

Supporting A (list of microscope parts)

(all prices 2004)

Name	Company	Item#	Quantity	Total price	# in Fig. 1
Green laser pointer	E-bay	N/A	1	\$50	1
100x/1.25NAobjective	Edmund	NT43-909	1	\$185	6
Eyepiece	Edmund	NT36-130	1	\$57	17
Dichroic 550dextr	Chroma	107447	1	\$175	11
Barrier filter HQ555lp	Chroma	55652	1	\$250	12
100% Al mirror	Chroma	21004	1	\$100	13
50/50 Al mirror	Chroma	21000	1	\$100	15
Plan-convex lens	Thorlabs	LA1608	1	\$19.50	5
Plan-convex lens	Thorlabs	LA1433	1	\$18.30	14
ND filter set	Thorlabs	NEK01	1	\$440	4
B/W CCD camera	Supercircuits	PC164C	1	\$120	19
Video capture card	ATI	TV wonder VE	1	\$50	
Breadboard	Thorlabs	MB12	1	\$162	
Mount with mirror	Thorlabs	KM100-E02	2	\$224	3
Z translator	Thorlabs	SM1Z	1	\$164	7
Translation stage	Thorlabs	MS1	1	\$160	2
XY translator	Thorlabs	HPT1	1	\$149	7
Slip plate positioner	Thorlabs	SPT1	1	\$51	
Cage plate	Thorlabs	CP02	3	\$48	
30 mm cage cube	Thorlabs	C6W	3	\$177	
Rotatable cover plate	Thorlabs	B3C	3	\$66	
1" optic mount	Thorlabs	B5C	3	\$87	
Extension rod	Thorlabs	ER8	2	\$19.50	
Extension rod	Thorlabs	ER6	1	\$8.50	

Extension rod	Thorlabs	ER4	7	\$50.75
Extension rod	Thorlabs	ER2	2	\$12
Post holder	Thorlabs	PH2E	3	\$69
Post	Thorlabs	TR1	3	\$15.75
Table clamp	Thorlabs	CL2	3	\$19.50
V-clamp	Thorlabs	VC1	1	\$34
Clamping fork	Thorlabs	CF125	3	\$24
Prism mount	Thorlabs	PM1	1	\$9
1" stackable tube	Thorlabs	SM1L20	2	\$36
1" stackable tube	Thorlabs	SM1L10	1	\$15
1" stackable tube	Thorlabs	SM1L05	2	\$26.50
Tube coupler	Thorlabs	SM1T2	1	\$16.80
Objective adaptor	Thorlabs	SM1A3	1	\$17.50
Camera "C" mount	Thorlabs	SM1A9	1	\$18
Centering hole	Thorlabs	CPA1	1	\$8
3V DC power supply	RadioShack	273-1662	1	\$14.99

Microscope total: \$ 3267.59

Supporting items (optional spectrometer)

Spectrometer	Ocean Optics	USB2000	1	\$2500	
Optical fiber	Ocean Optics	QP200-2-VIS-NIR	1	\$175	17
SM1 fiber adapter	Thorlabs	SM1SMA	1	\$26	
Translating lens mount	Thorlabs	LM1XY	1	\$128	16
1" stackable tube	Thorlabs	SM1L05	1	\$13.25	

Microscope + spectrometer total: \$6109.84

Supporting B (modifications)

(a) Objective

The as-manufactured Edmund Scientific objective (NT43-909) has a ~6 mm diameter back input aperture, which is a small for TIR. In the limit of an infinitely narrow excitation beam, an evanescent field at the substrate/sample interface will appear when the beam is shifted off-center from the objective's axis by $f \cdot \tan \alpha$, where f is the focal length of the objective and α is the TIR critical angle [$\alpha = \arcsin(1/1.515) \cong 41.3^\circ$ for a glass-air interface]. Since, the Edmund objective's focal length equals 2.74 mm, the displacement required to achieve TIR is $2.74 \text{ mm} \times \tan 41.3^\circ \cong 2.4 \text{ mm}$. However, because the laser beam has a finite ~1 mm diameter (after modification, see below), the evanescent field first appears at a beam displacement of $2.4 - 1/2 = 1.9 \text{ mm}$ and achieves its maximum intensity when the beam shifts $2.4 + 0.5 = 2.9 \text{ mm}$ off axis. To accommodate this, the radius of the objective's back input aperture must be larger than $2.4 + 1 = 3.4 \text{ mm}$. Since the radius of the objective's input aperture is ~3 mm, its size is widened to ~4.2 mm (radius) using a 21/64" drill. This can be done by hand since the aperture is made of plastic.

(b) Laser pointer

The commercial green laser pointer used in the current study produces a ~3 mm diameter output. This size, while fine for either the confocal or widefield epi-illumination modes, is slightly large for the creation of a pure evanescent TIR excitation. In this regard, because the diameter is wider than optimal (~1 mm diameter, see above),

part of it will not be incident on the substrate/sample interface with angles above the TIR critical angle (41.3°) even when the beam is displaced laterally off the objective's axis by 2.9 mm (see above). As a consequence, much of it will not undergo total internal reflection and simply passes through the interface unattenuated. Both the purity and efficiency of the TIR excitation are therefore reduced.

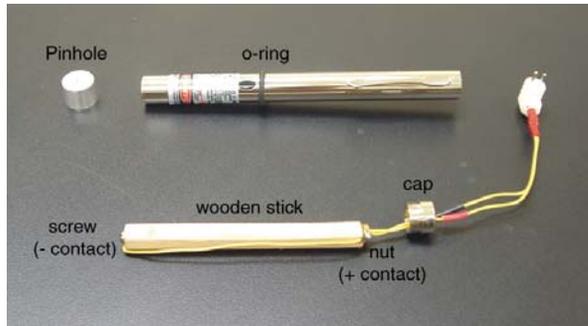


Fig. A

A ~ 1 mm (diameter) pinhole placed in front of the laser can be used to decrease the size of the output, permitting all of the incident excitation to sample angles greater than the TIR critical angle. Whereas the pinhole used in our apparatus is machined from aluminum (see Figure A), an easier way to achieve the same effect is to punch a 1 mm hole in a piece of heavy stock, black paper which can subsequently be glued to the front of the laser pointer. A numbered drill (#66) can be used to create this opening.

The laser pointer is also powered by two AAA batteries and can operate continuously for ~ 1 hour before the batteries die. To expand the amount of time available for our experiments, we have modified the pointer to accept power from a 3V DC wall mounted transformer (273-1662, Radio Shack). This is accomplished by drilling a hole (#31 drill) into the cap covering the battery compartment whereupon wires passed through the hole are connected to either end of a wooden stick having the same diameter and twice the length of a typical AAA battery. A negative contact is created by attaching one wire to a

metal screw secured to the far end of the wooden insert. The positive contact is achieved by attaching the other lead to a metal nut glued to the end facing the metal cap. A crude on/off switch is implemented by using a 3/8" viton o-ring to hold down the pointer's on/off button. See Figure A for more details.

(c) (X,Y,Z) sample stage

The (X,Y,Z) sample stage is assembled from two separate Thorlabs stages, a (X,Y) stage (HPT1) and a Z translator (SM1Z). A spacer (actually a threaded ring from a

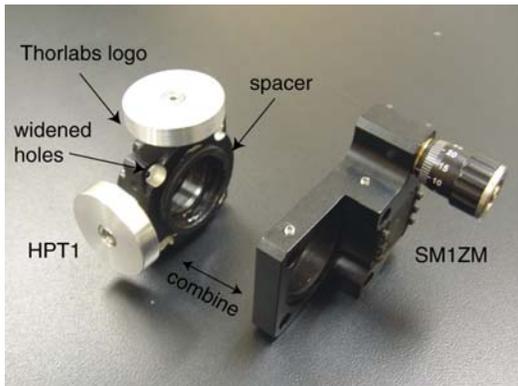


Fig. B

SM1T2 tube coupler) is used to combine the two stages because the SM1Z translator has three small protruding screws preventing their direct connection. First the spacer is glued to the center circular aperture of the SM1Z stage on the side opposing the micrometer (screw in the micrometer to push up this aperture if needed). Next before the HPT1 stage is attached to the spacer (we recommend doing this with both stages having rods passed through all four corners to allow centering of the stages relative to each other) all four corner holes of the HPT1 are widened with a 1/4" drill. The purpose of this is to reduce any friction along the z direction caused by the HPT1 when vertical adjustments are made

with the SM1ZM. The final 3D translation stage should have the micrometer on the bottom and the HPT1 Thorlabs logo facing up (see Figure B).

(d) Mirror, dichroic mounts

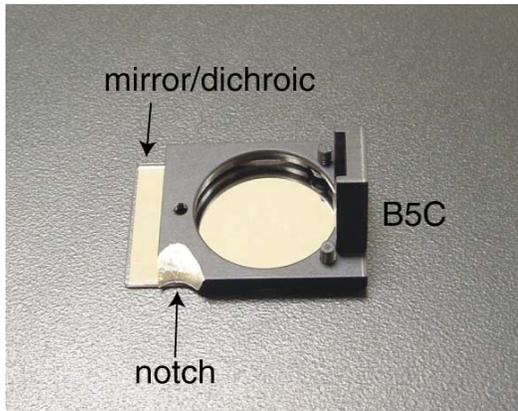


Fig. C

Three Thorlabs B5C 1” optic mounts are used to secure the dichroic beamsplitter and the two (50/50 and 100% reflective) mirrors. Before these elements are glued to the mounts, however, one edge of each B5C is filed down to a 45° angle. Without such modifications, the B5Cs will prevent one of the four rails from being inserted (or conversely the rail, already present, will prevent the B5C from being rotated to a 45° angle). In each case, the upper right corner of the BC5 is filed down (BC5 upright, front facing the viewer, see Figure C for exact orientation). Note that the dichroic and mirrors must be glued with the correct surface facing out (facing the table in Figure C. The dichroic will also have a beveled edge or mark indicating its top surface). Use small drops of 5 minute epoxy on each of four corners, allowing the optic to be removed if needed. Furthermore, the dichroic and mirrors must be secured flush against the top surface of the B5C. In this respect, there are two small plastic screws which should be avoided since otherwise an undesirable tilt will be built into the unit.

(e) Slip plate positioner

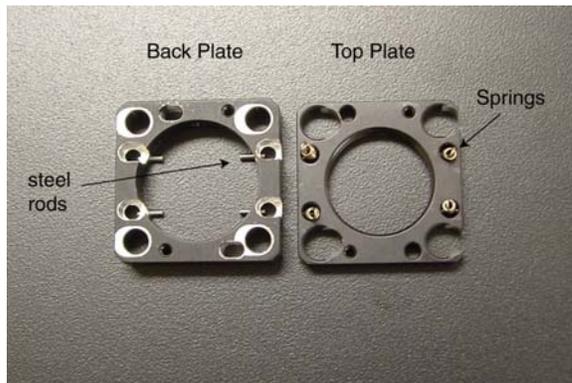


Fig. D

The Thorlabs SPT1 slip plate positioner holds the 75 mm plan-convex lens used for widefield epi-fluorescence and widefield objective-based TIR illumination. In its default configuration, the slip plate positioner has enough lateral displacement to accommodate all needs of the widefield epi-fluorescence mode. However, it does not have enough displacement to allow widefield objective-based TIR because the lens must translate ~ 2.9 mm in order to allow the incident beam (1 mm diameter) to sample angles above the TIR critical angle. Modifications are therefore made to the SPT1 by disassembling the unit (a small steel piece and hammer can be used to push in the 4 steel rods holding the springs in place) and widening the four through-holes on the top plate of the positioner by ~ 2.5 mm (see Figure D). A milling machine with a ($5/16''$) bit is recommended for this task, however, either a file or drill can be used instead. On the opposing back plate, four small holes (see Figure D) have been extended horizontally by the same amount using a $3/16''$ milling bit. In all cases we recommend that both pieces be securely fastened to a vise during their modification. Once all holes have been widened, the unit is reassembled with the four springs left unattached.

(f) Optional fiber bundle chuck

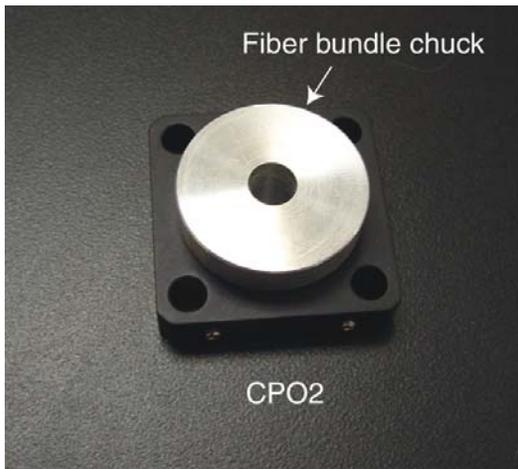


Fig. E

For transmitted light illumination, a QTH lamp and fiber bundle are used (OSL1, Thorlabs). The end of the fiber bundle (1/4" diameter) is held in place with an aluminum chuck (1" diameter, Fig. E) that fits into the center of a Thorlabs CP02 30 mm threaded cage plate. The aluminum chuck has an inner diameter slightly larger than 1/4" into which the fiber bundle is inserted. This cage plate is then mounted above the sample (X,Y,Z) stage (described above), allowing brightfield illumination. However, we have also successfully employed a flashlight to illuminate the sample and the actual choice of illumination source used is left to the reader.

(g) Miscellaneous modifications

Fig. 2 (main text) shows the microscope with an optional fiber-based spectrometer. However, the basic microscope has an eyepiece instead of fiber coupler. Therefore, the translating lens mount (LM1XY) and 0.5" SM1L05 tube are not present, having been replaced by a 1" SM1L10 tube. Since the outer diameter of the Edmund eyepiece is smaller than the inner diameter of this tube, we wrap it in electrical tape to increase its

diameter, ensuring a snug fit. A SM1RR retaining ring, screwed into the tube beforehand, holds the eyepiece at the correct height.

Supporting C (Assembly and alignment)

Assembly

(a) Excitation optics

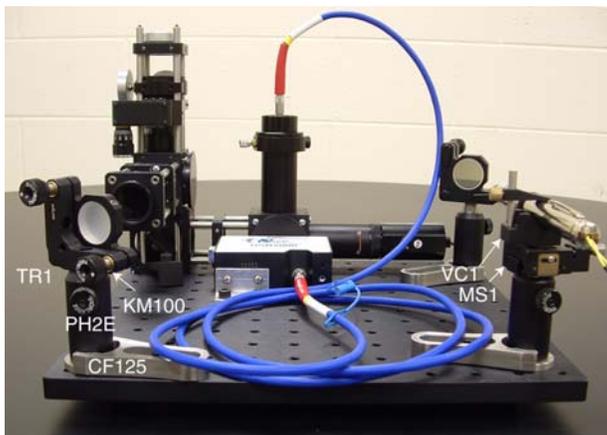


Fig. F

First the 1D translation stage (MS1, Thorlabs) is secured to a TR1 (1") post. Then the V-clamp (VC1, Thorlabs) is attached to the translation stage using a 8-32 set screw and a washer. In our case, the washer has a 0.072" thickness. These pieces are then mounted on a PH2E post holder and clamped to the breadboard with a CF125 holding fork. Next mirrors are added to the two KM100 mounts which are attached to TR1 posts. Both mirror mounts/posts are then inserted into PH2E post holders and are secured to the breadboard with CF125 clamping forks. We recommend positioning the laser and the mirrors in the configuration seen in Figure F. Note that the V-clamp/1D translation stage onto which the laser pointer is eventually attached must translate the laser beam only

laterally, meaning that the angle at which the laser hits both mirrors and enters the microscope must not change. Rotate the TR1 post (with attached laser pointer/v-clamp/translation stage) within the PH2E to achieve this.

(b) Microscope

At the structural heart of the microscope are 2 Thorlabs (C6W) cubes stacked on top of each other. One of them is rotated 90 ° relative to the other (see Figure G for correct orientation). Four extension rods (2 ER8, 1 ER6 and 1 ER4) are inserted vertically to

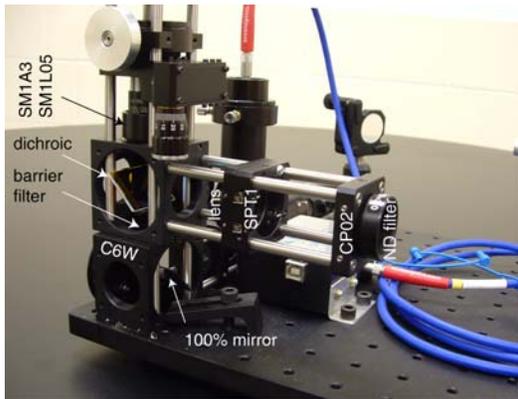


Fig. G

connect both cubes. The two 8 inch rods (ER8) (A and D, Fig. 2 main text) completely traverse the length of both boxes, whereas the 6 inch rod (ER6) (B, Fig. 2 main text) stops at the top of the second box, and the 4 inch rod (ER4) (C, Fig. 2 main text) stops at the top of the first cube. These rods are secured in place using set screws and are arranged in the order shown in Fig. 2 (main text). A SM1RR retaining ring, inserted into the top threaded opening of the bottom cube, holds the HQ555lp barrier filter. Modified B5C mounts with the dichroic and 100% reflective mirror are attached to B3C plates which are then inserted into both cubes (note that the dichroic will have a beveled edge or mark indicating the top surface which should always face the incoming excitation). The

dichroic is placed inside the top cube and the mirror is inserted into the bottom cube. (see Figure G for correct orientation)

Four additional 4 inch extension rods (ER4) are screwed into the top cube horizontally. A SPT1 slip plate positioner is then inserted through the rails and holds a 75 mm focusing lens mounted in a SM1L05 optic holder (in Figure G the curved surface of the plan-convex lens should face the incoming laser beam). A CP02 30 mm threaded cage plate is then added to the end of the rods and is used for holding mounted neutral density filters (NEK01).

The objective is now attached to the top threaded aperture of the first C6W cube using a SM1L05 optic holder. A SM1A3 RMS thread adaptor has been inserted into this piece allowing the Edmund objective to be attached to the SM1L05. Next the HPT1/SM1Z 3D stage is inserted over the objective and fastened into place using set screws.

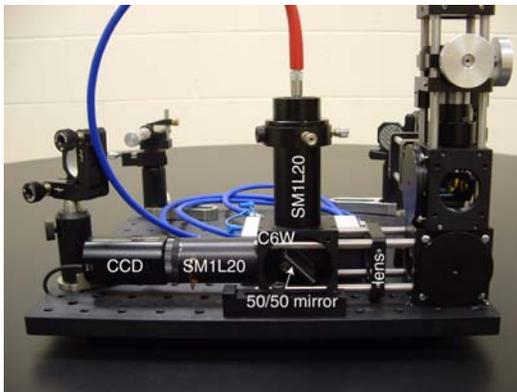


Fig. H.

Attached to the bottom cube are 4 more horizontal extension rods (2 ER4 and 2 ER2). The shorter rods face the inside of the breadboard, and the longer rods face out (see Figure H). A +150 mm lens inserted into a CP02 cage plate and held in place with two SM1RR retaining rings is then passed through all four rods (note that the curved surface of the plan-convex lens faces the incoming light from the 100% reflective mirror). Next

a third C6W cube is inserted through the rods and is secured in place with set screws. Move the mounted 150 mm lens ~1" away from the front of the last C6W cube and lock it into place.

A B5C with an attached 50/50 mirror is mounted on a B3C plate and all elements are inserted into the last C6W cube at a 45° angle (see Figure H). Two 2" tubes (SM1L20) are then screwed into the cube as shown in Figure H. A SM1A9 c-mount adaptor is glued to the end of the bottom tube after which a low cost CCD (PC164C) is attached. On the vertical tube a SM1T2 coupler is mounted to the exposed end and a SM1L10 1" tube is screwed into this piece. A SM1RR ring is inserted into the SM1L10 and the modified eyepiece (see Appendix B) is added. The SM1RR ring holds the eyepiece at the correct height so that images on the CCD and eyepiece are simultaneously in focus (i.e. they are parfocal).

Alignment

Alignment of the microscope is relatively straightforward. To find the correct orientation of the two Al mirrors, the laser beam is centered on the rails using a Thorlabs CPA1 alignment tool. The CPA1 is first placed near the outermost edge of the rails and the beam is centered on its aperture by manually rotating the mirrors and/or using their horizontal and vertical adjustment knobs. The CPA1 is then moved to the far end of the rails near the dichroic and both mirrors are adjusted to center the beam on the alignment aperture again. This two step alignment is repeated iteratively until the beam passes through the CPA1 at both positions, hitting the dichroic.

The angle of the dichroic beamsplitter is then adjusted to 45°. To determine the correct orientation, however, a CP02 cage plate is inserted through the vertical rails with the CPA1 centering piece rested against it [remove the objective and the (X,Y,Z) sample stage if present]. Since the axis of rotation of the B3C/B5C does not necessarily coincide with the height of the incident excitation, the reflected laser beam may not be centered on the CPA1 even though the angle of the dichroic is 45°. To correct for this, adjust both the position of the B5C and the orientation of the B3C. Paper shims can be added to the B3C if additional tilt is needed. At this point the laser beam should pass through the CPA1 aperture at all three positions. With the laser beam centered, the CP02/CPA1 is removed and the (X,Y,Z) stage is inserted. A 1" diameter circular coverslip is added to the sample stage and is secured by sandwiching it between two SM1RR retaining rings.

When the laser is on and incident on the coverslip, approximately ~4% of the excitation will reflect back into the microscope. This reflected light will be used to align the remaining collection optics. If present, remove the HQ555lp barrier filter (12, Fig.1) from the top of the second cube. It has an OD>6 at 532 nm and will attenuate the green light needed for this part of the alignment procedure. The filter can be re-inserted later. With the reflected beam hitting the 100% Al mirror, rotate it to approximately 45°. (The mounted +150 mm lens should be ~1" from the third C6W cube) Now turn on the CCD and adjust the angle of the B3C/B5C/mirror until the reflected spot can be seen. Try to center it, inserting paper shims into the B3C if needed. Once centered, lock the mirror into place. In this position, the beam should be more or less collinear with the bottom horizontal rails connecting the last two cubes. This can be checked using the CPA1 alignment tool. (Note that perfect alignment is not critical here)

Next the angle of the 50/50 Al mirror is adjusted to 45° in order to bring the beam collinear with the combined SM1L20 and SM1L10 tubes (remove the eyepiece or LM1XY translator if present). Center the beam through the tubes visually, using paper shims inserted into the B3C if needed. The eyepiece can now be reinserted.

When this part of the alignment is complete, the Edmund objective is inserted into the microscope. Check that the green light exiting the objective does not tilt much and that it appears symmetric about the objective's axis. If not, the alignment procedure described earlier is repeated. Next, immersion oil is added to the objective (~2 drops) and the coverslip is brought into the objective's focal plane by looking for images of dust with the CCD under brightfield illumination. The coverslip is correctly positioned when the dust is in focus and the 532 nm spot size is simultaneously the smallest. This can be checked by moving the sample in and out the focus using the SM1ZM micrometer on the (X,Y,Z) stage. If instead of an eyepiece, a fiber coupler mounted on a (X,Y) stage (LM1XY) is used, the position of the fiber input is adjusted to maximize the intensity of the 532 nm light detected by the fiber-based spectrometer. At this point the barrier filter should be inserted.

Once the microscope has been aligned, the transition from confocal to TIR excitation is achieved by simply displacing the laser beam laterally off the center axis of the objective (+75 mm lens removed) using the MS1 translation stage. The micrometer is turned until nearly all of the excitation light is reflected at the substrate/air interface. When this happens a second beam will appear nearly collinear with the incident beam indicating proper alignment of the TIR excitation. To achieve widefield TIR illumination, the +75 mm lens is reintroduced (barrier filter removed) and is moved laterally (using the

SPT1 slip plate positioner) until an elliptical excitation spot appears centered on the monitor. Now, to adjust the size of the excitation area, the +75 mm lens is moved along the rails to change its distance relative to the objective. The barrier filter can now be reinserted.

Supporting D (optional spectrometer)

For acquisition of fluorescence spectra from strongly emitting samples, a fiber-based spectrometer can be attached to the microscope in place of an eyepiece. This is accomplished by removing the 1" SM1L10 tube from the top of the 2" SM1L20 attached vertically to the last C6W cube. Then a (X,Y) LM1XY stage is screwed onto the remaining tube with the Thorlabs logo facing up. To build a fiber mount, a SM1SMA fiber adapter is inserted into a SM1L05 0.5" tube at a depth just below the top surface and is secured using a SM1RR retaining ring. This fiber mount is then attached to the LM1XY stage. A multimode fiber (QP200-2-VIS-NIR) connects the microscope with a fiber-based Ocean Optics spectrometer. Lateral adjustment of the fiber input is used to maximize the signal seen by the unit.

The spectrometer can be used with all modes of the microscope. The best performance, however, is achieved using the confocal mode. This is because the size of the excitation spot (and corresponding collection area) is diffraction-limited. Hence, the resulting image of the fluorescence, magnified $\sim 100\times$, can be completely sampled by the 200 μm fiber. By contrast, when the excited area is large, as occurs in widefield epi-illumination ($\sim 21 \mu\text{m}$ diameter) or in widefield TIR ($\sim 20 \times 30 \mu\text{m}^2$) not all the emission is collected by the fiber since the magnified image in either case ($\sim 2 \text{ mm}$ diameter) greatly

exceeds the fiber's core diameter. We therefore recommend using the confocal mode for acquiring fluorescence spectra of samples where possible.

Supporting E (CCD camera comments)

The PC164C is a low cost CCD that can be purchased from Supercircuits (www.supercircuits.com). The actual manufacturer of the unit is Korea Technology and Communications (KT&C) who also sells the unit under the part number KPC-350BHEX. The web site for the manufacturer is www.ktncusa.com. The Sony CCD chip at the heart of the PC164C is believed to be the Sony ICX254AL EXview HAD chip. The actual wavelength dependent quantum efficiency of this chip is not known at present.

Supporting F (Freeware comments)

A convenient image analysis program that can be downloaded as freeware is ImageJ. We recommend downloading the package and accompanying add-ons from <http://www.uhnres.utoronto.ca/facilities/wcif/download.php> as this site bundles a number of useful movie processing plug-ins. The main web site for ImageJ, however is found at (<http://rsb.info.nih.gov/ij/>). Alternatives to ImageJ include Scion Image for Windows users (<http://www.scioncorp.com/>) as well as NIH image for Mac users (<http://rsb.info.nih.gov/nih-image/>).

The ATI video capture card we have used saves movies in mpg format. To read these files into ImageJ, they are first converted into avi animations. We have done this using other movie editing freeware such as VirtualDub (<http://www.virtualdub.org/>). A nice feature of VirtualDub is that it allows cropping of the mpg files to make the

corresponding avi file sizes smaller. Once in avi format, the animation are analyzed using ImageJ's "stack" functions.