Spectral imaging for brightfield microscopy

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ABSTRACT

Molecular medicine now requires molecular pathology, defined as the image-based detection of specific genes, transcripts, proteins and other macromolecules. Because of the need to interpret molecular data in context, simultaneous assessment of more than single species is desirable. While fluorescence has traditionally been used for high-resolution multiplexed molecular imaging, clinical practitioners express strong preferences for non-fluorescent, brightfield multicolor methods. However, brightfield typically utilizes only one color at a time, and if more than one molecular target is to be analyzed, serial sections are made and a different probe applied to each slice. Such practice precludes assessment of co-expression on a cell-by-cell basis. Similar constraints apply to brightfield in-situ hybridization techniques. Double- and triple-staining procedures are rarely performed in non-research settings, not only because the wet chemistry can be difficult, but also because it can be challenging or impossible to determine visually where and to what extent different chromogens may physically overlap. Spectral imaging can be used to examine such double- and triple-stained specimens.

Two methods of acquiring spectral images are described, along with their application to multicolor immunohistochemistry and transmission in-situ hybridization (TRISH): 1) liquid crystal tunable filters, conveniently available for microscopy for the first time; and 2) a novel, spectrally agile light source. This source emits white light of any desired color temperature, or single 10-nm wavelength bands in the range 420 to 700 nm, or any combination of wavelengths with individual intensity control. Both methods are allied with a grayscale camera and appropriate algorithms to analyze multicolor samples of clinical significance. Spectrally unmixed images clearly separate signals linked to different chromogens, even with spectral and spatial overlap, with minimal cross talk. Intriguing challenges in matching mathematical algorithms to these specific problems remain: how many bands are enough? What are the optimal unmixing procedures? What automated tools can be applied to speed and simplify the procedures?

Keywords: spectral, imaging, tunable, illumination, segmentation, unmixing, immunohistochemistry, chromogens

1. INTRODUCTION

The advent of molecular medicine has led to demands on pathologist that they provide highly detailed information on individual patient's tumors. An accurate diagnosis, previously the major measure of a pathologist's prowess, is no longer enough; what is required now is predictive and prescriptive information that give precise risk estimates, and more importantly, therapy guidance.[1, 2] While expression arrays and other techniques working on tissue extracts are generating a great deal of excitement, the use of molecular probes in-situ may turn out to be more appropriate in typical clinical pathology situations. *In-situ* assays preserve the anatomical structures and give insight into regional heterogeneity. Already, imaging estrogen-receptor and Her-2/neu levels in breast cancer has become the standard of care, and demands for such assays will continue to increase. It has been estimated that over 500 new drugs are currently under development that will require the patient to be molecularly qualified prior to treatment (D. Rimm, personal communication). Reliable multiplexed image-based molecular assays are needed, and it appears that the imaging component rather than the reagents and labeling techniques has become the limiting factor.

1.1 Problems with current approaches

While fluorescence-based techniques are typically used for multiplexed microscopy, it is not the pathologist's choice for a variety of practical reasons. More popular in clinical applications than immunofluorescence, immunohistochemistry (IHC) is widely used for the detection of diagnostically or prognostically significant molecules in or on cells. In the past two decades the technique has become central to the practice of oncologic pathology since it can distinguish between

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look-alike lesions (mesothelioma vs. carcinoma, for example), or elucidate the cellular lineage of extremely undifferentiated neoplasms (lymphoma vs. other so-called "small blue cell" tumors).[3] IHC can also be used to highlight the presence of otherwise easily overlooked microscopic foci of tumor, such as micrometastases in lymph nodes, and can be used to measure levels of receptors or other markers, such as Her-2/neu, p53, ki-67, and a host of others.[4-7] Occasionally, double- or triple-staining single slides with different chromophore-coupled antibodies may be desirable, and as noted above, this demand for multiple markers will continue to increase. Triple-staining procedures are not often performed because of the effort involved; however, with the arrival of programmable staining systems, reproducible, complex, staining protocols may become more widely used. The chief difficulty that they present, however, is that it is hard to determine visually where and to what extent the different stains may overlap when co-expression of 2 or more analytes in the same cellular compartment occurs.

Even with computer-aided analysis of color images this continues to be a problem, as will be illustrated in this report. Typically, a painted-pixel, or rarely, a 3-chip, RGB camera is used to acquire images. It has proven difficult to do quantitative multicolor molecular imaging in brightfield with such RGB systems, as it is generally not possible to "unmix" two of more spectrally overlapping labels if they are co-localized, although some progress has been made in this area.[8, 9] Spectral imaging, that is, the generation of optical spectral information at every pixel of an image can overcome this difficulty, even in the presence of considerable spectral and spatial overlap with the chromogens used.

2. INSTRUMENTS AND IMAGE ACQUISITION

There are a number of different approaches that can be used to acquire spectral images. We will restrict our discussion to the two kinds we have employed to generate the images in this report, namely liquid crystal tunable filters and a spectrally agile light source. While these differ in their underlying technology, the resulting data are virtually indistinguishable, and the analysis discussion does not depend on the technique used to acquire the datasets. Whether the transmitted light or the illumination light is spectrally tuned, the procedure involved taking a number of monochrome images at each wavelength, and assembling such images in memory so that a spectrum is associated with every pixel. Such data cubes can be converted, for viewing purposes, into synthetic RGB images that accurately reflect the true colors of the original samples. All color images in this report are in fact synthesized from spectral datacubes.

2.1. Liquid Crystal Tunable Filters

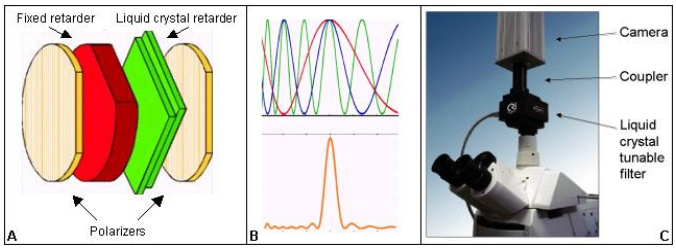

Figure 1: Principles of a liquid crystal tunable filter. (A) A liquid crystal element is inserted at each stage of a Lyot filter, allowing dynamic alteration of the location of peaks and valleys of each stage's periodic bandpass (B). The LCTF is positioned between a standard microscope C-mount and the camera, along with coupling optics to provide parfocality (C).

LCTFs use electrically controlled liquid crystal elements that transmit a certain wavelength band while being relatively opaque to others.[10] The rejection of the unselected wavelengths, without further manipulation, is of the order 10^4:1. The band pass can be as narrow as 1 nm or less, and the spectral range with a single device can range from 400 nm to 720 nm in the visible. Tunable filters operating in the NIR spectral region are also available.

The LCTF is based on a (multistage) Lyot filter into which, at each optical stage, a liquid crystal element is inserted. As shown in the accompanying figure (Fig. 1), the bandpasses from each state, interact to generate a region of constructive interference (the transparent region) while everywhere else at least one stage block light transmission. The liquid crystal elements provide the tunability, so that the bandpass can be set, randomly, to any desired wavelength within a tuning time of 50 to 150 ms.

While the position of the bandpass is actively tunable, its width is fixed and depends on the construction of the device. A typical bandpass in the visible is ~10 nm. Like the AOTF (acousto-optic tunable filter), the LCTF is polarization-

sensitive, which reduces the overall transmission by half. Fortunately, light in typical transmission microscopy is abundant.

2.2. SpectraLamp® Agile Spectral Light Source

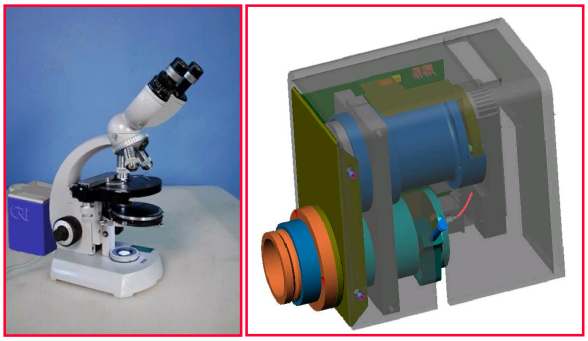

Figure 2: SpectraLamp® Agile Spectral Light Source. The image on the left illustrates where a SpectraLamp would be mounted (replacing the standard light source at the back of most research microscopes). Ordinarily, the microscope would also be equipped with a digital camera to take full advantage of the spectral imaging capabilities of the instrument. The right panel depicts the current operational prototype.

Instead of filtering the transmitted light after the sample, it is also possible to perform spectral imaging by illuminating the specimen with a series of narrow –wavelength band illuminants. The typical device used for generating spectrally pure light (from a broadband source) is a monochromator. However, these have the disadvantage that they cannot conveniently generate a white light suitable for routine microscopy viewing. However, CRI has created sources that are more flexible and can produce illumination of any desired pure wavelength (like a monochromator) or any selected mixture of pure wavelengths simultaneously, with white light output an easy option. The resulting images can be collected by a high-resolution gray-scale CCD camera and interpreted using appropriate algorithms and displays. While not covered in this report, it is possible to use software approaches such as projection pursuit, or principal components analysis to define specific illuminants. The sample can be then be illuminated with a precisely controlled mixture of wavelengths so that the image presented to the detector is a linear superposition of the sample properties at many wavelengths. Thus, spectral discrimination that would previously have required the collection of complete spectral cubes might require acquisition of as few as two or three matched spectral images per field, greatly speeding up acquisition as well as analysis procedures.

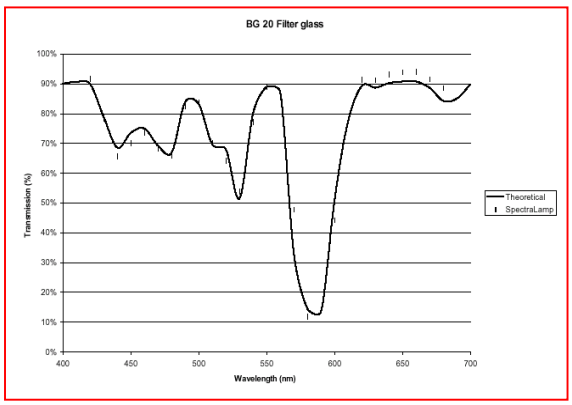

Figure 3: Spectral resolution and accuracy of SpectraLamp. A colored glass filter was imaged using the SpectraLamp and a spectrum of transmission as a function of wavelength obtained. The solid line is the manufacturer's published specification for the filter; the small vertical marks indicate the values obtained by the SpectraLamp at its 10-nm resolution.

Figure 2 shows the appearance and optical and mechanical layout of the present device. It is designed to mount onto most standard microscopes. Figure 3 illustrates the spectral performance of the CRI SpectraLamp device, which is designed to have 29 spectral bands, each 10 nm wide. It was used to image a particular glass filter with a complex spectral behavior. The SpectraLamp data (dotted points) are superimposed on the spectral curve provided by the glass filter manufacturer.

The samples (described below) were imaged using both the VariSpec tunable liquid crystal filter and the SpectraLamp source. Images were taken from 420 nm to 680 nm, spaced every 10 nm, with a 10 nm bandwidth. There was no perceptible difference in the spectral properties of cubes taken with either instrument.

3. SAMPLES

3.1. Immunohistochemistry

Immunohistochemistry is extensively used in pathology for the detection of diagnostically or prognostically significant molecules in cells or tissues. In the vast majority of cases,

only one analyte is probed for, and if more than one is sought, serial sections are made and a different reagent is applied to each slice. Most of the time, a counterstain is also employed to provide spatial context for the molecular reactions. Drawbacks of this limited approach include the fact that multiple slides (sections) need to be generated to examine multiple analytes. This procedure results in more slides and more handling steps than if the reagents could be multiplexed on a single slide. And if co-expression of antigens in a single cell is a question, as might be the case in certain hematologic malignancies or other oncologic situations, and if flow cytometry is not available or suitable, then double- or triple-staining single slides with different chromophore-coupled antibodies may be helpful. Triple-staining procedures are not often performed because of technical challenges, and probably more often because it is very difficult to determine where and to what extent the different chromogens may physically overlap due to co-expression. Spectral imaging can be used to examine such double- and triple-stained specimens and to tease out (probably semi-quantitatively) patterns of expression.

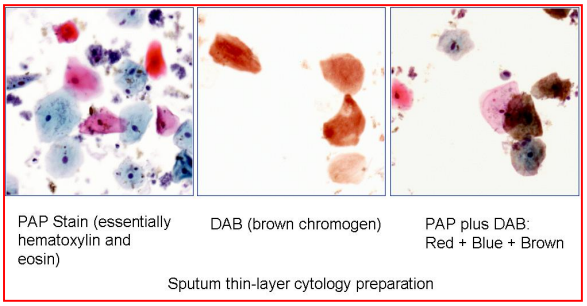

Figure 4: Panel of images illustrating the samples used for the spectral unmixing experiments. Lung sputum cells were stained with a standard Pap stain (largely red and blue colors), with an immunostain against a cytokeratin (using the brown DAB chromogen), or both Pap and DAB.

Another interesting modality is to superimpose immunostaining on top of conventional histological stains, such as hematoxylin and eosin, or, as will be shown here, a Papanicolaou (Pap) stain which allows both morphological assessment (because of the use of familiar histological reagents) as well as a molecular probe. In this case, the task is to separate the immunostain from the conventional stain, while avoiding cross-talk between these functionally distinct channels. Here we will examine a sample of lung sputum cytology (isolated cells) stained in several ways (Fig 4):

1) **Pap stain alone**, without a molecularly specific immunostain. In this case, the cells are stained predominantly with hematoxylin (blue, often nuclear) and eosin (pink, generally cytoplasmic), although both dyes can stain both compartments in this sample.

2) **Immunostain alone**. In this case, an antibody to a cytoskeletal protein detects only a subset of the lung cells in the sample. The antibody is coupled to an enzyme that converts a colorless precursor into a dense brown precipitate, here diaminobenzidine (DAB). Without a counterstain, it is hard to see the cells that do not express this particular protein.

3) **PAP plus DAB**. Blue, red and brown stains are present. The challenge here is to unmix the brown from the red and blue channels without cross-talk. We will use this sample to examine how many spectral bands appear to be necessary to accomplish complete separation.

4. ANALYSIS

4.1. Pixel-Unmixing

Regardless of the technology used to acquire the images, the task is to “unmix” spectrally mixed pixels occurring when multiple probes or stains co-localize. In fluorescence, because signals are emitted rather than transmitted, the signals adds linearly, and the observed spectrum is a linear mixture of the component spectra, weighted by the amount of each probe. A linear combination algorithm, with appropriate constraints, can be used to unmix the summed signal and, with an appropriate set of standards, and thereby quantitate the absolute amount of each label present.[11]

However, in brightfield, as opposed to fluorescence, multiple chromogens absorb light in a manner that generally follows Beer’s Law. The non-linear result makes direct analysis difficult. Fortunately, brightfield images can be converted to optical density (OD) using a straightforward mathematical procedure, yielding a dataset that is suitable for analysis using the same linear unmixing algorithms that work with fluorescence.

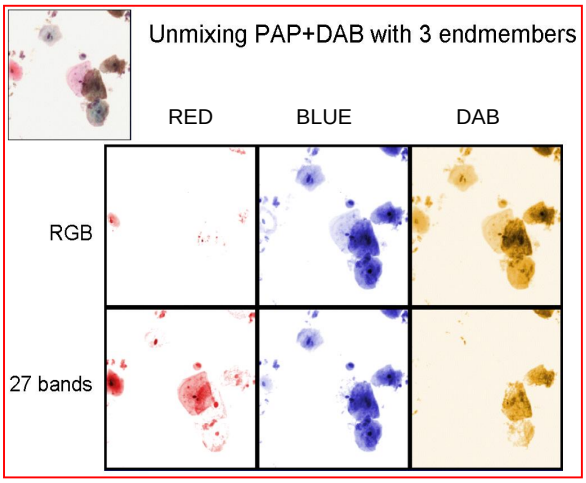

Figure 5: Unmixing a 3-color image using RGB or spectral data. The upper left inset (same as the right panel in Fig. 3) is a sample stained with PAP (red and blue) and DAB (brown). The top row of three images shows the result when the image was unmixed into red, blue and DAB channels using only RGB data. All of the red signal ends up in the brown channel. The lower row shows the result of unmixing using 27 spectral bands, allowing apparently correct unmixing into the 3 channels (red cells showing up in the red, but not the DAB channel).

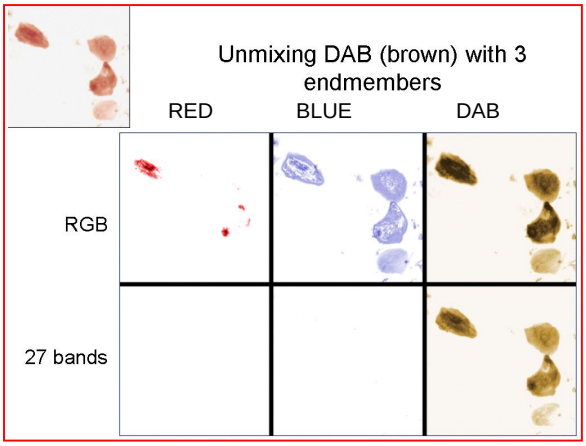

Figure 6: Unmixing a single color image (into 3 possible channels) using RGB or spectral data. A DAB-only image was unmixed into 3 possible channels (red, blue, DAB) using either RGB or spectral data. As can be seen, significant cross-talk was seen when only RGB data was used, but when all spectral bands are available, the DAB signal ends up exclusively in the DAB channel.

4.2. How many bands?

Spectral imaging involves the acquisition of a data set reflecting the spectral behavior of a sample at multiple wavelengths. One of the open questions is the number (and width or resolution) of the spectral bands that are required, since clearly, there is no good reason to take more images (with the consequent time, memory and storage penalties) than is necessary. One of the practical issues is: to what extent is spectral imaging even necessary? Can one accomplish what needs to be done with appropriate manipulation of standard RGB images? Such an approach has been recently described by Ruifrok et al, who use an RGB-variant of the spectral unmixing method used here. [9] In brief, the image is converted to optical density (OD) units by dividing, plane by plane, the transmitted signal by the illumination, and taking the negative logarithm of the quotient. Then, the "pure" spectra (also known as "end-members") of the component species (in RGB images, merely a 3-dimensional vector) are used to unmix.

In Fig. 5, we unmix the imaged sample using three spectral endmembers, namely red (eosin), blue (hematoxylin) and brown (DAB). The desired result has the unmixed channels containing only the appropriate signals. The inset image in the top left shows the actual appearance of the stained cells. All cells are either reddish or bluish, and in addition, a subset is stained with DAB, indicating the presence of a specific molecular marker. If one uses only RGB-spectral data and attempts to unmix into the three channels, one can see that while the blue channel appears to have accurately captured the blue signal, the DAB channel contains not only the DAB signal, but also most of the red as well. On the other hand, if one uses 27 spectral bands and 27-dimensional endmembers, it is possible to separate the red and brown signals with excellent spectral fidelity.

A different experiment (Fig. 6) has only DAB present in the sample, but allows the algorithm the opportunity to unmix into all three channels. In this case, using only RGB data it can be seen that the algorithm splits the DAB channel into both brown and blue outputs (and a little red), while performing the same procedure using a 27-band spectral data set, there is no detectable cross talk.

We have established that using 27 bands is evidently more powerful than simply RGB for this application. The question remains—what is the fewest number of bands that might be required? A systematic analysis of this has not been completed; however next figure demonstrates that the number is greater than might be expected.

In further analysis, the spectral data set was subsampled to see if the number of bands employed could be reduced while preserving an adequate unmixing result (Fig. 7). The image on the left is the original sample. The remaining panels

display only the unmixed red channel output utilizing the number of bands noted in the lower right of each panel. Note carefully the red cell partially covered by the brown (DAB-labeled) cell identified with the asterisk, and the bluish cell identified with the hash mark. Unmixing should reveal the partially covered red cell while the brown signal should disappear. Likewise, the blue cell should also largely vanish from the red channel, leaving only the appropriate light eosin-staining of the nucleus.

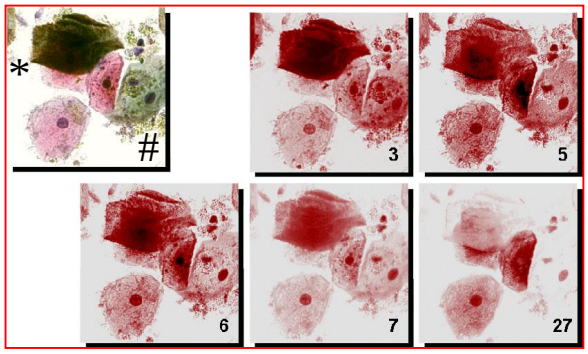

Figure 7: How many bands are enough? A Pap plus DAB image is unmixed using 3, 5, 6, 7 and 27 bands; the red channel only is shown. A brown cell (asterisk) and a blue cell (pound sign) persist in the red channel until 27 bands are used to unmix. The least number of bands required for this task was not determined, but in other, similar exercises, 14 bands (every other band in the spectral stack) were not sufficient to provide unmixing without cross talk.

Using 3 channels (RGB), it can be seen that the brown cell and the blue cell are still prominently featured in the red channel – an example of deleterious cross talk. Even using 7 channels (chosen manually, but with an eye towards using the most-likely-to-be-informative ones), one can see that both brown and blue cells have significant presence. Only when all 27 channels are employed do both of these signals disappear. Other analyses, not shown here, indicate that even every other channel was still not sufficient to allow optimal unmixing. However, it remains to be seen if the optimal bands had been chosen, what would have been the minimum number of exposures required. Further, would a 'structured' set of illuminants – rather than a band of wavelengths have caused improvements?

5. DISCUSSION

The images shown here were taken both with the VariSpec liquid crystal tunable filter and by the SpectraLamp spectrally agile light source. The SpectraLamp, operating in sequential narrow band illumination, gave identical spectral results to those generated by the VariSpec. We show that we can usefully unmix molecularly specific signals (linked to the brown DAB chromogen) away from standard, non-specific histological stains. While this is likely to be useful in certain pathology applications, it is desirable to optimize the data acquisition process in terms of speed, dataset size, analysis time and quality of results. In this report, we have begun to explore the specific requirements in terms of number of spectral bands required to accomplish accurate signal unmixing.

While theoretical considerations suggest that optimally, one can unmix *n* channels with as few as *n (*or *n+1*—assuming noise or scattered light is an additional channel) spectral bands,[12] actual performance will depend on the physical properties of the chromogens and on the signal-to-noise performance of both the labeling and imaging systems, as well as the level of sophistication of the analysis algorithms. Here we varied only the number of bands, while keeping bandpass width and algorithmic sophistication constant, arriving at the result that a significant number (more than 14) of bands appears to be necessary to achieve high-quality results. These results are essentially similar to those reported by Papadakis et al., who indicate that unmixing aqueous mixtures of a simple 2-chromogen system (hematoxylin and Fast Red) required at least 10 wavelengths for maximum accuracy. [13]

6. CONCLUSIONS

Spectral imaging, whether accomplished using liquid crystal tunable filters, spectrally agile sources, or other techniques, significantly outperforms color analysis approaches based on RGB imaging systems in samples relevant to current pathology practice. How much spectral resolving power is actually needed remains to be determined, but currently it appears that, up to a point, more spectral images are better, improving the ability of the present algorithms to separate overlapping signals with minimal cross-talk.

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