

Hyperspectral Imaging of Fluorescent Dyes

This white paper describes the use of a Resonon hyperspectral imager to monitor fluorescence dyes. Advantages of this approach include:

- Accurate removal of background fluorescence, which leads to excellent signal-to-noise ratios;
- Accurate spectral demixing of overlapping fluorescent signals, which enables dye multiplexing.

The work described here was done in collaboration with the Edward Dratz group at Montana State University with funding from the National Science Foundation [1].

1. Apparatus.

Resonon utilizes line-scan spectral imaging, where a scene is limited to one dimension with a slit, and then imaged off a diffraction grating to obtain spectral information for each pixel along the slit. A second spatial dimension is obtained by scanning the object as multiple frames are collected. Here, electrophoresis was used as the scanning mechanism, as illustrated in Figures 1 and 2. Proteins tagged with fluorescent dyes self-scanned as they migrated through the gel. This approach eliminated all moving parts, eliminated the need for a second scanning process after electrophoresis, and produced excellent signal-to-noise due to long integration times.

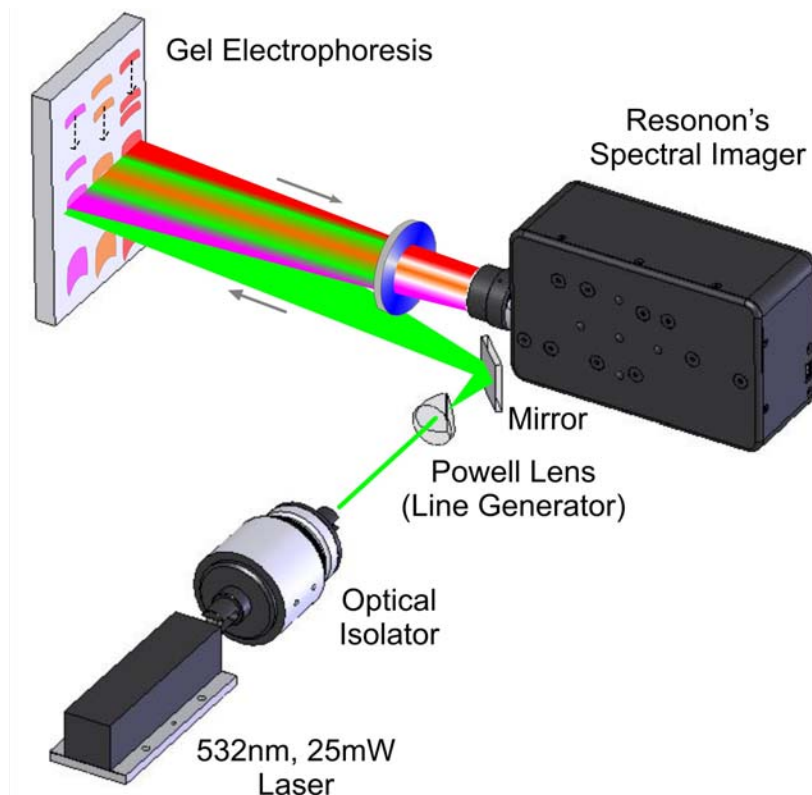


Figure 1. Schematic of electrophoresis scanning. The imaging spectrometer images a long, thin region on the gel that is illuminated with a line-source to excite fluorescence. The spectral signatures of fluorescently tagged proteins are recorded as they migrate through the field of view of the imaging spectrometer.

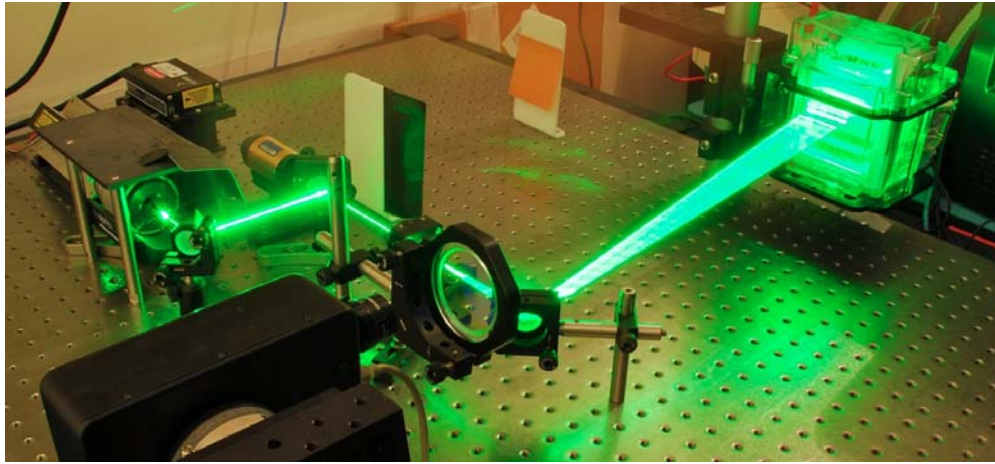


Figure 2. Photo of the apparatus used for electrophoresis scanning.

2. Spectral Demixing.

Four identical samples of proteins were prepared, each of which was labeled with a different fluorescent dye. These protein samples were loaded and run in separate lanes of a 1-D gel. The proteins migrated past the field-of-view of the imaging spectrometer to generate the hyperspectral image shown at the top of Figure 3 (shown in false color to differentiate between the dyes.)

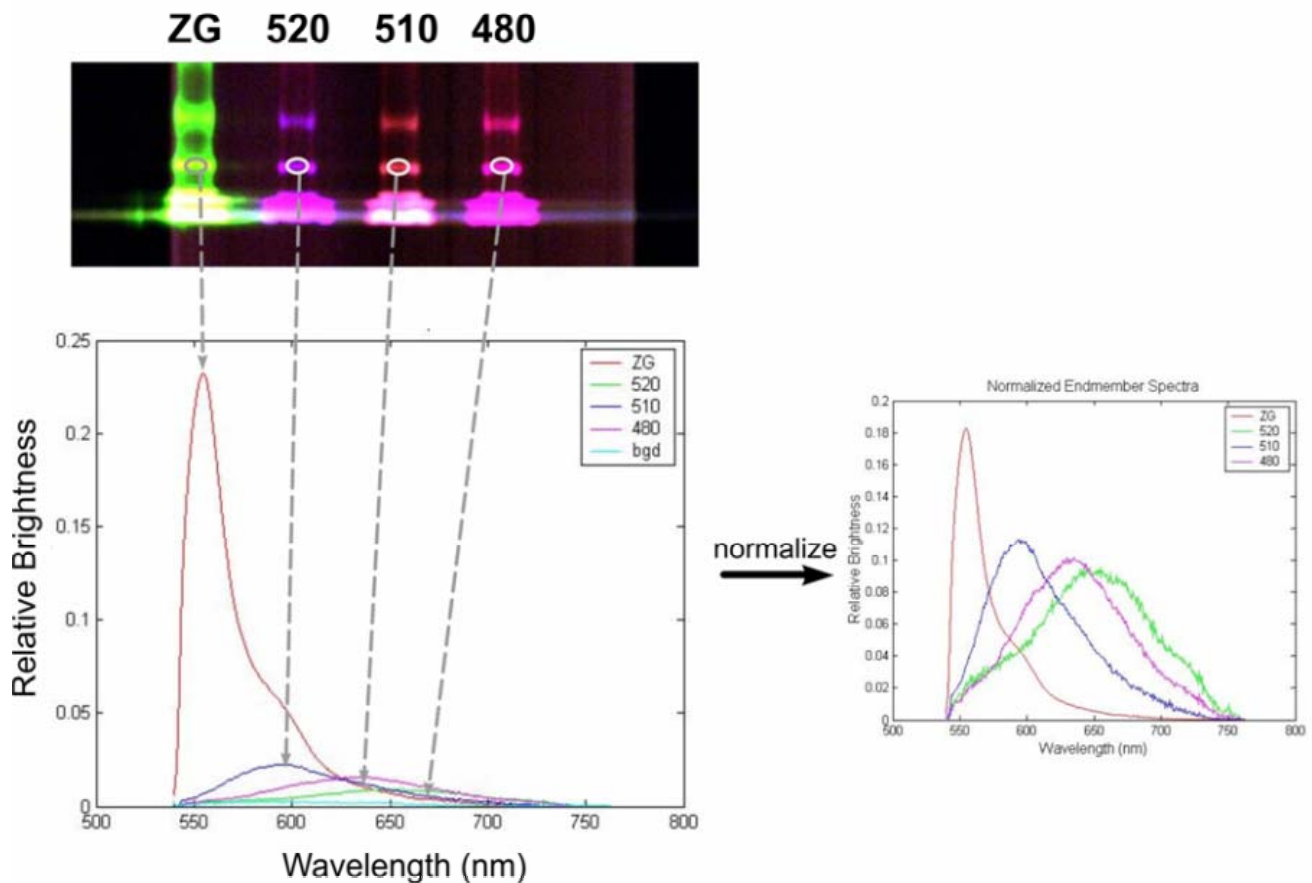


Figure 3. The top image shows a false-colored image of a 1-D gel with four differently dyed samples run in four different lanes. The spectrum from each dye is plotted below the false-colored image as indicated. The curve labeled bgd is the background fluorescent signal. Note that the ZG dye is much brighter than the other three dyes. Normalized plots of the dye spectra are shown to the right.

The average spectra from small regions of approximately the same protein concentration were measured and are shown below the false-color image of the separated proteins in Figure 3. Note that the spectral curves from the four dyes strongly overlap and that there are considerable differences in signal strengths between the dyes.

To demonstrate dye multiplexing, several dye mixtures were run in single lanes and imaged as described above. The spectra shown in Figure 3 above were then used as “basis vectors” to “demix” the spectral signatures from mixed samples pixel-by-pixel. The fitting parameter for each basis spectrum provides a measure of how much of that dye is present in any given pixel. As an example, demixing results from a two-dye, 50/50 mixture of proteins tagged with the strong dye (ZG) and proteins tagged with a much weaker dye (510) are shown in Figures 4 and 5.

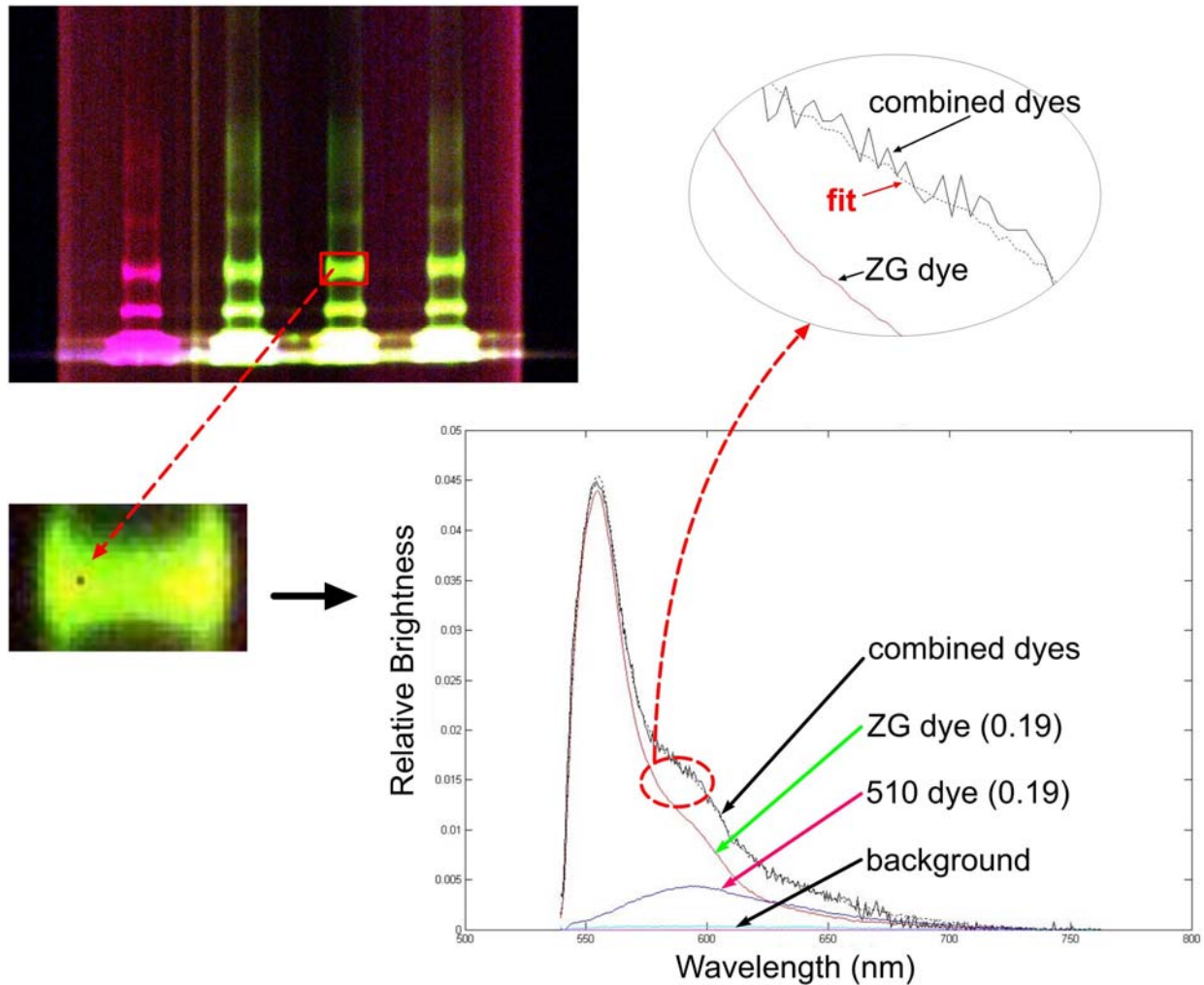


Figure 4. Results from two-dye mixture run with 50/50 mix of ZG and 510 dye tagged proteins. The measured spectrum for a single pixel was curve-fit using the basis spectra identified from single-dye runs (shown in Figure 3). The curve fit-result, based on a weighted addition of the basis spectra is very close to the measured spectrum as seen in the enlarged section of the graph.

The results shown in Figure 4 for the single pixel indicated are typical. Next, the fitting parameters for all pixels were calculated and plotted as a grey-scale density representation for the pixels in Lane 2 (counted from the right). Thus, there are four representations (i.e. images) for Lane 2, one for each dye. These results are presented in Figure 5. To provide a quantitative measure of the dye concentrations, a density profile is also plotted in Figure 5 that gives the average fitting parameter for each dye as a function of position down the gel lane.

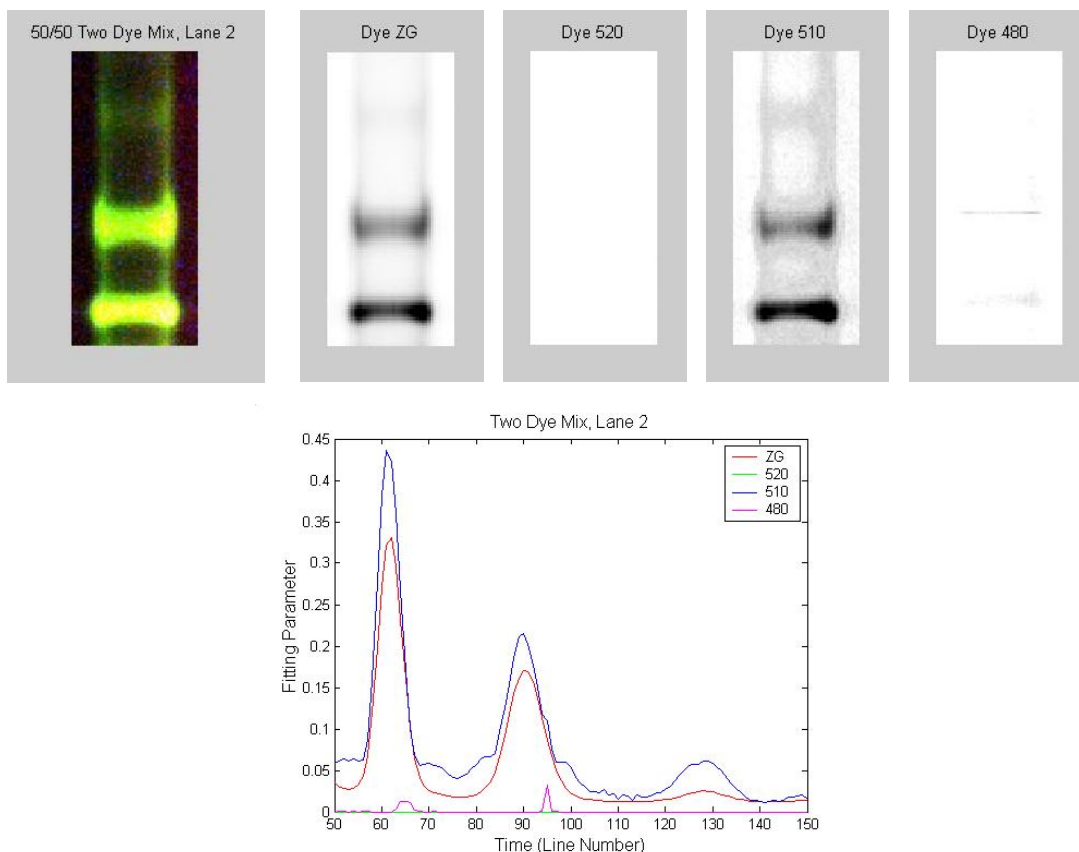


Figure 5. Two-dye mixture demixing results from a scan with a 50/50 mixture of ZG and 510 dyes. The top-left image shows a false-color image of the scan for reference. The four plots to the right are grey-scale representations of the four dye fitting parameters. The bottom plot shows the average fitting parameter profiles for each dye down the length of the gel lane.

For a 50/50 mixture, the fitting parameter profiles for ZG and 510 should be exactly the same, while the fitting parameters for 520 and 480 should be exactly zero. Neither of these is the case, although the general trends are correct. A detailed discussion of these discrepancies is beyond the scope of this paper, except to note that some discrepancies are inevitable given that the “basis” spectra were not taken from calibrated samples and there were noted differences in the amount of dyed samples loaded into the gel lanes during these proof-of-concept experiments. Similar results were obtained for three-dye mixtures, even when the three weakest dyes were used. The number of dyes that can be multiplexed is not yet known.

The results presented above demonstrate dye multiplexing, which will enable multiple samples to be run simultaneously in the same medium. These observations and conclusions are consistent with published work on hyperspectral imaging of fluorescent signals [2,3]. Electrophoresis scanning has no moving parts and therefore will be cost-effective and require very little maintenance.

References:

1. NSF Award number 011-0620581.
2. M.B. Sinclair, J.A. Timlin, D.M. Haaland, and M. Werner-Washburne, “Design, construction, characterization, and application of a hyperspectral microarray scanner,” *Appl. Opt.* **43**, 2079-2088 (2004).
3. A.M. Woodward, N. Kaderbhai, M. Kaderbhai, A. Shaw, J. Rowland, and D.B. Kell, “Histometrics: Improvement of the dynamic range of fluorescently stained proteins resolved in electrophoretic gels using hyperspectral imaging,” *Proteomics*, **1**, 1351-1358 (2001).