

## Procedure for Fluorescence Imaging with Example Craic Microspectrophotometer

Fluorescence can only be analyzed by MSP in reflectance mode. Either optical light source can be used. The laser source cannot be used for this mode. The Hg light source usually provides a stronger fluorescence signal in the VIS region than with the Xe source.

- 1- Switch on light source. Hg lamp (1) and/or the Xenon (2) for reflection.
- 2- Switch on Craic box, microscope, computer, Lambda Fire software and Prion Stage controller.
- 3- Place stage plate on stage to cover the transmission condenser. Activate light with RL switch. Select position 2 (%R, T2) on the filter cube turret. Place sample on stage.
- 4- Start with 10x or 20x objective to find the image using the microscope binoculars (preferred) or when Lambda Fire opens, can also use the live image monitor.
- 5- Optional: Get image in brightfield (TL, %T) using the Lambda Fire Auto brightness/contrast feature (Camera). Search for area of interest then switch to reflection mode.
- 6- Focus image, adjust Kohler illumination and then switch to the desired objective. Kohler Illumination – Reflected light: The brightness of the image may be lower in this mode. With image in focus, close down the upper Field Iris (F) part way. You will see the edges of the image darken and the center should brighten. Continue to close down the Field Iris until the edges of the iris become smallest and sharply focused. The sample should be close to focus at this point. Fine focus the image and change in the desired higher magnification objective. Check the Field Iris focus. Open the Field iris one half turn. Adjust the Iris (A) to desired brightness.
- 7- Use the menu selections at the bottom of the screen to set up data collection. Change folder to store images and data. The light source does not need to Optimize but you can try it to help you select an initial setting. It probably will not work in reflection mode since the light intensity in Fluorescence mode will be low. In Gears function, set the exposure time (10 to several thousand msec). You may need to experiment with exposure. The spectrometers usually can detect fluorescence detected with the Auto button selected. If the fluorescence is weak, you can experiment with increasing the exposure manually. Some detection is possible by adjusting the slider (camera menu) up to about halfway. Also you can select spectrum range to analyze. Select VIS or UV-VIS (300-800nm). Although you can select the entire spectrum (UV-VIS-NIR, 300-2000nm), there is limited usable data much beyond the VIS spectrum (spectrometer 1 range) for fluorescence.
- 8- Find the image area of interest. Focus again. Turn filter turret to the desired fluorescent cube (4,5,6). You should already know which cubes are in or have loaded an accessory cube into the condenser turret. The Lambda Fire monitor will most likely be black. In Camera mode uncheck Auto and incrementally increase the slider to the right. This increases the exposure time. At each step, wait a few seconds to see if the image appears. You want only enough exposure time to show an image on screen. Too much

exposure will greatly increase the time to get each image update and the camera may saturate (will not update at all).

9- Insert an aperture (1-6) over a fluorescent object. Perform a Dark Reference. You do not need to move the sample from the aperture area. Alternatively you can push off the R source shutter.

10- Press Collect Spectrum. Insert information for the file name. It is a good idea to be specific with all information included in the file name. These include: Sample Name, light source, type of imaging, turret position, magnification, aperture #, spectrum range, integration time, collection mode and counter.

Ex: Name-Xe-RLT4-100x-Ap4-VIS-1000ms-Fluo-1. Check that the magnification in menu is correct.

Select the type of data desired (fluorescence, absorbance, reflectance).

### Example: Fluorescent Fiber Analysis

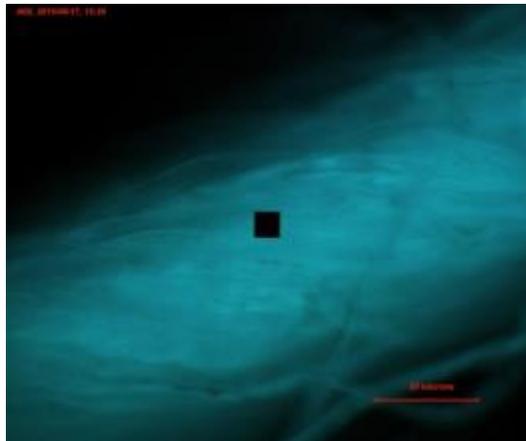
Light source: Hg (1 preferred) or Xe (2)

Stage: black plate

Microscope illumination: Push RL shutter on. Fluorescence can only be carried out in Reflected light.

Find Image:

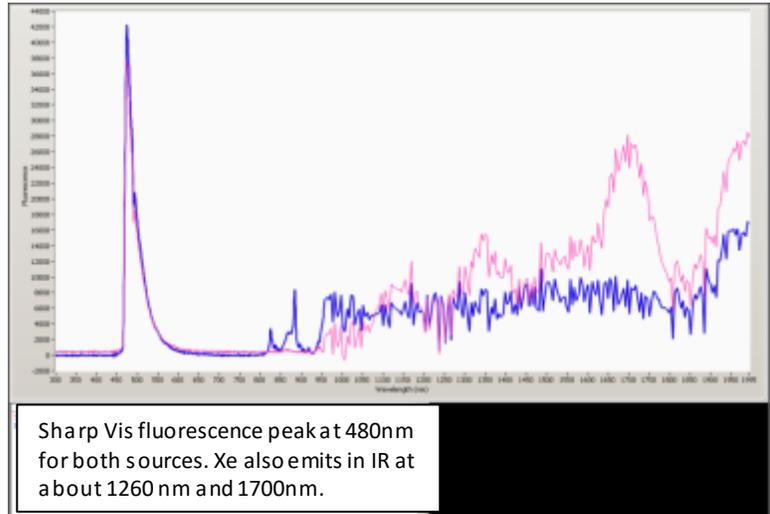
- a- Start in BF (Illumination turret T2= %R) with 10x or 20x objective.
- b- Start LambdaFire
- c- In Camera menu, use Auto or turn off to manually adjust the image illumination.
- d- Find and focus sample, select desired objective (sample should still be close to focus).
- e- Adjust Kohler illumination and move to desired location.
- f- Illumination mode Turret: Position 4-6. Have proper cubes loaded and select. If the excitation is correct, the object should fluoresce.



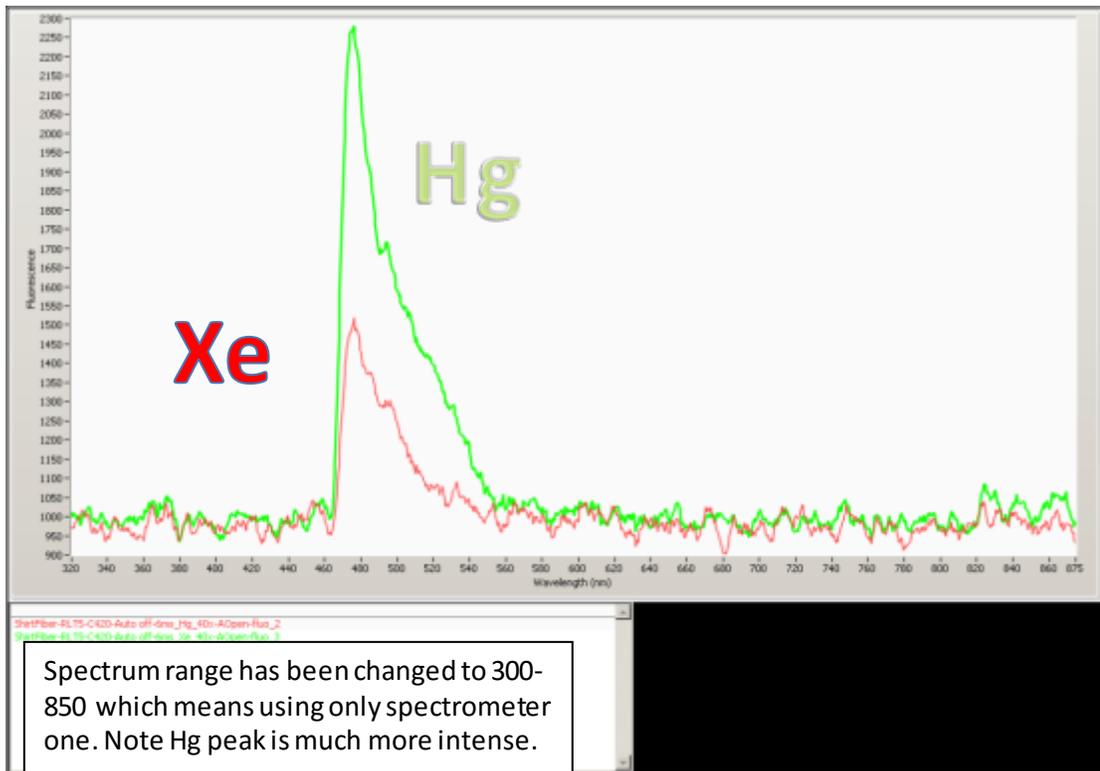
**Fluorescent synthetic fibers at 20x using 420nm cube.**

Lambda Fire setup:

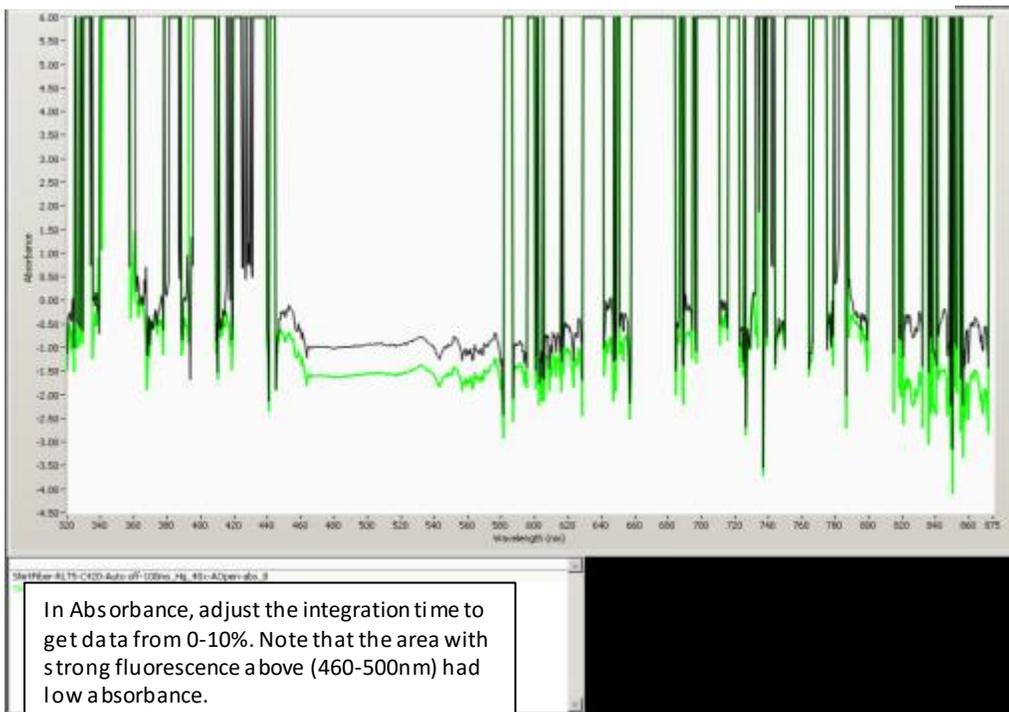
- a- Find area of interest and focus.
- b- In Gears menu and submenus, choose the spectrum range desired. The range can be further modified manually. Insert the integration time and number of exposures for one or both spectrometers. Press load and OK.
- c- Choose aperture that will just cover the area of interest.
- d- Go to My Folder to get your folder and subfolder.
- e- Optimize – Don't need to do this for fluorescence.
- f- Dark and reference – Fluorescence mode only uses the dark scan so it can be performed with aperture on the sample itself.
- g- Collect Spectrum- Select the magnification. Select the mode you want to sample (i.e., fluorescence, absorbance). Insert descriptive name with details. Push Enter.
- h- Change conditions, such as the imaging range or from fluorescence to absorbance. Note that it will be necessary to adjust the integration time to get units that make sense. Fluorescence mode will have high numbers depending on the intensity but set the integration to get sharp peaks and good separation from the background (signal to noise). Absorbance



**Full Range (spectrometer one and two) fluorescence spectrum from the image above using the Hg (blue) and Xe (red) light source in reflected light mode with 420nm excitation.**



New fluorescence spectrum from same fiber as above with some changed conditions.



Absorbance mode in the same fiber while still comparing Hg and Xe light source.