



Paving a New Path: Multispectral Imaging in Pathology

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The field of pathology began in its modern form as autopsy-based studies about a century and a half ago. Starting with the correlation between clinical medicine and the structural underpinnings of disease, first at the macroscopic level and, later, as techniques were developed to fix, section and stain tissue for microscopic evaluation, at the level of the cell and organelle.¹ This task continues in the present day and has expanded to include analysis at the molecular level; the pathologist is called upon not only for a correct clinical diagnosis as before, but often for prognostic or even theragnostic information as well. In addition, in the field of translational research, the pathologist can be asked to validate other forms of imaging and detection, some of which are molecularly based, and to play a role in guiding drug development through the various stages of clinical trials.²

A number of useful technologies have been added to the standard toolkit of pathology and pathology research, including gene and protein arrays, flow cytometry, Enzyme-linked immunosorbent assays, etc. Each of these methodologies provides a means of performing a multiplexed molecular analysis on a homogenized sample, either through the grinding up of a piece of tissue or on naturally homogenous samples such as serum, plasma or urine, for example. These multiplexed methods have proven themselves extremely useful both clinically and in research.³ In this light, the one-marker-at-a-time approach to the histochemical staining of tissue sections for imaging is seen as inadequate as the field expands. Immunohistochemistry (IHC), immunofluorescence (IF) and in-situ hybridization (ISH) techniques for DNA, mRNA and, recently, micro-RNA staining methods provide the foundation of molecular pathology, and also serve as an enticement to attempt multiplexed studies that would be able to extract the morphologic distribution of antigens/analytes of multiple markers simultaneously in the same tissue section.

There are at least three drivers to the adoption of multiplexed methods in pathology. The first is a practical one: if antibody panels⁴ assayed on serial sections can be multiplexed such that several probes could be applied on each slide, this would decrease sample handling demands and simplify the work-flow. It would also reduce the demands placed on scarce samples if multiple stains are required. The second driver would be to apply current multi-molecular phenotyping methods, especially with cell-surface markers, as is done now in flow cytometry, to a slide-based approach. Finally, we can anticipate that ongoing research in cancer and molecular biology, particularly addressing intracellular systems-biology approaches, will create a need to characterize multiple signaling molecules on a per-cell basis.

One factor which has limited the adoption of these multiplexed analyses in pathology is that visual assessment of a tissue section, or the use of colour cameras or whole-slide scanners, is unsuited to viewing samples which have multiple chromogenic or fluorescent markers on them. The human eye (or RGB camera) is unable to distinguish, for instance, a pure yellow fluorescent colour from a mixture of green and red. These limitations in viewing or imaging have kept the number of applications

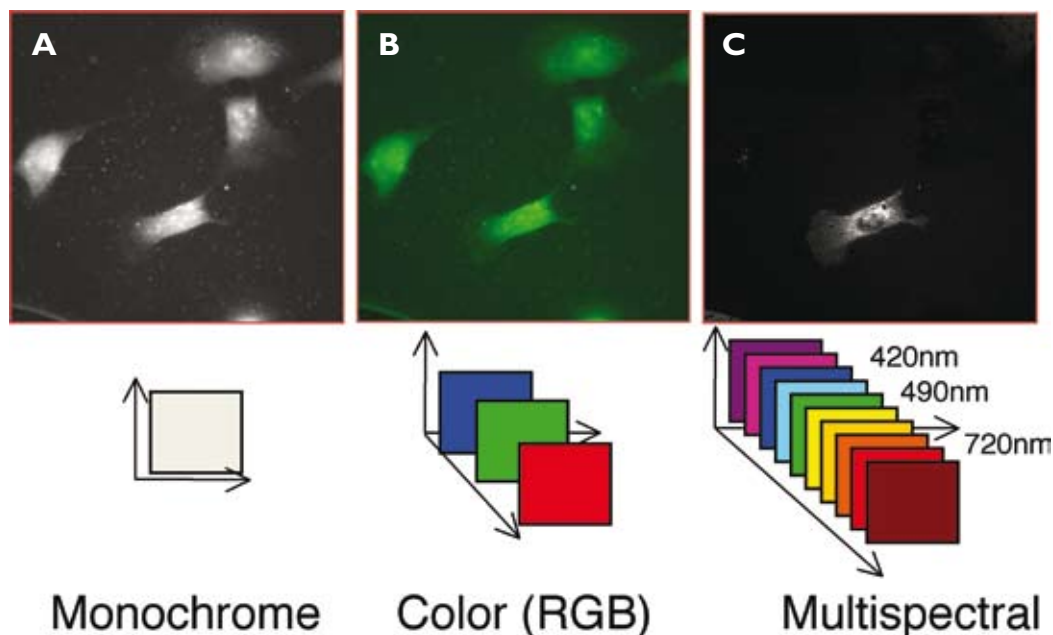


Fig. 1. Multispectral imaging (MSI) provides more information about the sample's composition than monochrome or standard colour (RGB) images. By providing full spectral information about a sample at each pixel in an image, MSI can differentiate signals which appear identical to the human eye, in this case separating the yellow fluorescent protein (YFP) signal from autofluorescence.

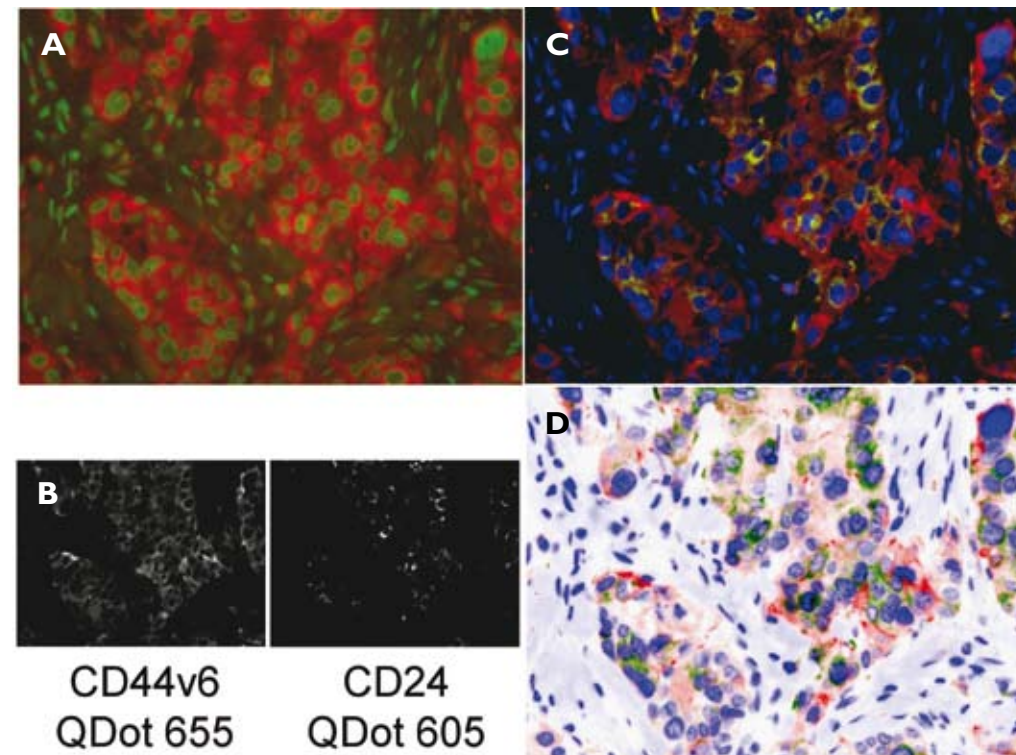


Fig. 2. Multi-label staining provides phenotypic information about a tissue section. Fluorescence images of a FFPE breast section labelled with CD44v6 (655-nm QDot), CD24 (605-nm QDot) and DAPI. A) Shows the RGB image of the sample, equivalent to what is seen down the oculars. B) Shows the unmixed images for CD44v6 and CD24. C) Shows a composite image of the DAPI (blue), CD44v6 (red) and CD24 (green). D) A simulated brightfield view of C. Fieldwidth: 280 μ m.

small, particularly for brightfield chromogenic IHC, resulted in multiplexed analysis being restricted to situations in which the analytes are not expected to co-localize or perhaps in which an otherwise useful counterstain may not be utilized.⁵ In addition, for IF methods, autofluorescence arising from formalin-fixed tissue can obscure important fluorescent labels and make visual assessment difficult.

Multispectral imaging (MSI) has been shown to be a perfect complement to these multiplexed IHC, IF and ISH⁶ methods, delivering high-quality images, often diffraction-limited in resolution, along with high-fidelity spectroscopic information about the sample on a per-pixel basis. The spectral information, when combined with spectral unmixing techniques for separating the components of a sample each into their own quantitative image, enables quantitative and qualitative, morphologically informed analysis of the component markers.⁷

There are many means of collecting multispectral data, ranging from simple bandpass filter wheel systems to complex multispectral laser-scanning

or two-photon confocal imaging systems. While this paper will focus on the pathology-related applications of multispectral data collected using a NuanceTM multispectral imaging system (CRI, Woburn, MA), it should be appreciated that many forms of multispectral data may be utilized similarly. However, given the relative complexity of many multispectral confocal imaging systems, it may not be easy to implement such methods in practice. Indeed, despite the availability of these modalities for a number of years, it is only recently that simplifications in hardware and advances in software have allowed MSI to make inroads into pathology research and practise.⁸

Given the high degree of autofluorescence found in many formalin-fixed tissue sections, MSI can also provide a simple contrast enhancement of up to 100-fold by separating, on the basis of their spectral signatures, tissue autofluorescence from the signal of interest. Figure 1 shows images of some cells on an autofluorescent background. Only one of the cells in the images is expressing YFP, the others glow only from their autofluorescence. Monochrome (Figure

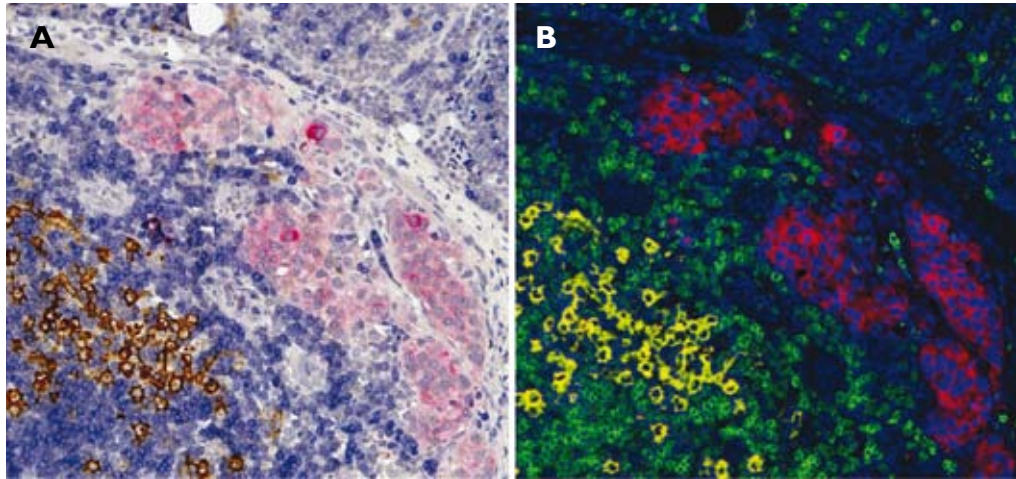


Fig. 3. Host immune response to breast cancer metastases in lymph nodes. A) Shows an RGB image of a FFPE lymph node section stained for CD3 (blue), CD1 (DAB), cytokeratin (red) and hematoxylin. B) Shows a simulated fluorescence composite image of the unmixed results from A. Fieldwidth: 350 μ m.

IA) and colour (Figure 1B) images are not able to show which cell is expressing YFP because the autofluorescence of the sample is being emitted at the same wavelength as the YFP. MSI combined with spectral unmixing can separate out the YFP signal, clearly revealing which cell is expressing YFP (Figure 1C). This contrast enhancement can also apply to multiple markers in a fluorescently labelled sample.

One example of a burgeoning new field in which the simultaneous assessment multiple markers is required is the study of cancer stem cells in solid tumors. Cancer stem cells (CSCs) are a subpopulation of cancer cells (found within tumors or hematological cancers) that possess characteristics normally associated with stem cells such as self-renewal and the ability to differentiate into multiple cell types. A theory suggests that such cells persist in tumors as a distinct population and cause relapse and metastasis by giving rise to new tumors.⁹ Development of specific therapies targeted at cancer stem cells holds hope for improvement of survival and quality of life of cancer patients, especially for sufferers of metastatic disease.

The first conclusive evidence for cancer stem cells was published in 1997 in *Nature Medicine*. Bonnet and Dick isolated a subpopulation of leukemic cells that express a specific surface marker CD34, but which lack the CD38 marker. The authors established that the CD34⁺/CD38⁻ subpopulation is capable of initiating tumors in NOD/SCID mice that are histologically similar to the donor.¹⁰ In

primary breast cancer, CSCs with a CD44⁺/CD24⁻ phenotype represent a minor population and are associated with self-renewal and tumorigenic potential. Unlike blood-borne diseases, which are amenable to flow cytometry multiplexed analyses, solid tumors present a more difficult problem. Figure 2 shows fluorescence images of a breast cancer specimen labelled with CD44v6 (655-nm QDot), CD24 (605-nm QDot) and DAPI. Also visible is considerable autofluorescence from the breast tissue itself. The unmixed images (Figure 2B) clearly show the distribution of each antigen, and the composite image (Figure 2C) provides a traditional fluorescence image of the sample. However, simulated brightfield representations of the sample (Figure 2D) may be preferable to many in the pathology community. In this image, each fluorophore is presented as if it was a chromogenic IHC marker, and the DAPI stain takes on the appearance of a hematoxylin counterstain.

Another example which requires multiple markers to be assessed simultaneously is in the host-immune response to metastases in lymph nodes.¹¹ More traditional grinding up of the sample and performing antigen analysis will not provide any information on the spatial relationship between different cell types reflected by their marker expression. Figure 3 shows images of a lymph node stained for CD3 (blue), CD1 (brown, DAB), cytokeratin (Fast Red) and counterstained with hematoxylin. Figure 3A shows a colour image of the sample. It is very difficult to visually distinguish between the blue CD3

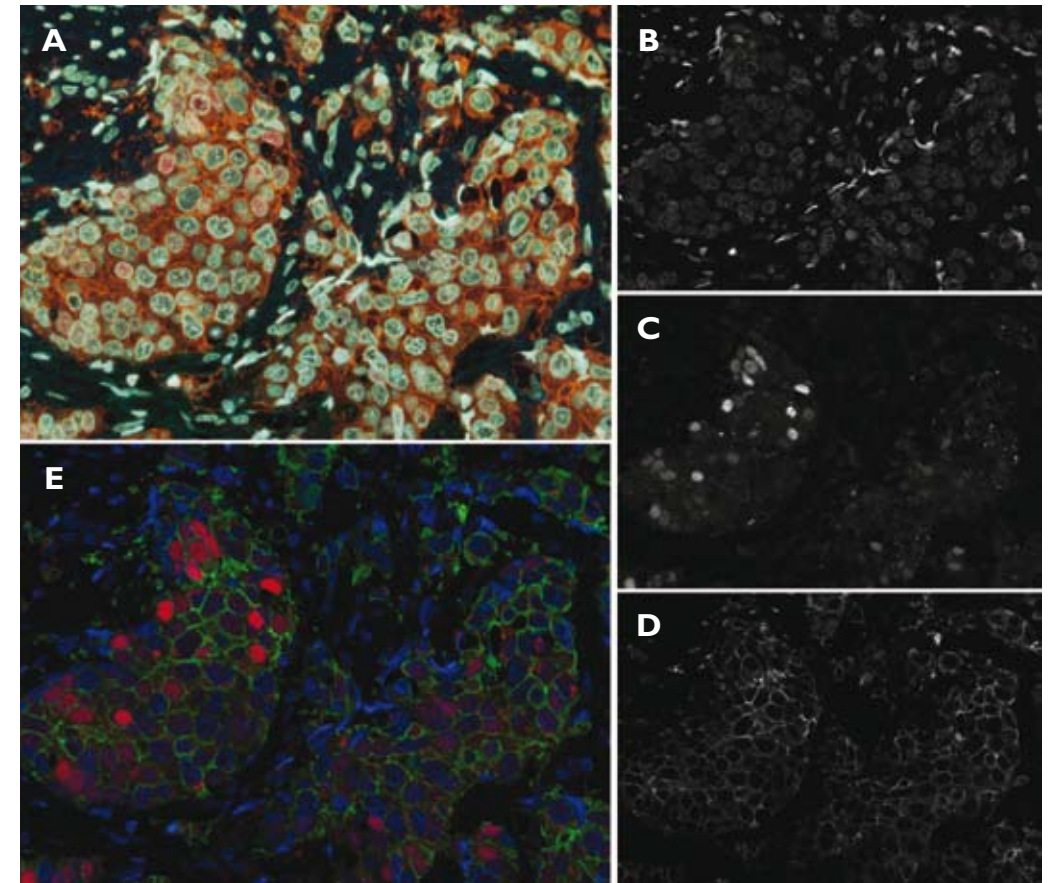


Fig. 4. Multiple clinically relevant markers can be imaged simultaneously using MSI. Fluorescence images of a FFPE breast cancer section labelled for EGFR (655-nm QDot), ER (605-nm QDot) and DAPI. A) Shows a standard RGB fluorescence image of the sample. B-D) Show unmixed images for DAPI, ER and EGFR, respectively. E) Shows a composite image with DAPI (blue), ER (red) and EGFR (green). Fieldwidth: 245 μ m.

stain and the hematoxylin counterstain. Figure 3B shows a simulated fluorescence composite image of the unmixed results from A where the three IHC markers and the spatial relationships among the respective cell types can clearly be seen. The CD⁺ cells are distributed some distance away from the cytokeratin⁺ tumor cells, a finding that would be lost if the sample had been homogenized before analysis.

Given some of the limitations of the IHC approach, that prevent a higher levels of multiplexing than what appears to be the practical limit of 3-4 simultaneous labels, fluorescence-based approaches may often be required. Recently, quantum dots have received a great deal of interest for multiplexed IF, and show great promise.^{12,13} Figure 4 shows fluorescence images of a breast cancer section labelled for EGFR (655-nm QDot), ER (605-nm QDot) and DAPI.

Figure 4A shows a standard colour image of the sample, which, as often is the case for multiplexed samples, does not contain a lot of visual information about antigen distribution and expression. Figures 4B-4D show the unmixed images for DAPI, ER and EGFR, respectively. Figure 4E shows a composite image of the sample, with DAPI in blue, ER in red and EGFR in green.

As previously mentioned, simulated brightfield images of fluorescently labelled samples may be the preferred visualization modality for the majority of pathologists. In addition to being able to show all of the markers in a simulated brightfield sample, MSI and its unmixed images can be used to demonstrate each marker separately. Figure 5 shows images of the same fluorescently labelled breast cancer section as in Figure 4, with Figure 5A being the simulated brightfield representation of a sub-region of Figure

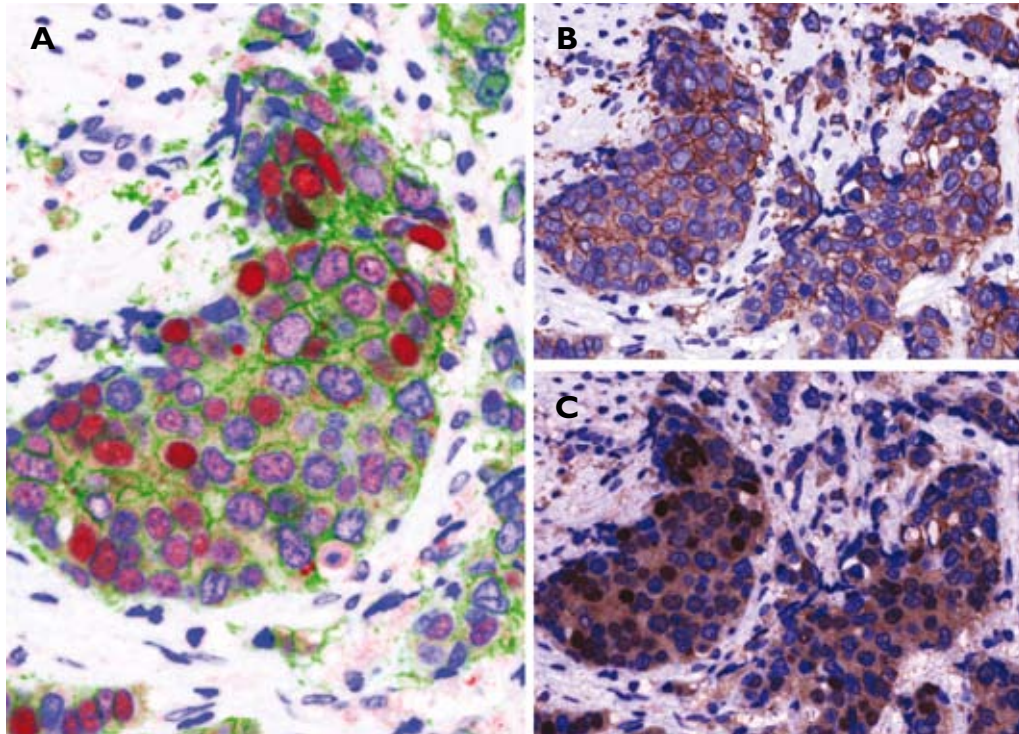


Fig. 5. Visualization of fluorescence images as simulated brightfield labelling improves legibility. Simulated brightfield of the same fluorescently labelled breast cancer section as in Figure 4. A) Shows a representation of a region of Figure 4E, with DAPI in blue, ER in red and EGFR in green. B) and C) show simulated DAB-stained images of the same sample, with B showing EGFR and C showing ER. Fieldwidth: 140 µm.

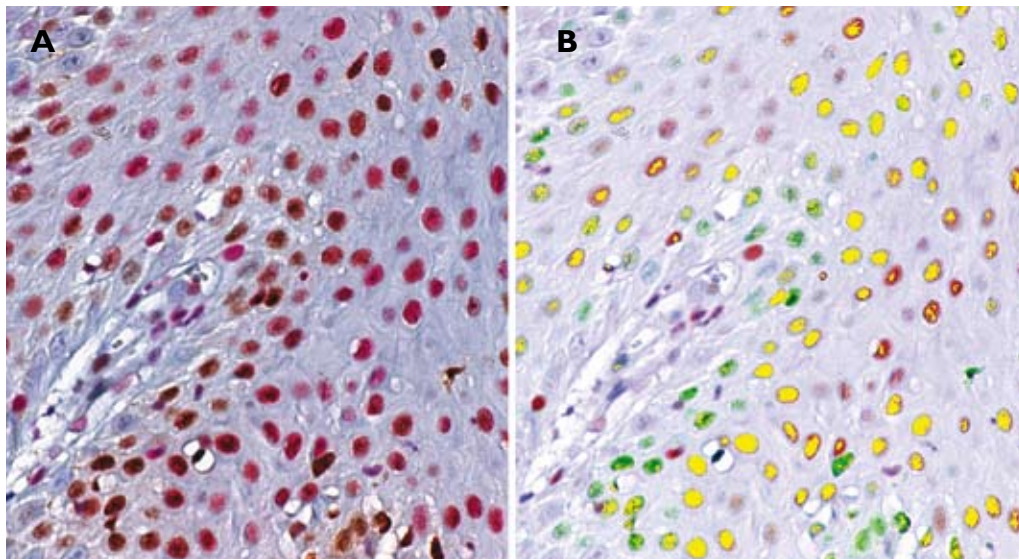
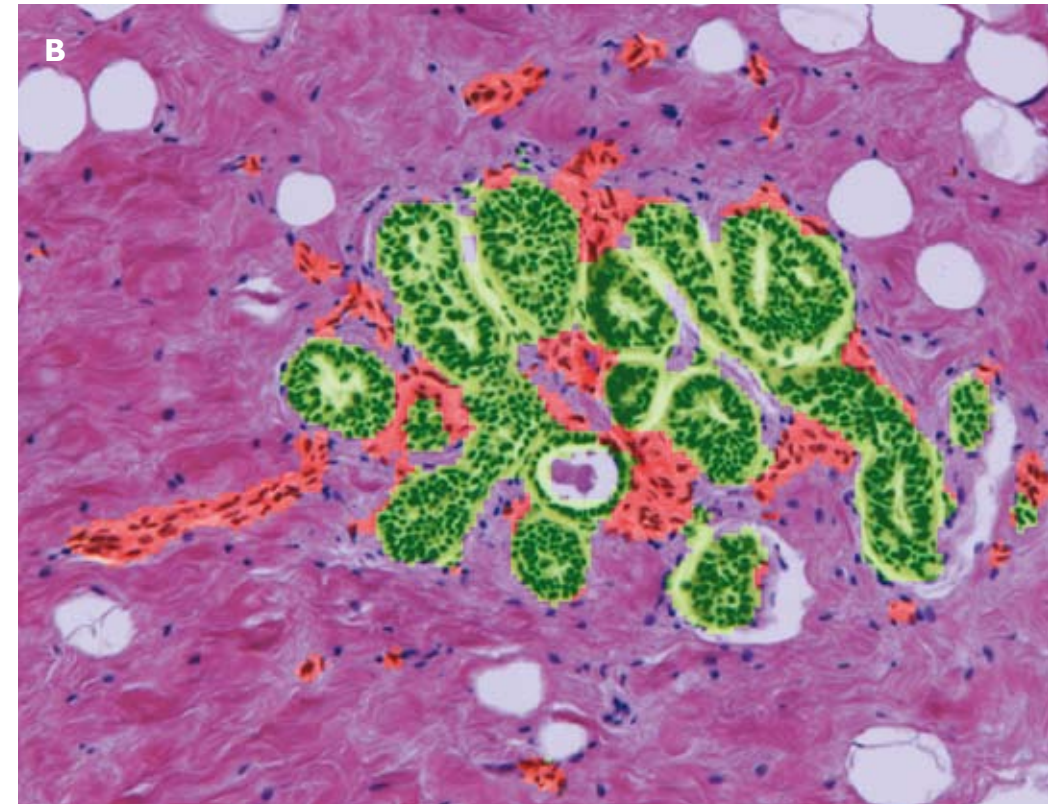
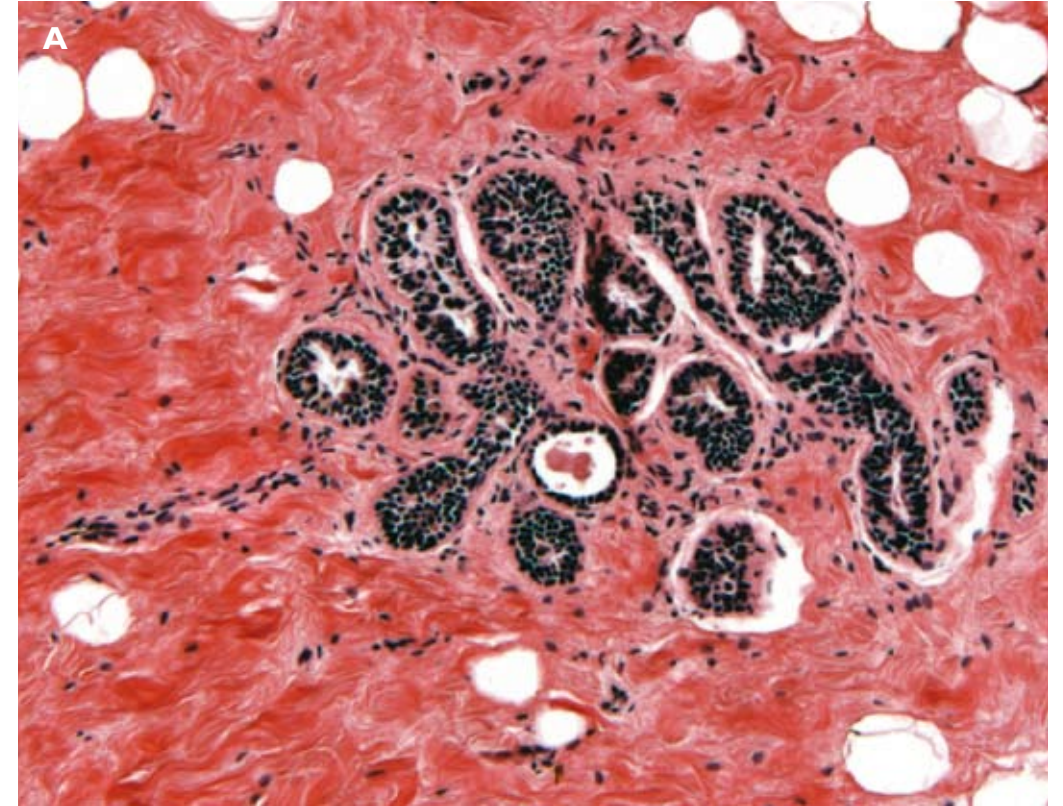


Fig. 6. Co-localization of multiple nuclear markers can be determined chromogenically. p21- and p27-labelled FFPE skin section counterstained with hematoxylin. A) Shows an RGB image of the sample. B) Shows a composite image of the same sample, with hematoxylin in blue, p21 in red and p27 in green. Pixels which contain co-localized p21 and p27 are shown superimposed in yellow. Fieldwidth: 140 µm.

Fig. 7. Automated segmentation by train-by-example machine-learning methodologies. H&E-stained breast section. A) shows a colour image of the sample. B) shows a train-by-example segmentation of the H&E image, differentiating breast (magenta) from lobules (green) from blood vessels (red). Fieldwidth: 500 µm.



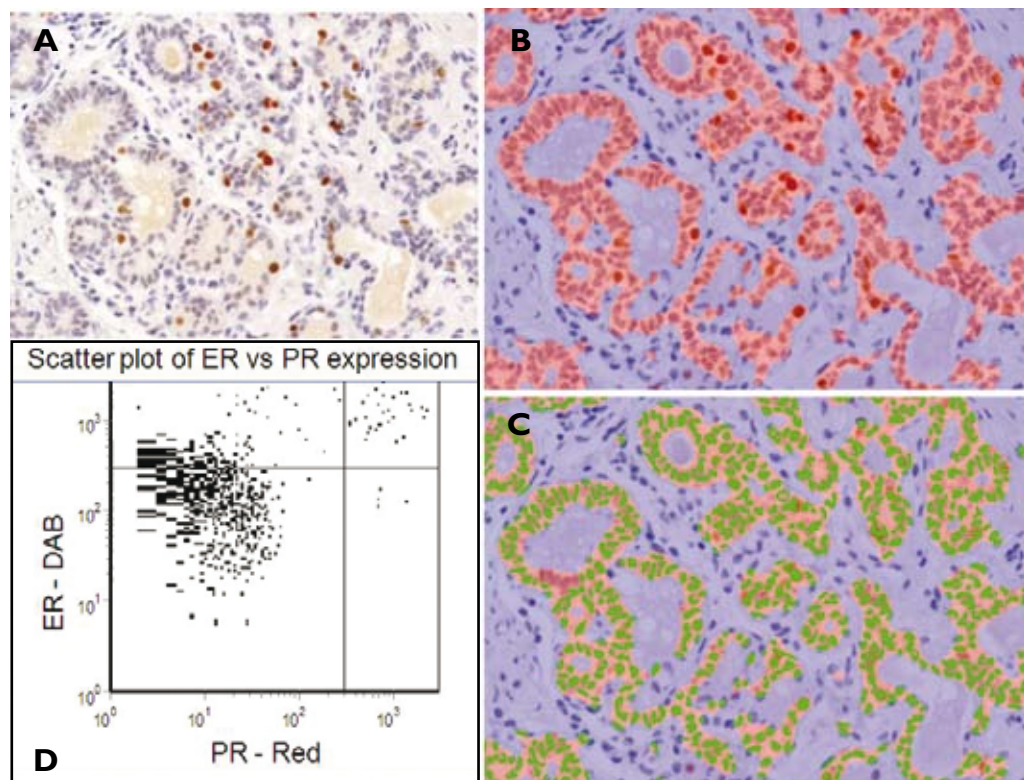


Fig. 8. Automated segmentation can provide flow-cytometry-like data on a per-cell basis. A breast cancer section dual-labelled for ER (DAB) and PR (Fast Red) and counterstained with hematoxylin. A) shows a colour image of the sample. B) shows the segmentation results for cancer (red) and normal tissue (blue) superimposed on the image. C) shows the nuclear segmentation (in green) added. D) Shows a plot of the ER and PR expression levels on a per-cell basis in the segmented cancer region. Fieldwidth: 385 μ m.

4E. Here, DAPI is shown in blue, ER in red and EGFR in green. Figures 5B and 5C show simulated DAB-stained images of the same sample, showing EGFR and ER staining, respectively. It is simple in software to be able to switch among all of the views of the sample in Figures 4 and 5, providing a flexible means of visually assessing multiple antigen distributions in a single section.

For many applications, a more quantitative approach to antigen expression and co-localization may be required. One means of addressing this is with pixel-based co-localization and manually drawn regions of interest (ROIs) for quantitation. Figure 6 shows images from a skin section labelled with p21 (DAB) and p27 (Fast Red) and then counterstained with hematoxylin. Figure 6A shows the colour image of the sample. Visual differentiation between p21-labelled, p27-labelled and co-localized nuclei may be difficult if not impossible. However, Figure 6B shows the unmixed composite image of the same sample, with hematoxylin in blue, p21 in red and p27

in green. In this case, however, pixels that contain co-localized p21 and p27 are highlighted in yellow, greatly easing visual assessment.

MSI has been used to analyze up to 4 IHC markers in brightfield or up to 7 fluorescent markers simultaneously.⁶ This can provide a means of determining the distribution of each of these markers individually or in any combination. However, for many applications in pathology and pathology research, a more automated and quantitative approach is desirable. To fully extract the information present in stained cells and tissues, it is necessary to have an integrated capability that will allow for coordinated image acquisition, automated region-finding and sub-cellular compartment delineation, visualization, calibration, quantitation, and reporting.

Recently, powerful and easy to use machine-learning software has been developed that combines largely textures-based¹⁴ neural network-derived segmentation algorithms with a learn-by-example

interface.^{15,16} It can be used to create a classifier that can distinguish between cancer, normal tissue, stroma and inflammatory infiltrates, to use one reasonable palette. Training can be extended over multiple examples in order to encompass the variability in the sample set. Not unsurprisingly, classifiers developed at one magnification should be applied only on images taken at the same magnification, and similarly, classifiers developed on one kind of lesion (e.g., prostate cancer) will not necessarily perform well on other cancer types. On the other hand, such classifiers can accommodate considerable variation in phenotype within a broad cancer class. Once a classifier is developed and validated, it can be re-used on subsequent images with reasonable robustness (unpublished data supported by results from over 3,000 images of intraductal breast cancer specimens). Training can be quite rapid, and good classifiers can be developed within an hour or so by end-users without particular computer or mathematical expertise.

An example of the kinds of segmentation that can be achieved is shown in Figure 7. While visual morphological segmentation is relatively easy for in this example, most automated methods will generally find this task extremely difficult. However, progress in this field is rapid; Figure 7B shows the results of CRI's train-by-example software, demonstrating segmentation into breast tissue (magenta), lobules (green) and blood vessels (red). This same kind of segmentation can work for immunostained samples as well. An important feature which contributes to robustness of results is the ability to perform the segmentation on an unmixed hematoxylin plane; this approach allows the classifier to base its output only on morphological parameters without relying on perhaps variable immunostains for guidance. An example of this kind of segmentation can be seen in Figure 8, which combines MSI with automated image segmentation of a breast cancer section dual-labelled for ER (DAB) and PR (Fast Red) and counterstained with hematoxylin. In addition to performing the morphological segmentation (8B) and cellular (nuclear) segmentation (8C), the software is able to extract per-cell expression levels from each nucleus within the cancer region, which enables the display of the data in a flow-cytometry-like scatter plot (8D).

Another development that will affect the practice of pathology is the advent of whole-slide imaging instruments, some of which are fast enough to enable the conversion of pathology to a largely digital discipline.¹⁷ MSI can readily be adapted to at least some of these slide scanning technologies, and preliminary work has indicated that this combination will indeed be practical and useful. Having digital information for an entire specimen will allow for statistically valid quantitative tissue segmentation and quantitation techniques operating in a largely hands-off fashion. Pathologists would then perform a review and validation function, along with integration of this slide imaging data with other sources of information as part of an overall clinical assessment.¹⁸

In summary, multispectral imaging (MSI) will provide pathology with many of the tools it needs to generate the complex information now being requested of it. While the methods and technologies involved may currently seem conceptually and practically challenging, the reality is that by the time the next generation of instruments and software tools emerge, MSI will be viewed as a routine addition to standard care practices. Prognosis, therapy selection and therapeutic monitoring will in many instances involve determinations of multiple analytes in unhomogenized, spatially intact tissue specimens, with resolution sufficient to measure expression in individual subcellular compartments. None of today's competing phenotyping technologies (expression arrays, serum proteomics, in-vivo imaging, e.g.) generate equivalent spatial and molecular precision.

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