorescence microscope from conventional Airy unit 1.0 pinhole descanned detection to a non-descanned detector immediately following the objective lens. Selecting and switching detection paths is straightforward for the automated hardware of current high-end microscopes. If the user has selected illumination wavelength(s), filter(s), or dyes that are not co-optimized, the experiment may yet fail. Even harder to identify is whether

Notable among the academic sites, PhotoChemCAD 2.0 has over 200 dyes and a downloadable database/graphing program, and the USC-A Chemistry UV-vis Database displays absorption spectra of many dyes and indicators used in clinical histology and pathology. Our Fluorescent Spectra graphing/calculator site presents dyes, filters, and illumination data from many of these and additional sources. PubSpectra is our free download site which uses Microsoft Excel files as standardized human/machine readable format with over 2,000 biomedical spectra. The principle that data is not subject to copyright provides a framework in which all scientific data should be made freely accessible. © 2006 International Society for Analytical Cytology

Key terms: fluorescence spectra; dyes; fluorescent proteins; filters; PubSpectra

installed optical components are performing as good as possible (2,3). Lenses get grunged, spectrometer leaves get stuck, filters become solarized, and dyes photobleach, quench, or interact with each other to form nonfluorescent compounds. Full knowledge of the optics, reagents, and specimen can enable users to get the most out of their experiments. While some microscope performance data is, or can be summarized as a single number, many parameters vary over the spectral range utilized in research, develop-

Grant sponsor: City of Hope National Medical Center; Grant sponsor: Saban Research Institute; Grant sponsor: University of Arizona; Grant sponsor: Chroma Technology, Inc.

*Correspondence to: George McNamara, Ph.D., Staff Scientist, Division of Cancer Immunotherapeutics and Tumor Immunology, Beckman Research Institute, City of Hope National Medical Center, 1500 E. Duarte Rd, Duarte, CA 91010.

Carl A. Boswell, Ph.D., Molecular and Cellular Biology, University of Arizona, 1007E. Lowell St, Tuscon, AZ 85721-0106.

E-mail: gmcnamara@coh.org, Geomcnamara@earthlink.net, cboswell@email.arizona.edu

Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/cyto.a.20304

ment, and manufacturing. This range encompasses the UV absorption maxima of nucleic acids and proteins and many small organic molecules, visible range defined by our eyes (380–700 nm), to the near infrared of specialized imaging modes (IR-VEC-DIC, silver grain quantitation, near infrared fluorescence and, *in vivo* bioluminescence) and most multiphoton excitation microscope illumination lasers. Performance—or lack thereof—of lenses in the infrared have been commented on a quarter century ago (4).

There has been no systematic effort to make photonic data available. This contrasts with the genome and microarray fields where data release is standard. Several enlightened companies and academics provide spectral data on their web sites (Table 1) or by personal request, while others, most notably the major microscope companies, have not systematically posted data. The major source of biomedical fluorescence dyes, Invitrogen/Molecular Probes, has led the way by providing both a graphical spectra viewer and ASCII text file download capability for many of its dye products. The two major optical filter companies, Chroma Technology and Omega Optical, have provided static or interactive graphs, respectively, and both provide ASCII text file download capability for many of their filters. Many other companies provide, for marketing purposes, spectra graphs of limited utility. Fortunately, graphs can be reverse engineered to numeric data by inexpensive software such as Un-Scan-It (<http://www.silkscientific.com>). Of particular significance, the major microscope companies have not systematically provided optical transmission curves or parfocality curves for their optics, thus limiting the ability of users to profile their entire microscope system from light source to specimen to detector. Keller shows a few graphs from a few Zeiss lenses (5) and Olympus and Zeiss have recently posted transmission curves for a few of their objective lenses (Table 1). The spectral confocal microscope manufacturers have limited and simplistic performance criteria that mask how irreproducible performance is between imaging sessions and between instruments (2,3).

As more multiphoton excitation microscopes are used, obtaining manufacturer's optics performance data will become ever more important because the utility of any dye or dye combination may be compromised by inefficient/suboptimal transmission of the objective lens and/or accessory lenses and filters. Camera and photomultiplier manufacturers often provide quantum efficiency curves as marketing graphs, but not as data. Several academic groups, notably Lindsey's PhotoChemCAD and Goldsmith's UCSA Chemistry UV-VIS Database, have acquired spectra of dozens to hundreds of dyes and made the data available on the Internet. Tsien and Campbell have provided spectra on request and Tsien has systematically included new fluorescent protein spectra graphs in publications. In this communication, we summarize the content of several Internet sites. We focus on our graphical web server, which integrates the available datasets with a common display, enabling a user to co-graph dye and filter spectra and to calculate performance indices. The data site, PubSpectra, includes over 2,000 spectra, with the data in standard Microsoft Excel spreadsheet format, which is human/

machine readable, and empowers the user to use the data. Once users have downloaded the data, they can perform their own calculations and generate graphs in Excel or other software packages.

SPECTRA WEB SITES OVERVIEW

There are a variety of web sites that display dye spectra, filter sets, and light sources, of which several are listed in Table 1. The PubSpectra website has an index file that includes the full reference plus summary data for every dye, filter, and light source spectra. Table 1 notes which major sites data has been added to PubSpectra. Our collaboration grew out of CB's original Spectral traces site, http://www.mcb.arizona.edu/IPC/spectra_page.htm, with a small set of dyes, filters, and light sources focusing on the capabilities of a Nikon PCM 2000 confocal system. The Invitrogen/Molecular Probes site offers both an interactive viewer and a static graph/text data download site. The Invitrogen data is spectrofluorometer text file output. The BD Biosciences web interactive graphing site evolved from an academic flow cytometry facility site. The Zeiss/Bio-Rad CellSciences has both an interactive single photon interactive graph server and a static source of multiphoton absorption cross-section graphs. Our graphing and data sites are the most comprehensive and are discussed below.

Commercial Sites

Of the microscope manufacturers, Zeiss and Olympus have filter and a few objective lens transmission spectra available on their sites. Leica has JPEG graphs of 40 of their filter sets, along with a table listing characteristics of their objective lenses. A PDF file with Nikon filter set spectra is available from a dealer. Most fluorescence microscopy filter sets are now produced by Chroma, Omega, or Semrock, but the four microscope manufacturer's filter data enlightens the older literature.

Chroma Technology uses static graphs for each filter set and most filter set pages include text download links. The Omega Optical site features their Curv-o-matic interactive graph server and most filter sets have the data available for download. Semrock has filter sets for fluorescence and other applications, as both web graphs and ASCII text file downloads. Schott Glass' web site formerly had a Windows program and database (Filter 99) with spectra of their filters, such as the classic BG38 near infrared blocking filter. More recently Schott has posted Filter 2004 as a Microsoft Excel file. Schott has also posted an Excel file with performance data for over 100 types of glass (optical glass catalog, Excel file), along with technical data on glass refractive indices, dispersion and fluorescence. A nice feature is that their entire Optical Glass Filters sub-web can be downloaded as a single Zip file and extracted onto the users computer (Optical Glass Filters—Data sheets, calculation prog. [http://www.us.schott.com/optics_devices/english/download/optical_filters_download_vers.zip]). Schott filter spectra graphs are posted on many photography, astronomy, and filter supply companies websites.

Table 1
Spectral Web Sites

Host	URL	Spectra graphs	Data	Data in PubSpectra
Carl Boswell/George McNamara (Fluorescent spectra & PubSpectra)	http://www.mcb.arizona.edu/IPC/fret/default.htm (introduction)	Interactive	Text notes	Y
	http://www.mcb.arizona.edu/IPC/fret/indexb.html (graph)	-	Excel sets	(source)
	http://home.earthlink.net/~pubspectra/ (download site)	-	Excel sets	(source)
Carl Boswell (Spectra Traces)	http://www.mcb.arizona.edu/IPC/spectra_page.htm	Interactive	n/a	n/a
Invitrogen/Molecular Probes	http://probes.invitrogen.com/resources/spectraviewer	Interactive		Y (next row)
	http://probes.invitrogen.com/servlets/spectra	Static	Text	Y
	http://probes.invitrogen.com/resources/education	n/a	Tutorials	n/a
BD Biosciences	http://www.bdbiosciences.com/spectra	Interactive	-	
	http://www.bdbiosciences.com/pharming/en/protocols/Fluorochrome_Absorption.shtml (descriptions and graphs for 11 dyes)	Static	-	
Zeiss/Bio-Rad Cell Sciences	http://microscopy.bio-rad.com/fluorescence/fluorophoreDatab.htm	Interactive	-	
	http://microscopy.bio-rad.com/products/multiphoton/Radiance2100MP/mpspectra.htm (multiphoton spectra from Dr. Warren Zipfel, Cornell Univ.)	Static	-	
Zeiss objective lenses	https://www.micro-shop.zeiss.com/us/us_en/czshop.php?cp_sid=244937fedf3&cp_tpl=main (257 lenses 12/2005, a few with graphs) http://www.micro-shop.zeiss.com (registration)	Static (BMP's)	-	
Zeiss filter sets	http://www.zeiss.de/C12567BE0045ACF1/Contents-Frame/119C8228E3B53862C125694A004CCFE0 (42 filter sets with PDF graphs)	Static (PDF's)		
Olympus objective lenses	http://www.olympusamerica.com/seg_section/uis2/seg_uis2.asp	Static (GIF's)		In progress
Olympus filter sets	http://www.olympusamerica.com/seg_section/uis2/seg_uis2_mirror.asp	Static (GIF's)	Text (CSV's)	In progress
Leica objective lenses	http://www.fluorescence-microscopy.com/LMSAccessories.nsf/905ad14d696d62c7c1256fc50032d57a?OpenView (196 lenses listed, no spectra)	(no spectra)		
Leica filter sets	http://www.fluorescence-microscopy.com/LMSAccessories.nsf/03fcd3e17cbae46ec1256fc5002e1904?OpenView (15 JPG files of 40 filter sets listed)	Static (JPG's)		
Nikon filter sets	http://www.techinst.com/pdfs/FluorescenceFilterBlocks2005.pdf (30 filter sets, Hg, Xe lamps)	Static (PDF)		
Chroma Technology	http://www.chroma.com/products/index.php	Static	Text	Y
	http://www.omegafilters.com/front/curvomatic/spectra_062904.php	Interactive	Text	Y
Semrock	http://www.semrock.com/Catalog/BrightlineCatalog_Microscopy.htm	Graphs	Text	Y
Schott	http://www.us.schott.com/optics_devices/filter/english/us/index.html (Filter 2004 Excel file/web download site)	Excel data, PDFs	Save as Text	Y
PhotoChemCAD	http://www.photochemcad.com	Windows program	Database/Text	Y (v1)
Goldsmith/UCS-A UV-VIS Database	http://www.usca.edu/chemistry/spectra (rename .p7b to .spc; open .spc files with Galactic DataViewer or Spekwin32 (8); use SpekWin32 to Save As text)	Download	Text (Spekwin32)	Y
Spekwin32 (8)	http://www.agdal.chemie.uni-konstanz.de/menges/spekwin/spekwin_beschr_en.html	Windows program	Database (minimal)	

Table 1
Spectral Web Sites (continued)

Host	URL	Spectra graphs	Data	Data in PubSpectra
R.Y. Tsien & R.E Campbell	http://www.tsienlab.ucsd.edu/Publication.htm http://www.chem.ualberta.ca/~campbell/publications/publications.html http://www.tsienlab.ucsd.edu/SUBWEB%20-%20Samples/ (sample request information) http://www.tsienlab.ucsd.edu/SUBWEB%20-%20Samples/Constructs.htm (available FPs)	PDFs	Graphs in PDFs	Y
Molecular Expressions	http://www.microscopyu.com/tutorials/java/fluorescence/fpfilterfinder/index.html http://www.microscopyu.com/articles/fluorescence/filtercubes/filterindex.html http://www.olympusmicro.com/primer/java/fluorescence/opticalpaths/index.html	Interactive Static Interactive (Olympus filter sets)	- - -	

Non-Profit Sites

PhotoChemCAD is a download site for the PhotoChemCAD 2.0 program and database with over 240 dyes (6,7). A major strength of PhotoChemCAD is the wealth of data on porphyrins, a research interest of the site's principal investigator, Prof. J.S. Lindsey. The PhotoChemCAD version 1.0 data are in PubSpectra and the new 2.0 data will be added. The PhotoChemCAD database includes the ability to graph spectra and calculate fluorescence resonance energy transfer (FRET) efficiency between dyes. The Goldsmith/UCSA site displays absorption dye spectra, and with file extension surgery and third party programs (PhotoChemCAD 2.0 or Spekwin32) access to the data. The download files have a .p7b extension. These can be opened in Galactic DataViewer (which at one time was freely available). The .p7b extension can be changed to .spc and the files opened in SpekWin32 (8), which includes a few spectra. Fifty-eight UCSA spectra from thirty-five dyes are available in PubSpectra. Prof. Roger Tsien has developed many fluorescent proteins in the past decade. Nearly all his lab's peer-reviewed publications are on his web site, along with information on obtaining plasmids. We contacted Prof. Tsien, and he and Prof. Robert Campbell provided data for many fluorescent proteins. We used Un-Scan-It to digitize more recent spectra (fruity fluorescent proteins). The Molecular Expressions interactive spectra are scattered among different sub-sites, three of which are listed in Table 1. We contacted many of these—and other—sources, obtained comprehensive data sets, organized them into a common format in Microsoft Excel, and set up our own interactive web graph server and PubSpectra data download site. The PubSpectra site and data sets include full references to the source of each spectrum.

FLUORESCENT SPECTRA WEB SITE

The authors' own site, Fluorescent Spectra, An interactive, exploratory website, has the most comprehensive spectra database. The site grew out of CB's Spectra Traces site combined with GM/TDC's collection of fluorescent dye and filter data assembled to evaluate filters to purchase for a new image core facility. The Fluorescent Spec-

tra site can graph a multitude of fluorescent dyes, lamps, lasers, excitation filters, dichroic mirrors, and emission filters. The default graphing page displays these data over the range of 300–850 nm and can display any number of spectra. The user selects a data source by moving the cursor over a data type button, and then picking a component from a drop down list. Moving the cursor over a trace highlights that trace and displays the trace name and data type over the graph. Clicking on a trace turns it off. Dyes may have one, two, or three trace components depending on whether emission, absorption and/or excitation data are available. Lamps, lasers, and filters are single traces. Traces are color coded by type, with color legends next to the drop down list boxes used for selection. Clicking on the Notes button opens a text field that summarizes spectral maxima, dye category, and references. The Notes text can be copied to any text editor. These notes, and much additional data, are available in the PubSpectra index and spectra files. Figure 1A illustrates the range of dyes that can be graphed together in the Default graph viewer. The dye names above each trace were added for publication. When using the website, the name for any trace appears above the graph when the cursor is moved over the trace. Figure 1A includes excitation and emission spectra of DAPI, Alexa Fluor 350, Alexa Fluor 488, Cy3B, Alexa Fluor 594, Alexa Fluor 647, Alexa Fluor 700, and Alexa Fluor 750. The Alexa Fluor dyes are available from Invitrogen; Cy3B from GE Healthcare (formerly Amersham Biosciences); DAPI is available from many suppliers.

Calculating System Efficiency

The Fluorescent Spectra site also offers a Calculation mode (Fig. 1B–1F). In Calculations mode, only one dye, one light source, either lamp or laser, and one triplet of excitation filter, dichroic mirror, and emission filter can be selected at one time. By selecting one of each type, the web site calculates and displays numerical performance data for the light source, dye, and filter triplet. The user can click the Save Results button to save the data to the Notes field to copy and paste. The user can then change one or more fields, then copy and paste the recalculated results for comparison. The complete calculations are only

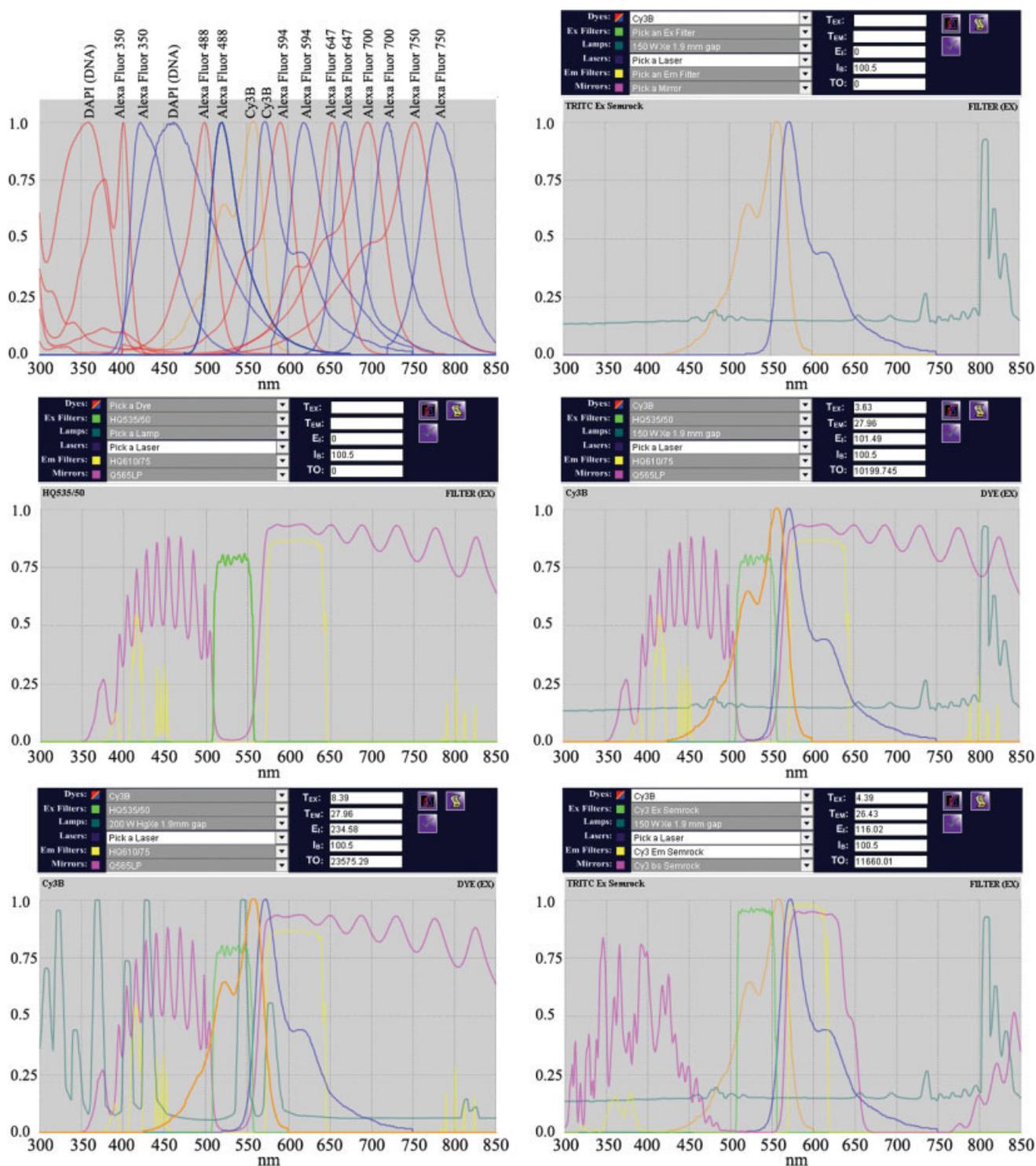


FIG. 1. **A** shows the Fluorescent Website Graphing page display of eight fluorescent dye excitation and emission traces from a wide spectral range of dyes. **B-F** show the Fluorescent Website Calculations page displays for Cy3B, Xenon or Mercury/Xenon arc lamps, and various filter sets (discussed in text). The figure axes text has been enlarged from the website to improve readability. The Y-axis scale is normalized to the spectral peak (dye excitation and emission traces), or fractional transmission (filters). Lamp spectra are normalized to the peak emission of the 200 W HgX1 1.9 mm gap arc lamp data from OptiQuip (Ushio bulbs). Web site laser lines display wavelength position, but do not display relative power.

performed if a dye has extinction coefficient (QY) and quantum yield (ϵ) values associated with it in the underlying database. The extinction coefficient is how well a dye absorbs at its absorption maximum. Quantum yield is the ratio of photons emitted as fluorescence and the photons absorbed. These values should be appreciated for what they are, and not accepted as constant under all conditions. Each is defined as a photophysical parameter from a spectrophotometer or spectrofluorometer. For example, fluorescein is most often reported to have an extinction coefficient of $81,000 \text{ M}^{-1} \text{ cm}^{-1}$ at 490 nm (ϵ_{490}) and QY of 0.9 at pH 9. Measurements need to be done in dilute solutions (absorption < 0.05, lower is better, if the instrument is sensitive enough) to avoid running afoul of the Beer-Lambert Law at which point the spectrum will be marred by self-absorption (dye dimers and multimers may also form, producing quenching and/or new spectra). The performance of a free dye in solution in its optimal solvent at its optimal pH may not be relevant when bound to an antibody, avidin, or nucleic acid probe, or in a different pH or ionic environment. For example, a preparation of IgG antibody (150 kD) with 10 fluorescein molecules will not be 10 times brighter than one fluorescein per antibody (maximum fluorescence is at ~3 fluorescein:IgG). Similar sublinear gains are observed with GFP multimers, e.g. of 2xGFP, 3xGFP, 4xGFP vs. 5xGFP fusion proteins (9). Conversely, other dyes increase dramatically in fluorescence when bound to an appropriate target. DAPI, Hoechst 33258, Hoechst 33342, and most other DNA dyes (Yo-Pro-1, To-Pro-3, and other "o-Pro" family members) have weak fluorescence in solution and become brightly fluorescent bound to DNA, both because of solvent shielding and binding leads to a high local dye concentration at the DNA. The signal-to-noise ratio of many DNA counterstains is often limited more by the autofluorescence of the specimen, optics, and immersion oil, than by free dye. Some "DNA" counterstains can bind to RNA. This is typically handled by using DNase or RNase to keep only one type in a tissue. Where information is available, the conditions under which the values were determined are noted.

The calculations performed on the web site are defined below [Eq. (1-5)].

$$\text{Excitation Throughput}(T_{\text{EX}})T_{\text{EX}} = \text{lamp output} \times \text{filt} \times 1 - \text{DM} \times \text{F1. dye ex} \quad (1)$$

$$\text{Emission Throughput}(T_{\text{EM}})T_{\text{EM}} = \text{F1. dye em} \times \text{DM} \times \text{Em filt} \quad (2)$$

$$\text{Instrument Efficiency}(E_1)E_1 = T_{\text{EX}} \times T_{\text{EM}} \quad (3)$$

$$\text{Brightness Index}(I_B)I_B = (\epsilon \times \text{QY}) / 1,000 \quad (4)$$

$$\text{Theoretical Output(TO)TO} = \text{IE} \times \text{BI} \quad (5)$$

DM = dichroic mirror QY = fluorescent dye quantum yield ϵ = dye Extinction coefficient

If either QY or ϵ or both are not available, a value of "1" is used in the TO calculation. If a laser line is selected, it

replaces lamp output in Eq. (1). However, lamp output results should not be compared to laser line results because the former is a continuum of wavelengths while the latter is a single wavelength (see below).

Equations (1) and (2) use the spectral curves of each component involved to calculate the products. Calculations, in effect, provide the integral under overlapping curves. Excitation throughput (T_{EX}) multiplies the lamp spectral output and the excitation filter spectrum (practically, the bandwidth of the shortpass or bandpass exciter filter), and 1-dichroic mirror transmission spectrum (the "1-" is because the dichroic is acting as a reflection surface, not transmission), then multiplied by the dye excitation spectrum (absorption spectrum if the excitation spectrum is not available). This product involves both the instrument (lamp and filters) and dye. Emission throughput (T_{EM}) multiplies the fluorophore emission spectrum by the dichroic beamsplitter transmission spectral curve multiplied by the emission filter spectral curve. Instrument efficiency (E_1), for the selected fluorophore, is the product of the excitation and the emission throughputs.

The (I_B) was popularized by Tsien and Waggoner (10). A dye with very good ϵ and QY will have a $I_B \sim 100$. Brightness index is the product of the dye extinction coefficient and fluorescence quantum yield. Brightness Index is a convenient way of indicating how good a fluorophore can be but is not the whole story. We emphasize that I_B is not a photophysical constant. I_B can vary with solvent, dye-target, and dye-dye interactions. This can be seen with FLAsH (fluorescein bis-arsenate) bound to proteins containing CCXXCC amino acid sequences. Griffin et al. (11) reported $\epsilon = 41,000$ and QY = 0.2 for the original generic CCXXCC sequence, resulting in $I_B = 8.2$. Adams et al. (12) found higher affinity peptides (core: CCPGCC) and quantum yield of the FLAsH:peptide rose to QY = 0.5 for $I_B = 20.5$ (the web site uses the Griffin ϵ). Tsien (13) mentioned the evolution by FACS screening for peptides that produce brighter fluorescence of FLAsH or the related ReAsH (best as of January 2005: FLNCCPGCCMEP).

Theoretical output is intended to let the user compare TO's when varying one or a few parameters. This is explored in Figure. The numbers should be taken as approximations both because of real life performance of the dyes and optics, and because we have not exhaustively validated the output of the calculations. Users who need hard numbers are advised to download the spectral data from PubSpectra (all the spectra on the Graphing site are on PubSpectra, the converse is not true) and perform calculations in Microsoft Excel or other spreadsheet/graphing programs. The Graphing site includes "dummy" filter, mirror, and dye (constant value 1.0 each) to allow some items to be maximized across the spectrum and allow efficiency to be determined for widely disparate components.

Figure 1 shows how different instrument parameters can be compared both graphically and numerically. Figure 1B shows the spectra of Cy3B (GE Healthcare) and a Xenon arc lamp. The Cy3B $I_B = 100$. Xenon arc lamps have nearly uniform light output from <300 to >700 nm. This feature often simplifies filter selection: a 20 nm wide exci-

tation filter will transmit twice as much light as a similar spectrally shaped 10 nm wide filter. Figure 1C has spectral traces for the Chroma HQ535/50 excitation filter, Q565LP dichroic mirror and HQ610/75 emission filters. Both the Q565LP (pink trace) and HQ610/75 (yellow trace) filters have significant transmission at less than 500 nm. Because the HQ535/50 (green trace) excitation filter is the first filter the light reaches, wavelengths <500 nm are blocked before they reach the dichroic mirror, specimen, dichroic beamsplitter again, or emission filter. Chroma and other manufacturers also offer "out of band blocking" (and other options) for a small additional fee.

Figure 1D combines the data of 1B and 1C, with $TO = 10,200$ for Cy3B, this Xenon arc lamp, and combination of the three Chroma filters. Figure 1E shows that switching to a Mercury/Xenon arc lamp more than doubles the theoretical output to $TO = 23,575$. This is because Mercury ions have an emission peak in the range of the excitation filter. The comparison is not perfect because the Xenon arc lamp is 150 W and the Mercury/Xenon is 200 W. TO 's are useful starting points to evaluate dyes and hardware, but the user needs to recognize limits. In real life use, the brightness at the microscope depends on the size of the arc (the Xenon arc lamp whose spectrum is traced has a 1.9-mm gap vs. 2.2-mm gap for the Mercury/Xenon arc) and the alignment of the arc lamp, its mirror image, the focus of real and mirror image arc, and age of the bulb will affect light output. Many current arc lamps eliminate alignment, arc position, and focus issues by coupling the lamp to the microscope with a liquid light guide or fiber optic (for example, Sutter Instruments LS300 Xenon lamp and EXFO X-Cite 120 metal halide lamp—age and kinking of the light guide or fiber can affect throughput, as can focus of the input and output optics).

Figure 1F graphs the Semrock Cy3 filter set (matched excitation, dichroic mirror, and emission filters) excited with the Xenon arc lamp, $TO = 11,660$ vs. Mercury/Xenon arc lamp $TO = 26,934$. How high can TO go? Ideally, for a single dye experiment, we would like to collect all emission photons. This can be done by using simple long-pass filters for the dichroic and emission filters. Using the Chroma HQ535/30 excitation filter, the Q565LP dichroic mirror and a Chroma E570LP emission filter results in $TO = 14,147$ for the Xenon arc lamp and $TO = 32,699$ for the Mercury/Xenon arc lamp. The ultimate in performance on the emission side is to do away with the emission filter. Using the same excitation and dichroic mirrors with the Dummy Em filter results in $TO = 17,420$ for the Xenon and $TO = 40,262$ for the Mercury/Xenon arc lamp. However, longpass or no emission filter may permit near infrared light (heat) to reach the detector. This can be especially problematic with Xenon arc lamps (the Sutter Xenon LS300 with liquid light guide avoids this problem with a cold mirror in the arc lamp housing, which prevents near infrared light from cooking the light guide). One spectral imager that benefits from no emission filter is the Lightform Inc.'s PARISS, whose spectral dispersion configuration is such that any residual excitation light or heat goes to a different part of the detector from the emis-

sion signal (see Lerner, this issue). The fourfold difference between typical bandpass emission filter and no emission filter should be weighted against possible excitation light leaks as well as the reality of broad autofluorescence and that most experiments involve multiple fluorophores that need to be separated with sequential filter sets. The Fluorescence Spectra site enables users to evaluate these needs both qualitatively on the Graphing page and quantitatively with the Calculations page.

PUBSPECTRA

PubSpectra is the web download site for our complete set of data in Microsoft Excel. As of August 2005, the PubSpectra website had 1,260 dyes and fluorescent proteins, 770 filters, and 85 lamps and miscellaneous spectra available for download. Most of the data was obtained on request. In particular, we cite Molecular Probes, Chroma Technology, Omega Optical, Jonathan Lindsey (Photo-ChemCAD), Dyomics, Jack Goldsmith (UCS-A Chemistry UV-VIS Database), and Robert Campbell/Roger Tsien as being major data providers. The PubSpectra website and contents lists all the data sources. In addition to obtaining numerical data on request, the U.S. Supreme Court's observation that data is not subject to copyright (14) provided the rationale to digitize spectra from published graphs. We used Un-Scan-It 5.0 and later the color-capable version 6.0, from Silk Scientific, Inc. (Orem, UT, <http://www.silkscientific.com>). The choice of Excel was pragmatic in that it was easy to add data, normalize all spectra to common units (0.0–1.0), zero out any negative and missing values, and add various details into the header. To make the spectral values easy to keep track of, the spectral data are in rows 200–1200 (row 200 = 200 nm, etc.). The first 179 rows are the header, of which only the first 99 are currently in use (rows 100–179 of some sheets are being used to evaluate ways to reorganize the header information; rows 180–200 of a few datasets contain short wavelength spectra). The first 99 header rows are reproduced in transposed form (columns instead of rows) in the index file.

In addition to the PubSpectra web site, the Excel files were provided to J. Paul Robinson for inclusion on the PUCL 2005 Microscopy 3 CD-ROM distributed at the Microscopy Society of America meeting in August 2005 (<http://www.cyto.purdue.edu/flowcyt/cdseries.htm>—the CD files are McNamaraBoswelldyes.zip, McNamaraBoswellfilterslampsothers.zip, McNamaradata.zip, MPmicro.doc, MPmicro.pdf, and SpeissBestvatermultiphoton.zip). We anticipate that all PubSpectra related data will be distributed on PUCL microscopy CD-ROM's.

The PubSpectra website is the host for links to several additional resources.

The Szollosi/Gabor FRET Calculator Excel file (<http://home.earthlink.net/~fluorescentdyes/McNamara20050709FRETJanosSzollosiHorvathGaborFRETcalculator.xls>) enables an user to take fluorophore spectra from PubSpectra file(s) and compute FRET R_0 values. Our goal is to incorporate a similar option for FRET calculations into the site. GM modified the original worksheet by adding a table

computing R_o values for ranges of dipole orientation ($Kappa^2$) vs. refractive index. Many researchers use $Kappa^2 = 2/3$, assuming time averaged random orientation of donor and acceptor dipoles. While this is an adequate assumption for small fluorophores diffusing freely in a low viscosity solution, it is still an assumption. Many publications that calculate R_o fail to mention what refractive index value they used. Others seem to pick any number from 1.33 (water) to >1.5 (immersion oil? solid protein?). The most useful set of values for mammalian cell cytoplasm is 1.34–1.38 (15). Johnsen and Widder (16) reviewed refractive indices for various mammalian organelles (plants, protozoa, bacteria, and archaea being less publicized). Additional data and references can be found in the refractive index section of McNamara (17).

The Fluorescent Proteins Data Excel file (see <http://home.earthlink.net/~pubspectra> Non-Spectral Data Links section) contains excitation and emission maxima, extinction coefficients and quantum yields, data on amino acid sequences, relationship to earlier fluorescent proteins, and primary reference (see PubSpectra for spectral data on some of these fluorescent proteins, mostly obtained from Tsien and colleagues). The Fluorophore Data Table Excel file contains 4,950 rows of fluorophore data, including excitation and emission maxima, extinction coefficients and quantum yields, chemical name, molecular weight, references and dye manufacturers (a few fluorescent protein data are also present). These two data sets have been collated from primary, review literature, text and specialized books, and researcher and supplier web sites. We believe these Excel files are the most comprehensive, freely available collection of data available on the Internet for these categories. The Objective Lens Tables Excel file lists basic facts on microscope optics from each of the four major manufacturers (Leica, Nikon, Olympus, and Zeiss). Unfortunately, the most useful data, spectral transmission and parfocality curves, of microscopy objective lenses and other optics is mostly not available (we plan to add the available Olympus and Zeiss transmission curve data to PubSpectra during 2006, along with PMT, CCDm human, and other visual response curves). The PubSpectra Excel files are stored in compressed ZIP files to speed downloads and conserve space (several commercial, shareware, and freeware unzipping software are available for each major computing platform). The Excel files are currently scattered over several personal websites, to save money. This is in keeping with the low cost of the entire project: Except for one financial donation by Chroma Technology, all of these efforts were done as part of our everyday work or on our own time (the University of Arizona was especially generous with A.G. and J.R.'s time for web development). We welcome others reposting the files on their own sites (please refer the Fluorescent Spectra and PubSpectra sites and tell us about your site).

Data Is Not Copyrightable

During the course of developing this data, one of us had an epiphany while reading in Lessig (18) about a U.S.

Supreme Court decision: data is not subject to copyright (14). Text and commentary about Feist can be found on many legal web sites by doing a Google search. Indeed, the broad availability of the text of Supreme Court decisions is because they are not subject to copyright. The Feist decision reaffirmed the U.S. Copyright act of 1976 that "there can be no copyright in facts". The basis for the Feist decision can be found in the U.S. Constitution.

Several hundred of the PubSpectra traces were digitized by un-scanning. Some of this was because digitizing one or a few traces from well organized published figures is easy. Other data was digitized because the corresponding author never responded, was unable to find, or declined a request for data (contrary to grant public sharing requirements for NIH and NSF funded projects, see 19). Several companies and researchers have been generous with making spectral data available, while others of the microscopy, flow cytometry, and spectroscopy communities not. This contrasts with the floods of data made available by the genome and microarray communities. Ironically, both of the latter communities have been successful because of the same light sources, optics, fluorescent dyes, and detectors used in fluorescence microscopy. We encourage all researchers to publish numerical spectral data as supplementary data to their online journal article and/or post the data on their own web site. We also encourage editors and reviewers to ask that all data be included as supplemental material at the time of manuscript review. The principle that data is not subject to copyright provides a framework in which all scientific data should be made freely accessible.

CONCLUSIONS

Other articles in this issue focus on particular instruments, fluorophores, techniques, and algorithms for spectral imaging. We describe Internet resources for finding spectra, and describe the use of our Fluorescent Spectra Graphing/Calculator web site in the context of typical microscopy projects.

ACKNOWLEDGMENTS

We thank Chroma Technology for their donation to the University of Arizona for Fluorescent Spectra web site development. We are grateful to our many data sources for data. Thanks to Erich Zeiss for pointing us to the Olympus objective lens spectra site.

LITERATURE CITED

1. Sandison DR, Williams RM, Wells KS, Strickler J, Webb WW. Quantitative fluorescence confocal laser scanning microscopy (CLSM). In: Pawley JB editor. Handbook of Biological Confocal Microscopy, 2nd ed. New York: Plenum; 1995. p 39–53.
2. Lerner JM, Zucker RM. Calibration and validation of confocal spectral imaging systems. *Cytometry A* 2004;62:8–34.
3. Zucker RM, Lerner JM. Wavelength and alignment tests for confocal imaging systems. *Microsc Res Tech* 2005;68:307–319.
4. Hugins WA. Near-infrared microscopy. *Science* 1980;207:359.
5. Keller HE. Objective lenses for confocal microscopy. In: Pawley JB editor. Handbook of Biological Confocal Microscopy, 2nd ed. New York: Plenum; 1995. p 110–126.
6. Du H, Fuh RA, Li J, Corkan LA, Lindsey JS. PhotoChemCAD: A computer-aided design and research tool in photochemistry. *Photochem Photobiol* 1998;68:141,142.

7. Dixon JM, Taniguchi M, Lindsey JS. PhotoChemCAD 2: A refined program with accompanying spectral databases for photochemical calculations. *Photochem Photobiol* 2005;81:212,213.
8. Menges FS. http://www.agdal.chemie.uni-konstanz.de/menges/spekwin/index_en.html; 2006.
9. Bulinski JC, Odde DJ, Howell BJ, Salmon TD, Waterman-Storer CM. Rapid dynamics of the microtubule binding of ensconsin in vivo. *J Cell Sci* 2001;114:3885-3897.
10. Tsien RY, Waggoner A. Fluorophores for confocal microscopy: Physics and photochemistry. In: Pawley J, editor. *Handbook of Biological Confocal Microscopy*, 2nd ed. New York: Plenum; 1995. p 267-279.
11. Griffin BA, Adams SR, Tsien RY. Specific covalent labeling of recombinant protein molecules inside living cells. *Science* 1998;281:269-272.
12. Adams SR, Campbell RE, Gross LA, Martin BR, Walkup GK, Yao Y, Llopis J, Tsien RY. New biarsenical ligands and tetracysteine motifs for protein labeling in vitro and in vivo: Synthesis and biological applications. *J Am Chem Soc* 2002;124:6063-6076.
13. Tsien RY. Building and breeding molecules to spy on cells and tumors. *FEBS Lett* 2005;579:927-932.
14. Feist Publications, Inc. v. Rural Tel. Serv. Co. 1991;499 U.S. 340.
15. Oheim M. Imaging transmitter release. II. A practical guide to evanescent-wave imaging. *Lasers Med Sci* 2001;16:159-170.
16. Johnsen S, Widder EA. The physical basis of transparency in biological tissue: Ultrastructure and the minimization of light scattering. *J Theor Biol* 1999;199:181-198.
17. McNamara G. Multi-Probe Microscopy. <http://home.earthlink.net/~mpmicro/MpMicro.zip>; 2006.
18. Lessig L. *The Future of Ideas*. New York: Random House; 2001. p 368.
19. Cech TR, Eddy SR, Eisenberg D, Hersey K, Holtzman SH, Poste GH, Raikhel NV, Scheller RH, Singer DB, Waltham MC. Sharing publication-related data and materials: Responsibilities of authorship in the life sciences. Washington, D.C.: National Academy of Sciences; 2003. p 170.