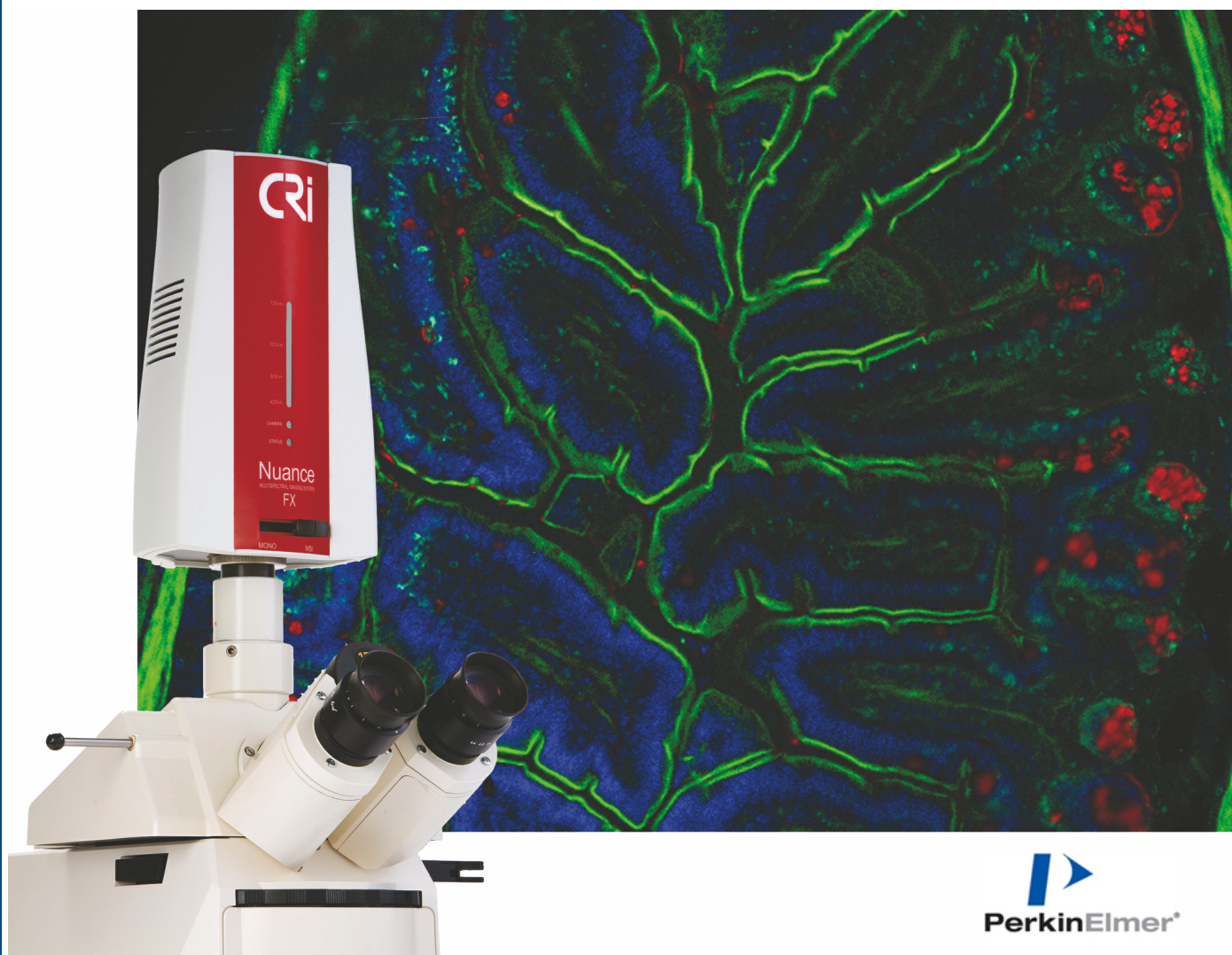# User's Manual for Nuance 3.0.2

PerkinElmer®

<u>Notice</u>

The information in this document is subject to change without notice and should not be construed as a commitment by PerkinElmer, Inc. PerkinElmer assumes no responsibility for any errors that may appear in this document. This manual is believed to be complete and accurate at the time of publication. In no event shall PerkinElmer be liable for incidental or consequential damages in connection with or arising from the use of this manual.

This manual describes system operation using Nuance version 3.0.2 software.

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US Patent 5,892,612; 5,953,087; 7,655,898; and patents pending.

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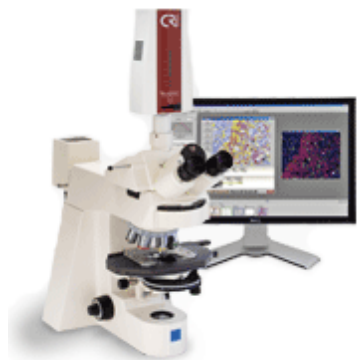
Multi-label Imaging Without Cross-talk

As biological imaging becomes more about studying complex systems rather than single events, **Nuance** multispectral imaging systems let you quantitate multiple molecular markers even when they are co-localized in a single tissue section, producing clear and accurate images of each individual label on a multi-label tissue section.

The unique and patented Real Component Analysis (RCA) tool automatically detects spectral components. View and quantitate signals as either single images, as overlays, or as a composite image showing all unmixed labels in high contrast. **Nuance** systems offer powerful spectral unmixing capability to remove autofluorescence, thereby dramatically increasing signal-to-noise and improving the accuracy of your results.

Getting Started — New Users

- Review the Introduction to Multispectral Imaging Theory and Concepts [7] to familiarize yourself with the concepts behind multispectral imaging technologies.

- Study the Getting Started with Nuance [17] section to familiarize yourself with the Nuance Hardware components and the software.

- Make sure you review the Method Development [31] section, which discusses methods for building accurate and reliable spectral libraries.

- You might also find it helpful to run through the Practice Exercise [37] to see how easy it is to spectrally unmix a counterstain from a target-specific marker.

- If desired, see the Practical Multiple Staining Pamphlet on the installation CD for a discussion on staining and imaging.

2 Introduction to Multispectral Imaging

This section provides an introduction to the theory and concepts that enable PerkinElmer's multispectral imaging systems to function as well as they do.

2.1 Light

Light, as used in this discussion, means the portion of the electromagnetic spectrum that is visible to the human eye. While the physical description of light can be highly complex, we will restrict this discussion to the wavelengths of light, and the interaction of that light with physical and biological materials.

The Electromagnetic Spectrum is illustrated in the figure below from radio to gamma ray frequencies. We are concerned with the visible wavelength range for purposes of this discussion, although the Nuance imaging system can operate out to 950 nm, into the so-called near-infrared wavelength range.
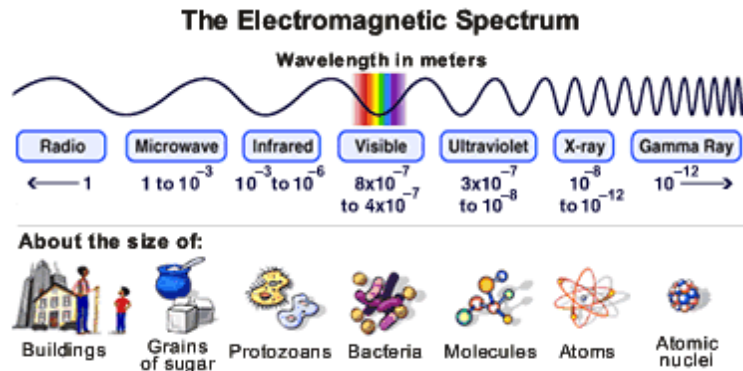
Figure 1: The Electromagnetic Spectrum

Although this illustration of the electromagnetic spectrum[1] suggests that the visible range of light covers approximately 400 nm to 800 nm, most humans realistically are limited to the range of 400 nm (deep violet) to 700 nm (deep red). Visible light makes up a very small portion of the entire electromagnetic spectrum.

Light is the transmission of energy. Visible light is associated with an energy level of about one electron volt per photon. As one moves to shorter wavelengths of light, the energy per photon increases. In the shorter ultraviolet wavelengths, which approach soft x-rays, the electron energy per photon increases to 50 to 100 electron volts. This energy content of light is useful when one wishes to induce a change of energy state in a material (i.e., cause a receptive molecule to undergo a series of energy additions and then relaxations, resulting in fluorescence).

(1) The Electromagnetic Spectrum illustration was prepared by NASA under Contract NAS5-26555 for the Education Group of the Space Telescope Science Institute's Office of Public Outreach. It is used here under public domain in accordance with NASA's contract.

2.2   Human Perception of Light Intensity and of Color

A Highly Adaptive Light Detector

The human eye is a highly adaptive light detector, becoming significantly more sensitive in low light, and less sensitive under bright light. This adaptive change is not instantaneous, and it takes some time for the eye to fully adjust to a new illumination level. This is the reason one needs to "dark adapt" by being in a darkened room for some time before observing weak fluorescence through a microscope.

While the eye can be very sensitive to low light levels and can also "see" in very bright conditions, it does not discriminate light levels very well. An individual has no "internal meter" that indicates the current sensitivity level setting for the eye. The eye also has a limited ability to discriminate levels of illumination ranging from the lowest level to the highest level at any given sensitivity. US Department of Defense research indicates that while some people can distinguish as many as 500 levels of gray, most humans can only distinguish approximately 30 to 35 levels of gray, ranging from black to white. This is relatively insensitive to the actual total illumination level, although the ability to discriminate gray levels does degrade in both very dim light and very bright light.

Response to Illumination

The eye's response to illumination is not a linear response, but more closely approximates a logarithmic function. The result is that while the human eye interprets differences between gray levels as "even steps," to achieve a two-fold brightening of the perceived gray level, the actual illumination level would need to increase significantly more than a simple doubling.

Contrasting the eye with a digital electronic sensor system, any sensor that has 8-bit resolution can detect 256 levels of gray. As the number of bits of resolution increase, the number of gray levels also increases. A 10-bit system gives 1024 levels and 12 bits yields 4096 levels of gray. Digital electronic sensors are linear in response to light levels.

Ability to Distinguish Colors

While the eye is relatively poor at discriminating gray levels (intensity), it is very good at distinguishing colors. Most individuals are estimated to be able to discriminate thousands of colors. The problem is, no two individuals see precisely the same color. In other words, each individual interprets colors slightly differently when viewing the same color. The basis for this is the way in which color sensors are arranged in the eye.

The eye contains three different types of color sensors, similar in response to the red, green and blue detectors in color cameras. Individual colors are composed of some combination of responses from these three different types of color sensors. While the general arrangement of these color sensors (cone cells) in the eye is reasonably standard, there are differences in the total number of each type of cone cell, and in the actual physical arrangement within the detecting cell layer (retina). These minor variations lead to the differences in perceived color between individuals, as does the actual way in which the individual's brain learned to interpret color(s).

2.3   Light Absorbance and Reflection

We perceive objects based on the way they interact with incident light (excepting light emitting objects such as light sources, and fluorescing or phosphorescing objects). Objects transmit, absorb, and/or reflect light; in most cases they exhibit a combination of **transmittance**, **absorbance**, and, **reflection**. The perceived color of a non-transmissive object is a direct result of both absorbance and reflection of light. An opaque object we would perceive as red is one that absorbs most wavelengths of light, except for red wavelengths that are reflected back to the eyes of the observer. An object we would perceive as red in transmission is one that transmits primarily red wavelengths, while absorbing or reflecting other wavelengths.

Generally speaking, absorbance and reflection of light are very similar phenomena. One can say that the light transmitted through a semitransparent object is the light that is not absorbed by the object. Note how similar this is to the definition of reflectance. Only the geometry is different. Reflectance is more complex than simple transmission due to variations in surface texture, transparency of the reflecting layers, and the characteristics of the opaque surface beneath the reflecting layers.

In brightfield light microscopy we have a controlled geometry, and are observing light that is transmitted through a specimen. Excepting a few pigments and inclusions, biological specimens are essentially invisible, unless we employ some absorbing dye, or specific optical arrangement to impart contrast. It is this need for contrast that led to the initial development of biological stains and stain protocols and subsequently to phase contrast and other optical contrast enhancing techniques.

The amount of light absorbed by a dye or stain in a specimen can provide a measurement of the amount of the absorbing material present. This is the basis of spectrophotometry. A basic law of spectrophotometry is the **Beer-Lambert law**, which states that the amount of material present (absorbance) is defined by the relationship:

Absorbance = -log(transmission) = (molar absorbtivity) x (path length) x (concentration)

For a given material, the molar absorbtivity, also called the molar extinction, is a constant, and therefore one only needs to determine the percent transmission of light through the specimen and the path length in order to calculate the concentration. The transmission is the amount of light without the specimen versus the amount of light with the specimen, and this is easily measured. Since transmission is based on a ratio, it is not sensitive to actual light level, assuming that the light does not change between the measurement of the "blank" or 100% level; and the "specimen" or sample measurement. It is this basic law that is used in solution spectrophotometry to determine the concentration of absorbing materials in solution.

The Beer-Lambert law has two specific requirements:

➢ The absorbing material must be homogeneous

➢ The absorbing material must be a true absorber of light, not a light scatterer

This first requirement poses problems for spectrophotometry through a microscope. The very reason one uses a dye on a microscope preparation is to see structure, and by definition, if one can see structure, then the dyed material is not homogeneous. While this may seem like an insurmountable obstacle to performing spectrophotometry through a microscope, the solution is simple. Microscope optics are characterized by their ability to resolve two points as separate. This is the resolution of a particular lens system. If the light detector element sees a smaller portion of the specimen than the lens system can resolve as separate points, then by definition, the spot being measured is homogeneous.

Using modern CCD or CMOS cameras for image collection, accurate spectrophotometry requires

that each individual pixel of the sensor see a portion of the specimen that is smaller than the lens resolution of the particular lens system being used. In practical terms, this means the camera pixels should be smaller than:

$$\text{(magnification) x 1.22 x (wavelength)} \div \text{(NAobjective + NAcondenser)}$$

The result of not having homogeneity of absorption at each pixel is an error called **distributional error**. Distributional error can be accurately defined mathematically, but the following illustration will suffice to understand this principle. Assume you have a light shining through an aquarium filled with water and some means of measuring the amount of light coming through the aquarium. If you place a small-capped bottle of ink into the aquarium, the measured amount of light will decrease by only a small amount. If you reach into the aquarium and remove the cap from the ink bottle and let the ink diffuse throughout the aquarium, the measured amount of light will decrease significantly. The total amount of ink in the aquarium is the same in both cases; it is only the distribution of the ink that is different. In practical microscope systems, distributional error can give errors of up to 40% if the specimen is not homogeneous at the detector element(s).

The second requirement of the Beer-Lambert law is that the material being measured must be a true absorber, and not a light scatterer. There are several reasons for this. One is that we cannot determine a molar extinction (the "constant" in the Beer-Lambert equation) for a sample with mixed properties. A more understandable rationale is based on the microscope optics. If a material scatters light, as the concentration of the material increases, more light is scattered, and this scatter can be outside the capture cone of the objective being used. Obviously, any light that is scattered in such a way as to not be seen by the objective cannot be measured. The consequence of this is that the detected signal is non-linear as the concentration of the mixed absorber-scatterer changes.

Assuming a specimen with a true absorbing chromogen, and an appropriate magnification for the detector being used, the Beer-Lambert law provides for true brightfield microscopy quantitation. After an image is converted to optical density or absorbance, each pixel is a true measure of light transmission at that pixel. This is because the background illumination is taken into account in the conversion. Absorbance images are therefore not constrained by total illumination level, and if the same specimen is imaged through identical optical systems at two different illumination levels, the resulting absorbance images should be identical.

There are several requirements that need to be met to achieve the accuracy inherent in optical density or absorbance measurements. It is necessary that the illumination be even over the entire image field. For microscope systems, this may not be the case, if the microscope illumination system is not carefully aligned.

Accurate measurements depend on some mechanism to perform a "flat field correction" for illumination inconsistencies. A common way to do this is to collect an image of the illumination field with no specimen in place (a blank field image). To ensure accurate data, any quantitative system should be able to meet the "test of the five positions". This involves selecting some object in a field of view, and then taking images of this object when placed in the center of the image field, and when placed at the top, bottom, left and right of the image field. In each of these five positions, the object should yield identical measurement results.

2.4 Fluorescence

Many biological and natural materials give off light of a particular color when exposed to light of another color. This property is a type of **luminescence**. If the emitted light occurs rapidly after illumination (around one-millionth of a second), the luminescence is called **fluorescence**. If the light emission takes longer than one-millionth of a second, the luminescence is called **phosphorescence**. Materials that exhibit fluorescence have proven extremely useful as labels or

indicators in many biological systems.

Fluorescence light emission is different than light absorption. Each fluorescent molecule generates light. We measure the total amount of light generated, and are not dependent on the interaction of the light with another material, such as a dye. While it would seem that fluorescence is much more amenable to accurate measurement than absorbed light, there are a number of factors that complicate such measurements. Fluorescence is emitted by a molecule in all directions, and most imaging systems are designed to capture light coming from a particular direction only. Therefore, there is no way to capture all of the light emitted by a fluorescent molecule with such a system. Additionally, fluorescence emission is influenced by the local environment, in particular by pH. The total amount of fluorescence will therefore depend on these local conditions of pH, as well as other surrounding molecules that may either enhance or quench some of the fluorescence energy. There is also the problem of obtaining identical excitation of all fluorescence molecules in a specimen, and this can be exceedingly difficult to achieve.

Fluorescence is an extremely sensitive technique, as it is much easier to visually assess or measure light emission against a dark background than it is to see a decrease in light intensity from absorption by a dye. Regardless of the sensitivity of fluorescence, the difficulty in establishing uniform excitation, and controlling for local environment effects makes quantitation of fluorescence emission difficult in biological preparations.

Stoke's Shift

Materials that fluoresce always emit light at a longer wavelength than the wavelength of the exciting light. As an example, rhodamine isothiocyanate can be excited by green light, and then emits red light. This difference between the wavelength of the exciting light and the emitted light is called the **Stoke's Shift** and is based on **Stoke's Law**.

A range of excitation wavelengths will excite fluorescence. This range of wavelengths is known as the **absorption spectrum**. The emitted light also covers a range of wavelengths, and this is known as the **emission spectrum**. Since the Stoke's Shift for most materials is not that great, there is generally some overlap between the excitation and the emission spectra. We will return to this point shortly as it does impact choice of emission and excitation filters in fluorescence systems. This figure contains an example excitation and emission spectra, showing Stoke's Shift and the overlap of the spectra.
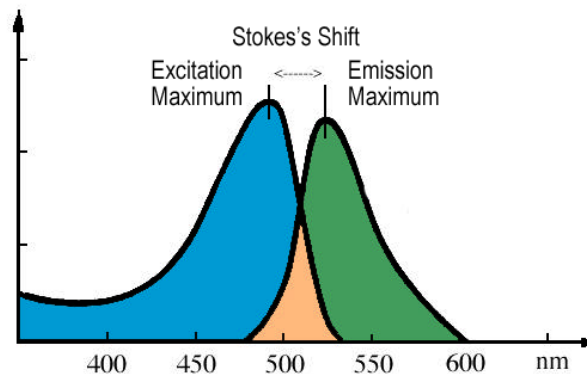

Figure 2: Stoke's Shift

Autofluorescence

Many biological materials are naturally fluorescent. In particular, many vitamins, some hormones, and a variety of biological enzymes and structural proteins are naturally fluorescent. These materials often fluoresce strongly enough to interfere with specific fluorescence labeling studies. Because of this unwanted background, or **autofluorescence**, both excitation light sources and emitted light paths are highly filtered in fluorescence systems.

On continued stimulation (illumination at the excitation wavelength), most fluorescent materials fade. While some specific preparation methods can reduce the rate of fading, and different fluorescent materials fade at different rates, all fluorescent materials eventually fade, and this effect is irreversible. For this reason, specimens should be illuminated only while aligning and focusing, and during actual image collection. At other times, the excitation light should be closed off.

Excitation and Emission Filters

Filters that are used for fluorescence excitation and emission are specifically constructed to have very narrow pass bands. They pass only a limited range of wavelengths of light. Restricting the excitation light wavelengths may reduce the amount of autofluorescence. Restricting the wavelength range of the emitted light helps minimize the amount of autofluorescence light that interferes with observing and measuring the desired specific fluorescence.
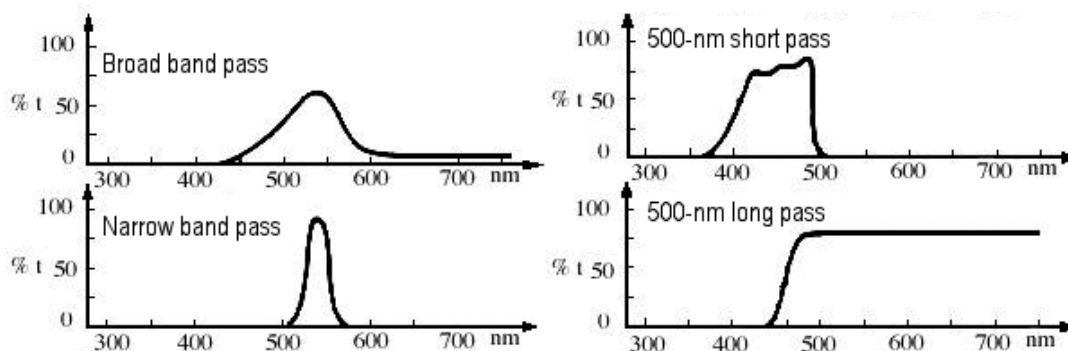
Figure 3: Excitation Filter Types

Excitation filters should be chosen to match the excitation maximum of the fluorescence label being used. The emission filter should match the emission maximum. In practical terms, the filter maxima may be slightly different from the ideal case, simply due to limitations of filter manufacturing, or to assist with autofluorescence reduction.

Specific excitation and emission filter combinations are available for most commonly used fluorescence dyes or labels. Nevertheless, regardless of how carefully one matches the excitation and emission filters to a given label, there will be some background autofluorescence, and this will reduce the perceived contrast between the "real" or actual label fluorescence and the specimen background.

2.5   The Multispectral Solution

The Nuance imaging system offers a unique solution to the problem of autofluorescence and selection of emission filters. Multispectral analysis is based on the fact that all fluorescent materials

produce a unique spectral emission. In other words, if you excite a material, and then examine the emitted fluorescence over a range of wavelengths, and record the intensity of emission at each point along the plotted curve of those wavelengths, you can generate an "emission spectrum" (like the green emission spectrum shown in the figure illustrating Stokes Law [11]). This spectrum is different for each specific fluorescent material.

Overlapping Emission Spectra

The complication is that for many fluorescent labels of biological interest, the emission spectra overlap significantly, and these emission spectra may also be obscured by autofluorescence from other constituents of the specimen. Often, autofluorescence is a strong (bright) broad signal that may obscure the specific fluorescence that the investigator wishes to see.
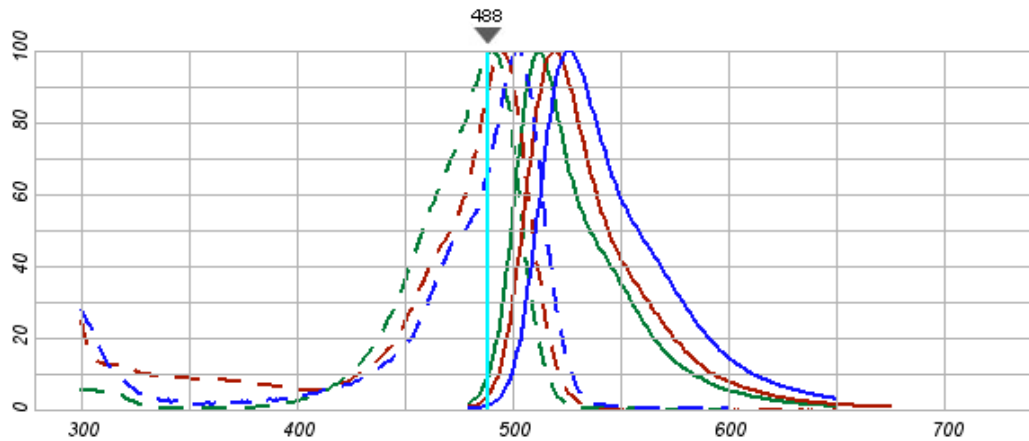
Figure 4: Overlapping Excitation and Emission Spectra

In this example of overlapping excitation and emission spectra, green is MitoTracker® Green Fn, Blue is Acridine Orange, and Red is Fluorescein isothiocyanate (FITC). The Cyan line at 488 represents the illumination light. Dotted lines are excitation spectra, and solid lines are emission spectra. (Illustration derived from Invitrogen™ Spectraviewer.)

There are three points to note in the graph shown above.

1. The excitation spectra overlap significantly with the emission spectra. This is why one needs to carefully select excitation filters. The goal is to prevent as much excitation light as possible from appearing in the emission spectra.

2. Even with distinct emission spectra, there is significant overlap in the emission spectra of these three dyes. Visual examination of such a mixture of fluorescence spectra would be unable to distinguish these three dyes as individual "colors." They would be seen as some combination of yellow and green by most observers.

3. The graphical display of spectra is normalized, and in actual practice, some fluorescent materials are much brighter than others.

As the graph illustrates, many labels of biological interest have emission spectra that are so similar that separation using narrow band filters is difficult or impossible. Multispectral analysis provides the solution to this problem and reduces the need for multiple expensive narrow-band emission filters. A single long-pass emission filter can replace a large collection of emission filters.

Multispectral Analysis

Multispectral analysis generates the spectral curves for the various fluorescent dyes or materials in a specimen. In addition, it generates a spectral curve for the autofluorescence that almost always is present to some degree. Using sophisticated algorithms, the contribution of autofluorescence to the image can be removed, and the individual fluorescence spectra separated. The result is a set of images representing each spectrum that contributes to the final image.

In other words, as illustrated in the graph above, multispectral analysis yields (1) an autofluorescence image, (2) a MitoTracker Image, (3) a Acridine Orange image, and (4) a FITC image. By removing the autofluorescence contribution to the image, the actual signals from the applied labels (MitoTracker, Acridine Orange and FITC) can be readily seen. If these individual images are recombined using highly contrasting colors to represent the location of each of the labels, a composite image of high contrast and readily observable colors can be generated.

The Nuance Imaging System

The Nuance imaging system's combination of unique hardware and sophisticated software makes powerful multispectral analysis possible. In a multispectral analysis, a series of images is captured at specific wavelengths. The range of wavelengths captured should cover the spectral emission range of the labels present in the specimen. The number of images within that range should be chosen to adequately define the emission spectral curve for each label. The result will be a series of images, called an "image cube," taken at specific wavelengths. The data within the image cube is used to define the individual spectra of both autofluorescence and specific labels.
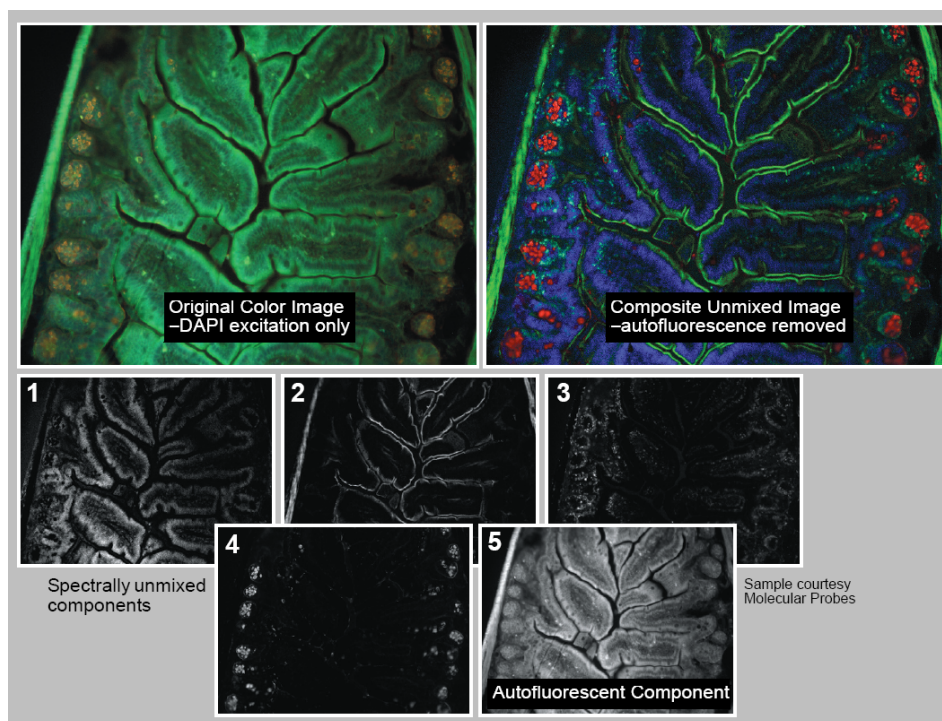
Figure 5: Mouse Intestine with Five Unmixed Fluorescent Elements

This figure shows a specimen (courtesy of Molecular Probes, Inc.) with poor contrast between the

five different fluorescent elements. A conventional color image is shown in the upper left. By acquiring a multispectral data cube and using the Nuance system's unmixing tools, a new Composite Unmixed Image can be created (shown on the right). Separate grayscale images representing each unmixed component are also created.

As the figure illustrates, many labels of biological interest have emission spectra that are so similar that separation using narrow band filters would be difficult or impossible. Multispectral analysis provides the solution to this problem, and in addition reduces the need for multiple, expensive and very narrow band emission filters. A single long pass emission filter can replace a large collection of emission filters. Multispectral analysis is able to separate all of these autofluorescence signals from the specific labels applied to the specimen, and provides the ability to localize each material present, and to detect weak specific labeling even in the presence of strong autofluorescence.

2.6   Nuance FLEX Multispectral Imaging

Nuance FX and EX imaging systems have the unique ability to narrow the bandwidth of the Liquid Crystal Tunable Filter by half. Flex technology lets you use the system in broad or narrow mode, which greatly increases the system's multiplexing capabilities for brightfield and fluorescence imaging. This figure shows LCTF transmission with and without Flex technology at 700 nm and 800 nm.
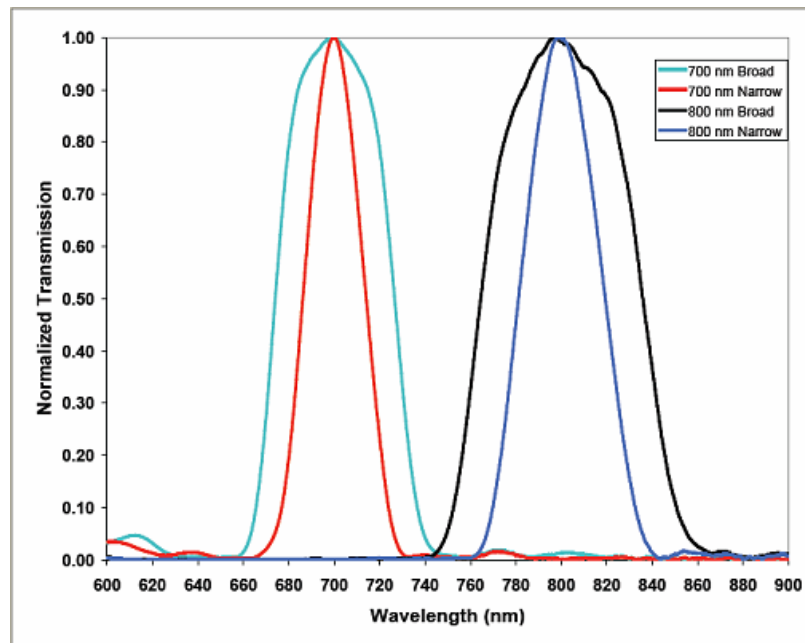
Figure 6: Comparison of LCTF transmission with and without FLEX

Use the system in narrow mode to obtain better resolution of closely spaced and overlapping emission spectra. Narrow mode can also increase the number of detectable chromogens or fluorophores in your sample with overlapping spectra. This can increase the dynamic range of your multiplexed signals. This figure illustrates the higher spectral resolution obtained with Flex technology.
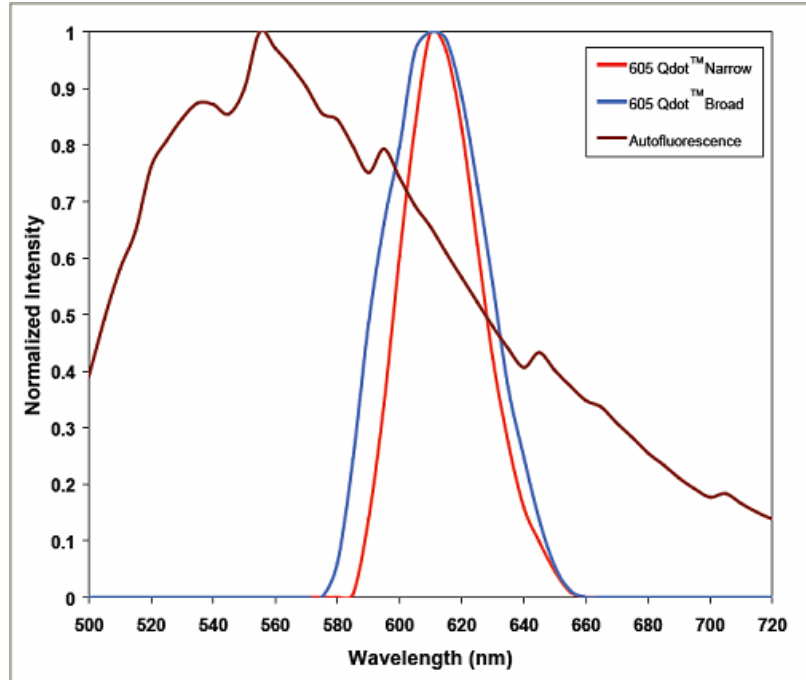
Figure 7: Comparison of 605 nm Qdot™ with and without FLEX

3 Getting Started with Nuance

PerkinElmer's Nuance multispectral imaging systems are high-performance multispectral imaging systems that can be installed on an upright or inverted microscope's C-mount camera port, on a copy stand, or on a camera tripod for field use. Properly configured Nuance systems can be used for applications as diverse as biomedical research, materials QA/QC, forensic analysis, and archeology. (Nuance systems are not intended for clinical or diagnostic use at this time.)

The patented liquid crystal (LC) tuning element functions as a high-quality interference filter that enables the transmitted light to be electronically tunable. This allows rapid, vibrationless selection of wavelengths in the visible or NIR range, and digital images (called image cubes) are captured at the specified wavelengths. The intuitive Nuance acquisition and analysis software performs spectral classification and unmixing of overlapping dyes or stains that may look indistinguishable to the naked eye but have differing spectral signatures.

The Nuance product family includes one general purpose model and two fluorescence-optimized models:

> The **Nuance VX for visible wavelengths** operates on or off the microscope in the wavelength range of 420-720 nm.

> The **Nuance FX** and **Nuance EX** operate on or off the microscope in either the 420-720 nm range (FX) or the 450-950 nm range (EX) and have electronically selectable bandwidths.

The topics in this section provide an introduction to the Nuance multispectral imaging system. This section includes a brief description of each of the system's hardware components and software features.

3.1 Operator and Equipment Safety

It is the responsibility of the purchaser to ensure that all persons who will operate the imaging system are aware of the following cautionary statements. As with any scientific instrument, there are important safety considerations, which are highlighted throughout this User's Manual.

General Cautionary Statements

⚠️ **READ AND UNDERSTAND THIS USER'S MANUAL BEFORE ATTEMPTING TO OPERATE, TROUBLESHOOT, OR MAINTAIN THE NUANCE IMAGING SYSTEM. READING THIS MANUAL FIRST MAKES IT EASIER AND SAFER TO OPERATE AND MAINTAIN THE SYSTEM.**

Operate the system on a flat, stable surface.

Do not drop the imaging module.

Do not expose the imaging system to prolonged heat above 40 °C.

Do not operate the system in an environment with explosive or flammable gases.

Do not subject the imaging system or its components to intense light from laser, focused arc or Hg lamp sources.

Do not operate the system in places where it may be splashed with liquid.

Use only a properly grounded power cable appropriate for the site where the system is installed.

Some cables and adapters supplied with the system have proprietary specifications. Do not connect components supplied by PerkinElmer using unqualified cables or adapters. Doing so could result in damage, and voids the Warranty.

Use only a properly grounded power outlet when connecting the system to power.

If you are using third-party mechanical components for the Nuance system, consult the System Specifications & Dimensions 104 topic.

Clean the Nuance imaging system periodically. This will help ensure optimal performance over years of use. See Cleaning the Nuance Imaging Module 21.

Caution: Service should be performed by PerkinElmer authorized and trained personnel only. Power must be disconnected from the system before servicing.

For Technical Assistance

If you experience any difficulty setting up, operating, or maintaining your imaging system, please refer to the Troubleshooting 102 section of this documentation. If that does not resolve your problem, contact your PerkinElmer representative. Office hours are 8:00 a.m. to 8:00 p.m. (Eastern Standard/ Daylight Time), Monday through Friday.

- Telephone: 800-762-4000 or +1 203-925-4602

- Fax: +1 203-944-4904

- E-Mail: global.techsupport@perkinelmer.com

3.2   About this Manual

This manual describes the use and functionality of the Nuance multispectral imaging system and the Nuance version 3.0.2 software. Operating instructions, functional descriptions, troubleshooting, illustrations, and other relevant information are contained in this manual.

Design Change Disclaimer

Due to design changes and product improvements, information in this manual is subject to change without notice. PerkinElmer reserves the right to change product design at any time without notice to anyone, which may subsequently affect the content of this manual. PerkinElmer will make every reasonable effort to ensure that this User's Manual is up to date and corresponds with the shipped Nuance multispectral imaging system.

Reproduction Disclaimer

This User's Manual is solely for the use of the owner and operator of the PerkinElmer Nuance multispectral imaging system. Any reproduction of this publication in part or in whole without the express written consent of PerkinElmer is strictly prohibited. Neither may this publication be made available for electronic download without the express written consent of PerkinElmer.

Help Menu in the Nuance software

The **Help** menu in the Nuance software provides quick access to the Nuance **Help Topics**. You can also view the **Filter Selection Guide** and the **Quick Start Guide**, both in PDF format. The **Help > About** menu item displays the current Nuance software version number as well as other important system information.

3.3 CE, CSA, and UL Testing and Certification

The Nuance multispectral imaging system has been tested by an independent CE testing facility, and bears the appropriate CE mark.

The Nuance system has been awarded the right to display the CSA mark.

The Nuance system has undergone tests to meet UL standards.

For more information, contact PerkinElmer.

3.4 Nuance Imaging Module

The Nuance Imaging Module contains all of the principal imaging components in a single compact enclosure:

- ➢ High-resolution, scientific-grade CCD imaging sensor
- ➢ Solid-state liquid crystal (LC) wavelength tuning element
- ➢ Spectrally optimized lens and internal optics
- ➢ Industry-standard C-mount (compatible with 1x C-mount camera tube)
- ➢ Industry-standard threaded mounting hole for securing the module to a camera tripod or other holder.

Wavelength LEDs indicate the current wavelength to which the LC-tunable filter is tuned. These LEDs scroll while the filter is exercising.

CAMERA LED is steady green when the camera is powered ON, there is a USB connection to the host computer, and the required camera drivers are installed.

STATUS LED is steady green when the LC-tunable filter is ready for MSI acquisition. Blinking green indicates the filter is busy. Red indicates an error condition.

MONO / MSI slider moves the LC tuning element into and out of the light path.

Microscope Interface is industry standard 1x C-mount.

Figure 8: Nuance Imaging Module (front view)

USB 2.0 Type B port is used to connect the camera to the host computer via the supplied USB cable.

+5V DC power connector is for use with the supplied power cord with transformer.

Industry-standard threaded mounting hole is for attaching the module to a camera tripod or other holder.

Figure 9: Nuance Imaging Module (rear view)

Workstation Computer

Nuance multispectral imaging systems require a computer with a level of performance appropriate for acquiring and analyzing digital multispectral data. Computers purchased from PerkinElmer that will be used with the Nuance system meet the performance requirements listed in System Specifications and Dimensions 104 If you decide to supply your own computer, be sure it meets the performance requirements as well.

Cleaning the Nuance Imaging Module

It may be necessary to periodically clean the outside of your Nuance imaging module. Follow these guidelines when cleaning:

➢ Spray a clean cloth with a cleaning solution such as 70% ethanol and wipe down the external surfaces of the imaging module.

➢ If dust particles are present on the optics, use a blow-off gun connected to clean dry nitrogen to blow dust away. Using canned air is not recommended due to the risk of spraying liquid compressant onto the optics, which will damage optical coatings.

➢ Do not touch optical surfaces with your fingers. Oils may damage sensitive coatings.

➢ If optical surfaces require more thorough cleaning, apply lens-cleaning fluid, spectroscopic-grade isopropyl alcohol, distilled water, or methanol to a lint-free lens tissue and drag-wipe the surface.

➢ Do not rub the surface because anti-reflection coatings can be scratched. If further cleaning is required, repeat with a fresh tissue and fluid after one pass, since contaminants need to be wicked away, not spread over the optical surface.

▬ Powering Up the Imaging Module

➢ Connect the power cable to the imaging module and plug it in to a surge protected electrical outlet.

➢ Connect the USB cable to the Nuance imaging module and the workstation computer.

➢ Blinking LEDs on the front of the unit indicate initialization is in progress. When the **STATUS** LED illuminates steady green, initialization is complete and the imaging module is ready.

3.5   Installing & Starting Nuance

The Nuance software requires an activated license number to run. If you already purchased the Nuance system with a computer, your software was installed and activated for you at the factory. To start Nuance, select **Start > All Programs > PerkinElmer > Nuance > Nuance 3.0.2**. Or double-click the **Nuance** icon on the desktop.

If you need to install and/or activate the Nuance software, refer to the separate Nuance *System Setup & Installation* card.

Power up the Nuance imaging module. (You can run the Nuance software without a connection between the computer and the Nuance imaging module. However, you will not be able to acquire images unless it is connected.)

▬ Evaluating Nuance Software

If you have not yet purchased a Nuance system but you want to evaluate the software, select the **Get Trial** option when the **Software Activation Required** dialog box opens. You will be allowed to use the software for 30 days before being required to purchase and activate the software to continue using it.

▬ Recovering a Lost Seat License

Nuance seat licenses are not transferable or recoverable. If your seat license is damaged or inadvertently destroyed, please contact PerkinElmer for a replacement license.

3.6 Understanding the Nuance Work Area

The Nuance work area includes a menu bar, a toolbar, control panels for acquiring and analyzing images, an image gallery, and thumbnails and data pages. This figure shows a sample of the work area with a cube and a set of unmixed images.
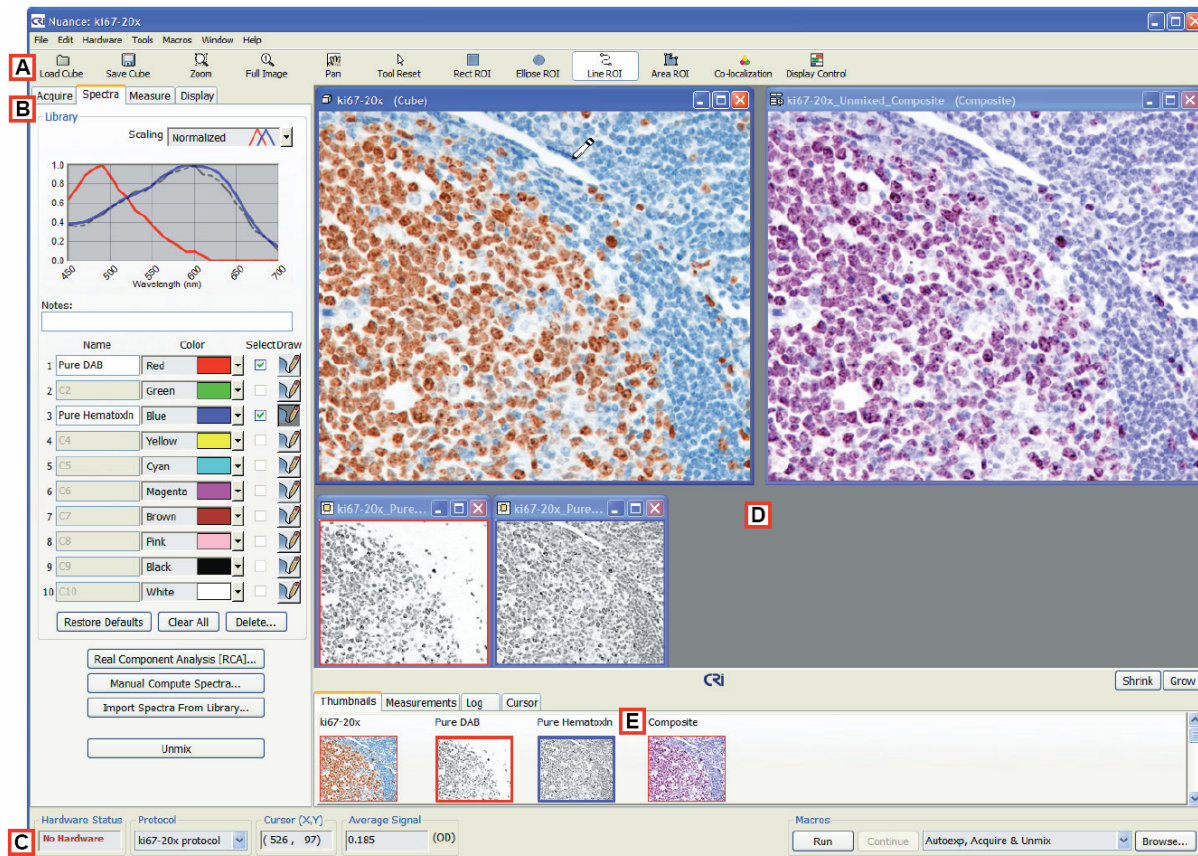
Figure 10: Nuance Work Area

A. Toolbar

The toolbar makes it easy to access a variety of functions including loading and saving cubes, adjusting the image zoom level, drawing measurement regions, and adjusting the display settings. The tools that are available on the toolbar will change based on the current action.
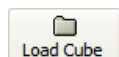
Click this button or select Open Cube from the File menu to open an image cube. See Opening an Image Cube [58].
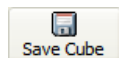
Depending on the active window, click this button to save an image or your multispectral dataset. See Saving Images and Image Cubes [54].
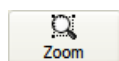
Use this tool to zoom in on an image. See Zooming In and Out [26].

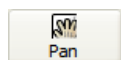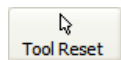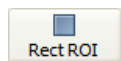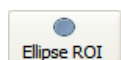|	
Full Image	Click this button to return the current image magnification to 100%.
Pan	Click this button to pan the zoomed image that is currently active. The pointer becomes a hand symbol. Pan the active image by clicking on the image and dragging it to the desired view within its window.
Tool Reset	Click this button to return the mouse pointer to its default pointer mode.
Rect ROI	When sampling spectral signals for your spectral library, click this button if you want to sample a rectangular region of pixels.
Ellipse ROI	When sampling spectral signals for your spectral library, click this button if you want to sample an elliptical region of pixels.
Line ROI	When sampling spectral signals for your spectral library, click this button if you want to sample a line of pixels.
Area ROI	When sampling spectral signals for your spectral library, click this button if you want to sample a freehand drawn region of pixels.
Co-localization	Click this button to open the Co-localization tool for co-localization staining detection and analysis.
Display Control	Click this button to open the Display Control to adjust the display settings (brightness, contrast, display scaling, etc.) for the current image. See also Customizing Spectral Displays 83.
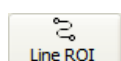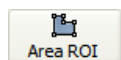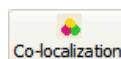
B. Control Panels

To select a control panel, click on its tab. The following panels are available:

➢ The **Acquire > Brightfield** panel includes all the controls needed to acquire new brightfield images and image cubes.

➢ The **Acquire > Fluorescence** panel includes all the controls needed to acquire new brightfield images and image cubes.

➢ The **Spectra** panel is used to create spectral libraries and unmix cubes.

➢ The **Measure** panel is used to find, measure, and adjust measurement regions in component images.

➢ The **Display** panel is used to change current display settings of images.

C. Status Bar

The **Hardware Status** box displays the current status of the camera: "No Hardware" or "Ready."

The **Protocol** drop down box displays the currently loaded protocol. It also lists all protocols opened during the current session with Nuance.

The **Cursor X/Y** and **Average Signal** boxes display the coordinate location and signal level at the cursor's current position over any image.

Use the **Macro** drop down list and associated buttons to select and run macros. Nuance comes with pre-recorded macros for acquiring and working with images, and these are listed in the **Macros** drop down list. Any additional macros that you create and save will be added to this list as well.

D. Image Gallery

The image gallery displays the live image (when in acquisition mode) and the current opened or acquired cube. The gallery also displays unmixed composite images and their component images, as well as any other images open in the software.

E. Thumbnails and Image Data Pages

There are four pages with tabs across the bottom of the image gallery. You can change the viewable size of these pages by clicking the **Shrink** or **Grow** buttons at the far right. These pages display the following types of information:

➢ The **Thumbnails** page displays a thumbnail of each image in the gallery. If you have more than one cube open, you can double-click any cube thumbnail to display the cube and its components in the gallery. If you have zoomed in on an image, the zoom view rectangle within the thumbnail shows the zoomed region. (You can drag this rectangle with the mouse pointer to pan the zoomed image.)

➢ The **Measurements** page displays the measurement data of measurement regions drawn on component images. See Quantifying Results 75.

➢ The **Log** page contains an action history of the current session. The log can be saved or cleared by clicking the buttons at the far right of the page.

➢ The **Cursor** page provides information about the image pixel at the current pointer location. For cubes, it displays the average signal. For composites, it displays absolute and percent contribution from each component. For other image types, it displays the pixel values.

3.7   Window Layout

Each window type has a home position on the screen. The cube lives in the upper left corner, the composite image lives in the upper right corner, and the component images are positioned in a grid starting from the lower left and working up and to the right.

Pressing Ctrl+L will force the windows to their home positions. In addition, many operations will force the windows to their home positions. These include resizing the thumbnail view, unmixing a cube, or activating a window from the thumbnail view.

Resizing an Image Window

There are a few ways to change the size of an image window or windows.

To manually resize a window:

Click and drag its window border to the desired size. The image inside the window resizes to fill the window, while also maintaining its aspect ratio.

To maximize the current window:

Click the window's maximize button. The window will fill the image gallery.

To expand or reduce a window in steps:

Select **Expand Window** (or press Ctrl+E) or **Reduce Window** (or press Ctrl+R) from the **Window** menu.

Zooming In and Out

You can zoom in to focus on details or zoom out to see more of an image.

Click the **Zoom** button on the toolbar. The mouse pointer changes to a magnifying glass. Use the magnifying glass to draw a box around the area you want to zoom.

You can also click the area of the image you want to zoom. Each time you click the image with the Zoom tool, the zoom increases. In addition, you can scroll the mouse wheel to zoom in.

To zoom out:

Click the Full Image button on the toolbar.

You can also right-click on a zoomed image and select **Zoom To Full Image** from the pop-up menu. You can also scroll the mouse wheel to zoom out.

Panning a Zoomed Image

When you zoom in on a particular area of an image, you might not be able to see other areas of the image.

To pan a zoomed image:

Do one of the following:

➢ Right-click on the image and select **Pan** from the pop-up menu. The mouse pointer changes to a hand icon. Click and hold the mouse button while dragging, or panning, to view the desired area of the image.

➢ Drag the zoom view rectangle that appears on the corresponding thumbnail image in the **Thumbnails** tab.

➢ Scroll the mouse wheel while holding down the Shift key (to pan horizontally) or the Control key

(to pan vertically).

▬ Panning All Images Simultaneously

Use the **Zoom** tool to zoom in on a particular region of the image cube. Next, hold down the Shift or Control key and drag the upper left-hand corner of the zoom view rectangle on the cube's thumbnail image. All images will pan simultaneously. If you want to return all images to the full image view, click the **Full Image** tool, then shift-click on the cube's thumbnail image.

Figure 11: Simultaneous Panning

3.8   Specifying Nuance Hardware Settings

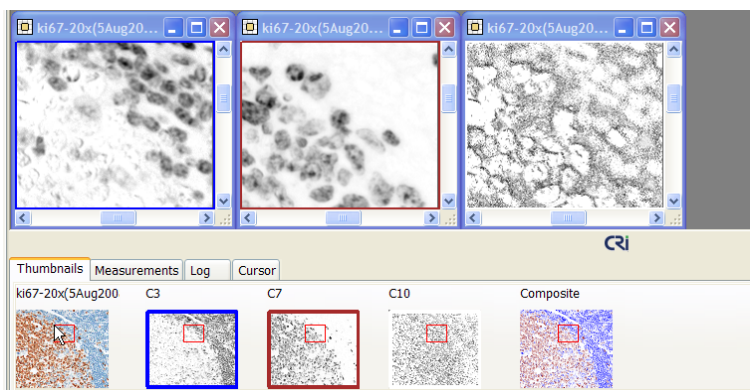You can specify a variety of settings and parameters for the camera in your Nuance imaging system. To access the camera settings, select **Hardware > Edit Hardware Settings > Camera**. A brief explanation of each setting is provided below:

➢ **CCD Bit Depth** lets you capture either 8-bit (256-level) or 12-bit (4096-level) grayscale images per wavelength. 12-bit images take up twice the memory and disk space of 8-bit images and can only be opened using scientific imaging applications, including Nuance software. In most instances, it is best to use the default 12-bit selection. This gives higher quality images. Many Microsoft® Windows® applications cannot open 12-bit images. If you want to capture images that can be displayed using non-scientific imaging applications, select the 8-bit option.

➢ **Gain** affects image quality because it modifies the CCD readout process. For images that have low intensities, consider increasing the gain value slightly to achieve images with brighter pixel values. Increasing the gain can also reduce the exposure time. Note, however, that higher gain settings can result in increased signal noise, which reduces image quality. Gain values of 1, 2, or 3 should be suitable for most applications.

➢ **Flip Image** rotates the live image stream and new acquired images 180 degrees. Select this check box if your images are upside down. (Previously acquired images are not affected.)

➢ **Check Dark Current** is used to calculate the amount of background noise present in the camera. This is calculated using a zero time exposure and no light reaching the camera.

➢ **Check Dark Frame** is used to calculate the amount of background noise present in the camera. This calibration is required for the Nuance software to support long exposure times (exposures greater than five seconds). This calibration is done at PerkinElmer and takes approximately 30 minutes to complete.

➢ **Frames to Average** is a useful feature for reducing noise in acquired images by increasing the signal-to-noise ratio. This value specifies the number of images the camera will take at each acquisition wavelength. The software then averages the values of those images to create a single image for each wavelength.

The signal-to-noise ratio increases approximately by the square root of the number of images averaged. For example, averaging four images per wavelength gives a 2x signal-to-noise ratio. This should translate into approximately a 2x reduction in the limit of detection.

➢ **Fluorescence/Brightfield Autoexpose target % of dynamic range** sets the autoexposure target values, and attempts to prevent saturation of pixels by restricting the exposure time to a percentage of the dynamic range of pixel values. The defaults are 70% and 80%, respectively.

Enter a percentage for the dynamic range for autoexposure in fluorescence and brightfield. For example, the default value of 70% for 12-bit (4096-level) images results in a maximum intensity value of 2867.

The **Maximum Exposure** settings let you specify maximum exposure times (in seconds) for fluorescence and brightfield:

➢ **User Defined Exposure** sets the maximum value that a user can enter in the **Exposure** edit box on the **Acquire** panels.

➢ **Auto Exposure** sets the maximum exposure that is allowed to be returned when the user autoexposes a fluorescence or brightfield image.

Scaling: Selecting a Measurement Pixel Unit

You can select *Counts* or *Scaled Counts per Second* as the measurement pixel unit. Select **Hardware > Edit Hardware Settings > Preferences**. In the **Measurement Pixel Unit** box, select one of the following options.

➢ **Counts**: Measurements indicate count levels as read from the camera, so the indicated value depends on the gain, binning, bit depth and exposure time used for a given measurement.

➢ **Scaled Counts/Second**: Measurements indicate count levels after scaling for the effects of exposure time, binning, camera gain, and bit depth, so the indicated value is essentially independent of these settings. This is represented as follows:

$$\text{Scaled Counts/Sec} = \frac{\text{counts}}{\text{full scale*}} \times \frac{1}{\text{exp}^{(s)}} \times \frac{1}{\text{bin}^2} \times \frac{1}{\text{gain}}$$

* full scale = 4096 for 12 bit acquisitions
256 for 8 bit acquisitions

Displaying Image Cubes with Enhanced Contrast

The **Display** panel and the **Display Control** window (see Advanced Display Controls 86) both include an **Enhance Contrast** check box that lets you display images with enhanced contrast. You can control whether this box defaults to checked or unchecked each time a cube is acquired or opened in Nuance.

1. Select **Hardware > Edit Hardware Settings > Preferences**.

2. Check **Display Image Cubes with Enhanced Contrast** for either **Fluorescence Images** or **Brightfield Images**, or both.

This selects how fluorescent and brightfield image cubes are shown when first acquired, or when first loaded from disk. You can adjust the enhanced contrast setting for an image by using the check box in the **Display Control** or the **Display** panel.

3.9 Calibrating Pixel Size

To take measurements using mm^2 units, the Nuance software needs to know the camera's pixel size relative to the current magnification. This procedure requires that you have already acquired a clear and focused monochrome image of a fiduciary at least 10 microns long, with the binning set to 1x1.

1. With the monochrome image of the fiduciary loaded, select **Hardware > Calibrate Pixel Size**. The **Distance Calibration** dialog box opens.
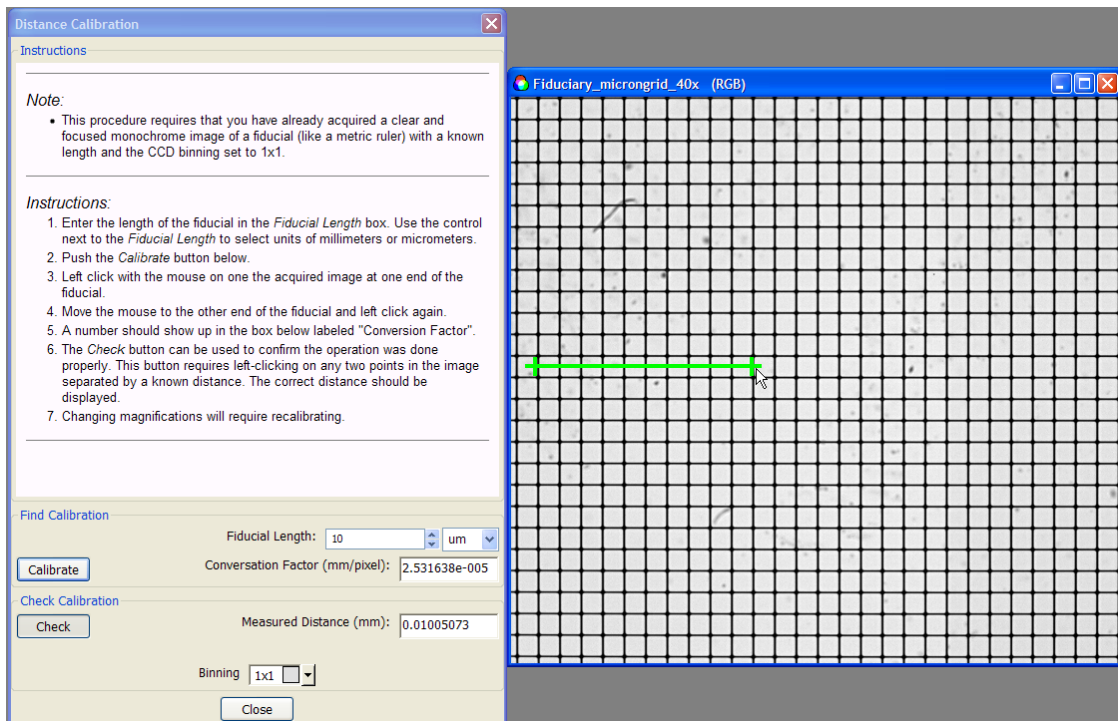
Figure 12: Calibrate Pixel Size

2. Position this dialog box on the screen so the image of the fiduciary is visible. For this example, a 1-micron by 1-micron grid was imaged at 40x magnification.

3. Select 1x1 in the **Binning** drop down box.

4. Select the units (µm or mm) and enter the length of the fiduciary. (For this example, we used 10 microns.)

5. Click the **Calibrate** button.

6. Click with the mouse pointer on the acquired image at one end of a 10 µm length.

7. Move the pointer to the other end of the 10 µm length and click again.

8. The number of millimeters per pixel displays in the **Conversion Factor** field.

9. You can use the **Check** button to confirm that the calibration is accurate. To use the **Check** function, obtain an image with points separated by known distances. Then click the **Check** button and click on any two points in the image separated by a known distance. The correct **Measured Distance** should be displayed.

3.10 Using Low Screen Resolution Mode

Nuance software only supports display resolutions of 1280x1024 or higher. However, some displays - especially some digital projector displays - do not support resolutions this high. The Nuance software has a special mode to support projectors or screens at 1024x768 resolution. Some software functions are not available in this mode.

Screen resolution is detected at startup. A dialog box warns if you are running in low-resolution mode. If the Nuance software is started, and the screen resolution is subsequently changed, the Nuance software will not detect it. If the Nuance software is run with a screen height of 800 pixels or less, it makes the following adjustments:

➢ In low-resolution mode, spectral libraries can only have five elements. This effects the **Spectra** and **Display** panels as well as the **Batch Mode**, **Import Spectral Library**, and **RCA** dialog boxes.

➢ The toolbar is removed from under the main menu.

➢ The **Sample ID** and **Notes** boxes are removed from the **Fluorescence** panel.

➢ The **Notes** box is removed from the **Spectra** panel.

➢ Plots on the **Manual Compute Spectra** dialog box are 4/5 their normal size.

➢ The spectral graph (for cube display) is removed from the **Display** panel.

➢ The status bar at the bottom of the screen does not display.

3.11 Reinitializing Nuance Hardware

When you start the Nuance software, it automatically detects the Nuance camera/imaging module hardware if it is attached to the computer via the USB 2.0 cable. If the Nuance software loses its connection to the hardware, make sure the USB 2.0 cable is properly connected to the imaging module, then select **Hardware > Reinitialize**. Nuance should detect and initialize the hardware. If this fails to reinitialize the connection, try restarting the Nuance software.

If the computer's power save feature is enabled, the computer will enter sleep mode if left inactive for a length of time. Turn this feature OFF. If the computer goes to sleep while it is connected and running the software, the connection with the camera/imaging module is lost. Reboot both the imaging module and the computer to reconnect.

Avoid this problem by making sure the computer is set to never enter sleep mode. This will prevent losing the hardware connection between the computer and system.

1. Click **Start > Control Panel > Power Options** to display the dialog box.

2. On the **Power Schemes** tab, select **Always On** in the **Power schemes** drop down box.

3. You can select any time period for the **Turn off monitor** setting. The **Turn off hard disks** and **System standby** options must be set to **Never**.

4. Click **Apply** and then **OK** and close the **Control Panel**.

4　Method Development

Method development is a fundamental component of any good experiment, and building spectral libraries is a key part of method development. This section discusses methods for building accurate and reliable spectral libraries. We will explain how to create and save libraries using a variety of sample specimens, and we will discuss their unique characteristics. The Unmixing Spectral Images 40 section discusses how to open libraries and use them to unmix images.

If you are new to the Nuance multispectral imaging system and have not already familiarized yourself with the rest of this manual, we suggest that you do so now. Then return to this topic for further study.

4.1　Methods for Building Spectral Libraries

You should create a spectral library for each of your experiments or series of experiments. Acquire or open the image cube you want to use to create the library. The Brightfield and Fluorescence Imaging 40 topics describe in detail how to acquire image cubes of your specimens.

There are a number of ways to build a spectral library. A few examples include:

➢ Using the **Real Component Analysis (RCA)** feature to automatically compute and unmix spectra (see Computing and Unmixing Spectra Automatically 58 for detailed instructions). This process adds spectra to the library, which you can modify as needed and then save.

➢ Using the **Manual Compute Spectra** feature, which lets you specifically sample spectral signatures from images and save them in a library (see Computing and Unmixing Spectra Manually 61 for detailed instructions).

➢ Adding spectra from other libraries, component images, or composite images of the same or similar specimens (see Importing Spectra Into a Library 36).

➢ Also, depending on the nature of your experiment and the types of dyes or fluorophores used, you may have imaged a single slide or two slides from which you will obtain the spectra of the pure autofluorescence and that of a single dye mixed with autofluorescence, if present. These can be used directly in the spectral library or after "pure spectra" calculation.

After building the library, save it as described in the Saving Spectral Libraries 35 topic.

⊟ Tips for Spectral Library Development

➢ There is no substitute for proper library spectra. If possible, use samples stained with single components for determining the spectra of chromogens or fluorophores of interest, using one or more sections for each label.

➢ The computed pure spectrum should be similar to, but not necessarily the same as, published spectra of that chromogen or fluorophore. Variations in the label's physical environment within tissue can cause spectral shifts.

➢ Verify that your libraries work correctly by testing them on a few positive and negative controls. Use critical judgment during this evaluation.

➢ Some chromogens slightly change optical characteristics with hematoxylin counterstaining. Use the **Manual Compute Spectra** tool to generate an appropriate spectrum after counterstaining.

➢ Unmix into white or pink for autofluorescence. Unmix into red, green and/or blue (or more colors)

for chromogens or fluorophores of interest.

➢ If you want to make a chromogen or fluorophore (i.e., autofluorescence) "disappear," assign it to the black channel or simply turn the layer off in the **Display Control** utility (see Advanced Display Controls 86).

➢ Make sure you save the library for later use.

▬ Obtaining Counterstain and Label Spectra from a Brightfield Sample

In this example, obtain the pure spectra of a chromogen and the counterstain present in the Ki67 data set. (The Ki67-20X data set comes with the Nuance software and is located in the Nuance sample data folder: *C:\Nuance Data\Images\Sample Data*. Double-click the *"ki67-20X.im3"* file to open the cube.) Remember, you must be able to identify the pure and mixed spectra of the specimen to compute pure spectra and provide proper libraries.

➢ Obtain the hematoxylin (counterstain) signal of the tissue background from an area of pure (blue) counterstain only (blue spectrum, Label #3 in the figure below).

➢ Obtain the mixed DAB signal plus hematoxylin from the nuclei that have both spectra (green spectrum, Label #2 in the figure below).

➢ In this example, use **Manual Compute Spectra** to calculate the pure spectra of DAB (red spectrum, Label #1). Save the spectral data in a spectral library.

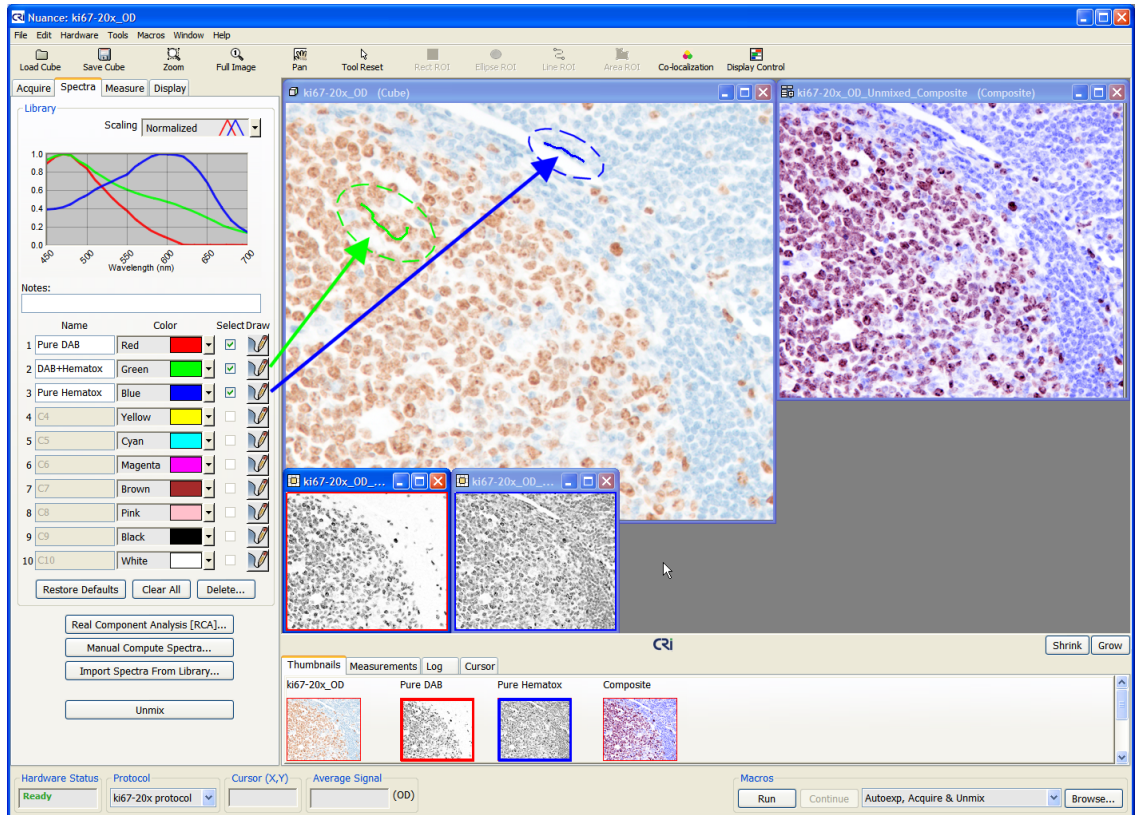(These steps are explained in detail in the Practice Exercise 37 and in the *Nuance Quick Start Guide*.)

Figure 13: Counterstain and Label Spectra from a Brightfield Sample

Notes:

➢ In this example, the counterstain signal is named "Pure Hematox."

➢ The **Zoom** [Zoom] tool can be used to help visualize the drawing tool on particular pixels.

➢ When computing pure spectra, observe the plots of the spectral curves in the Spectral Graph. Each plot should have a curve similar to that shown in the figure above. In this sample, the **Scale** option was turned off during Manual Compute Spectra.

➢ Compare computed pure spectra with that of known sources in published literature.

➢ Note that the unmixed hematoxylin signal reveals the pale-staining germinal center cells.

⊖ Obtaining Autofluorescence and Label Spectra from a Fluorescent Sample

In this example, obtain the pure spectra of the two fluorophores present in the kappa lambda fluorescence cube of tonsil tissue. (The kappa lambda data set comes with the Nuance software and is located in the Nuance sample data folder: *C:\Nuance Data\Images\Sample Data*. Double-click the *"kappa lambda tonsil 605 655.im3"* file.)

Remember, you must be able to identify the pure and mixed spectra of the specimen to compute pure spectra and provide proper libraries.

➢ Obtain the autofluorescence signal of the tissue background from an area of pure

autofluorescence (black spectrum, Label #9 in the figure below).

➢ Obtain the mixed 605 nm quantum dot fluorescence plus autofluorescence from cells that have both spectra (blue spectrum, Label #3 in the figure below).

➢ Obtain the mixed 655 nm quantum dot fluorescence plus autofluorescence from cells that have both spectra (yellow spectrum, Label #4 in the figure below).

➢ In this example, use Manual Compute Spectra to calculate the pure spectra of each quantum dot. Save the spectral data in a spectral library.
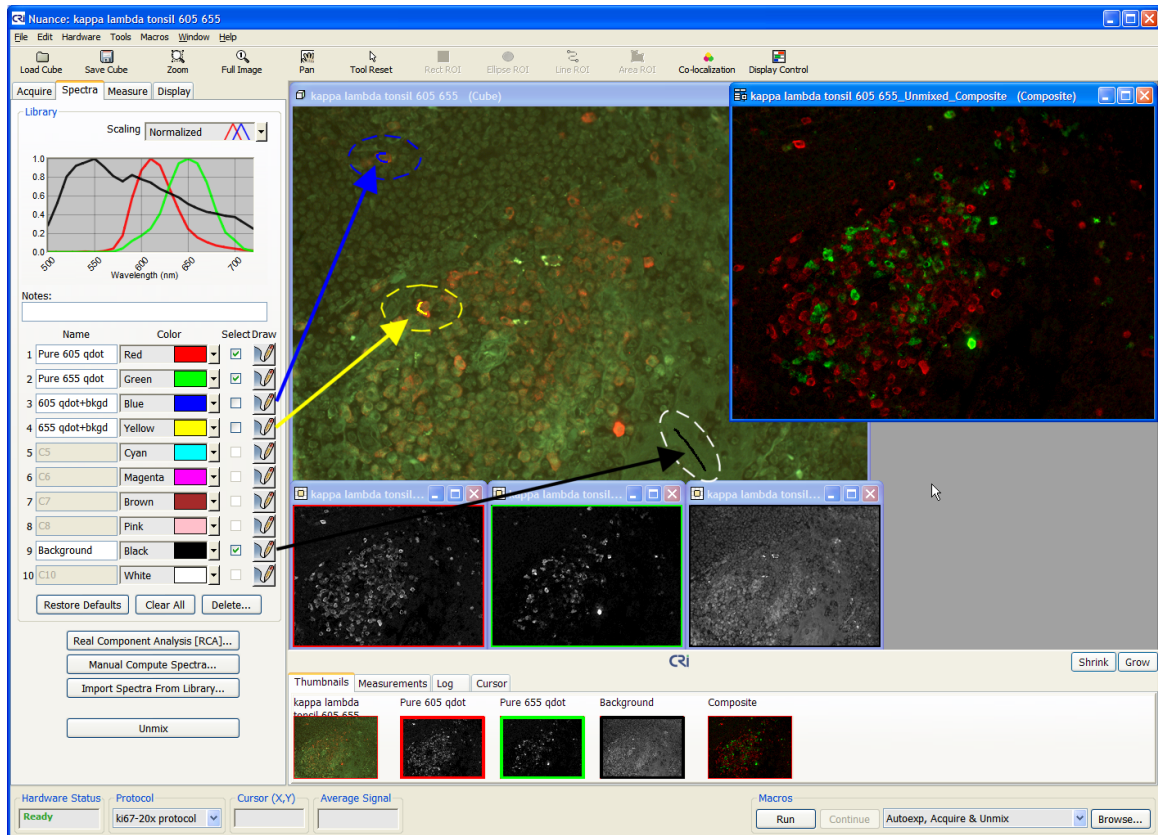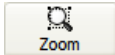
Figure 14: Autofluorescence and Label Spectra from a Fluorescent Sample

Notes:

➢ In this example, the pure fluorescence signals are named "Pure 605 qdot" and "Pure 655 qdot."

➢ The **Zoom** [Zoom] tool can be used to help visualize the drawing tool on particular pixels.

➢ Notice that the Blue and Yellow lines disappear, and Red and Green lines representing the pure 605 nm and 655 nm signals appear in the Spectral Graph.

➢ When computing pure spectra, observe the plots of the spectral curves in the Spectral Graph. Each plot should have a uniform Gaussian curve similar to that shown in the figure above.

➢ When computing pure spectra, adjust the **Scale** and **Fit Offset** options to obtain a spectrum that best fits the pure spectrum of the signal.

➢ Compare the computed pure spectra with that of known sources in published literature.

4.2   Saving Spectral Libraries

By saving your spectral libraries, you make them available for use again later when performing analyses on similar specimens. Instead of just saving the library, you could also save the Nuance protocol, which saves all of the Nuance acquisition and analysis settings (see <u>Saving Protocols</u> 35ˀ for how to save protocols.)

1. Select **File > Save Spectral Library**. The **Choose Spectral Library** dialog box opens.

2. Navigate to the folder where you want to save the spectral library and type a name in the **File Name** field.

3. Make sure "Spectral Libraries (*.csl)" is selected in the **Save as type** box and click the **Save** button.

If you want to be able to open the library in another application such as Microsoft® Excel® to review or graph its numerical data, you can save the library as a text file. Select the "Text Files (*txt)" option. Note however, this format does not include all of the information the .csl file includes (for example, it cannot save color or hardware settings) so do not use it to save and share your libraries.

4.3   Saving Protocols

The Nuance software saves its settings in protocols. Protocols are recipes for collecting and measuring data, and they contain the following information:

➢ acquisition settings including region of interest and binning, wavelength settings, filter selections, exposure times, and hardware settings.

➢ associated spectral library

➢ auto save options

➢ measurements options including threshold level and minimum connected pixels

Whenever you make changes to any hardware settings or acquisition settings, or edit the current spectral library, save those settings in the current protocol or create a new one. By saving your protocols, you make them available for use again later when acquiring image cubes of similar specimens.

1. Select **File > Save Protocol**.

2. In the **Choose Protocol** dialog box, select the name of an existing protocol if you want to overwrite it, or enter a new name in the **File Name** field.

3. Click **Save** to save the protocol.

4. Notice that the new protocol name appears in the **Protocol** drop down box on the status bar. If other protocols were loaded during the current session of Nuance, you can select and re-load them from the **Protocol** drop down box.

Note: When you exit the Nuance software, you will be prompted to save the current protocol if you have changed any hardware or software settings during the current session. Be sure the save the protocol if you want to use the new settings again.

4.4 Importing Spectra Into a Library

From an Existing Library

You can import individual spectra from other libraries.

Note: The spectral range and spectral spacing parameters of the library must be the same as current loaded spectral cube data.

1. Click the **Import Spectra From Library** button on the **Spectra** panel.

2. The **Choose Spectral Library** dialog box will prompt for the file name of a spectral library. Spectral libraries have a .csl file name extension. Spectral libraries from older versions of the software with *.txt file extensions may also be opened.

3. Double-click the name of the library to open it.

4. The **Import Spectra From Library** dialog box opens. It lists all the spectra stored in the selected library.

5. Make sure there is a check-mark in the **Select** column next to each spectrum you want to import. Select a **Location Number** and click the **Transfer to Library** button.
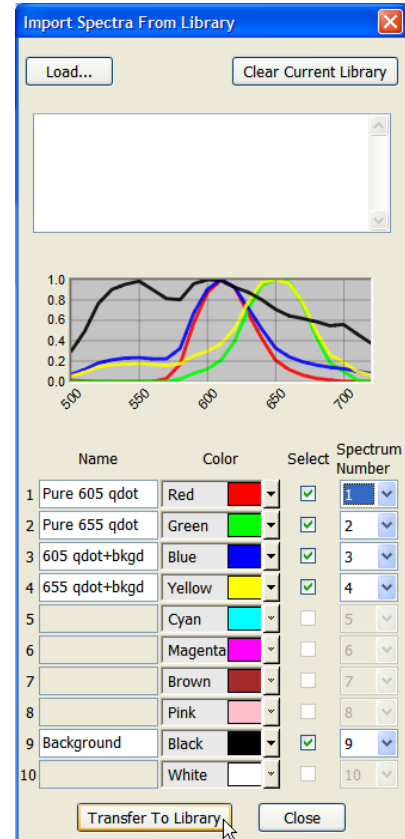

Figure 15: Import Spectra

6. If you want to load spectra from another library, click the **Load** button and select another library to load. The **Clear Current Library** button removes all spectra from the current library (same as the **Clear All** button on the **Spectra** panel).

7. Click **Close** when finished importing spectra.

From a Saved Component Image

You can import spectra from component images.

1. Open the Result Set (.umx) file that includes the component image from which you want to import spectra.

2. Right-click on the component image and select **Import Spectra** from the pop-up menu.

3. The **Import Spectra From Library** dialog box (see Import Spectra 36 figure) opens with the spectrum used to unmix the cube into that component.

4. Make sure there is a check-mark in the **Select** column next to each spectrum you want to

import, then click the **Transfer to Library** button.

5. If you want to load spectra from another library, click the **Load** button and select a library to load. The **Clear Current Library** button removes all spectra from the current library (same as the **Clear All** button on the **Spectra** panel).

6. Click **Close** when finished importing spectra.

⊟ From a Saved Composite Image

You can import spectra from composite images.

1. Open the Result Set (.umx) file that includes the composite image from which you want to import spectra.

2. Right-click on the composite image and select **Import Spectra** from the pop-up menu.

3. The **Import Spectra From Library** dialog box ⌐36⌐ opens with all the spectra used to unmix the cube.

4. Make sure there is a check-mark in the **Select** column next to each spectrum you want to import, then click the **Transfer to Library** button.

5. If you want to load spectra from another library, click the **Load** button and select a library to load. The **Clear Current Library** button removes all spectra from the current library (same as the **Clear All** button on the **Spectra** panel).

6. Click **Close** when finished importing spectra.

4.5   Practice Exercise

In this exercise you will open and analyze a brightfield image cube representing a lymph node specimen stained for Ki67. B-cells are on the left, T-lymphocytes are on the right, and all nuclei are stained. Some cells are positive (brown) for the Ki67 proliferation marker, and all cells have a blue hematoxylin nuclear counterstain. By using Nuance multispectral imaging, we will be able to see the blue counterstain underneath the brown Ki67 marker.

1. To open the sample Ki67-20X image cube click the **Load Cube** button and navigate to the Nuance sample data folder: *C:\Nuance Data\Images\Sample Data\.*

2. Double-click the *"ki67-20X.im3"* file to open the image cube.

3. This cube has not yet been converted to "optical density," which should be done prior to unmixing. Select **Tools > Convert to OD - Auto selects white spectrum**. (See [About Optical Density Images](42) for more information about optical density conversion.)

4. The Ki67 cube is now ready for unmixing.

5. Manually obtain spectra and use **Manual Compute Spectra** to unmix the cube:

   a. Select the **Spectra** panel.

   b. Obtain the hematoxylin (counterstain) signal of the tissue background from an area of pure (blue) counterstain only: Click the **Draw** button in row #3 and draw a line over some of the blue nuclei on the right. When you release the mouse button, a check-mark appears in the **Select** column in the Library. Change its default name from "C3" to a more descriptive name like "Pure Hematoxylin."

   c. Obtain the mixed DAB signal plus hematoxylin from the nuclei that have both spectra: Click the **Draw** button in row #2, then draw a line within a brown (mixed) nuclei on the left. A check mark appears in the **Select** column in the Library. Name this spectrum "DAB+Hematoxylin."

   d. Click the **Manual Compute Spectra** button to calculate the pure spectra of DAB and save the spectral data to the Library.

Figure 16: Manual Compute

   a. In the **Known Spectrum** group, click the blue "Pure Hematox" spectrum.

   b. In the **Mixed Spectrum** group, click the green "DAB+Hematox" spectrum.

   c. The computed pure DAB spectrum will display in the **Computed Spectrum** group.

   d. Select the **Scale** and **Fit Offset** check boxes. These functions fine-tune the spectral computation and will result in a better unmixing in this example due to the weakness of the signals that are being unmixed. See [Error Scaling When Manually Computing Spectra](63) for more about these functions.

2. Make sure the computed spectrum is assigned to row #1 and its name to "Pure DAB." Click

the **Transfer to Library** button and close the dialog box.

3. In the **Library**, make sure that only the Blue and Red markers (representing both of the pure spectra) are selected. Click the **Unmix** button to generate the properly unmixed set of images (shown in the figure below).
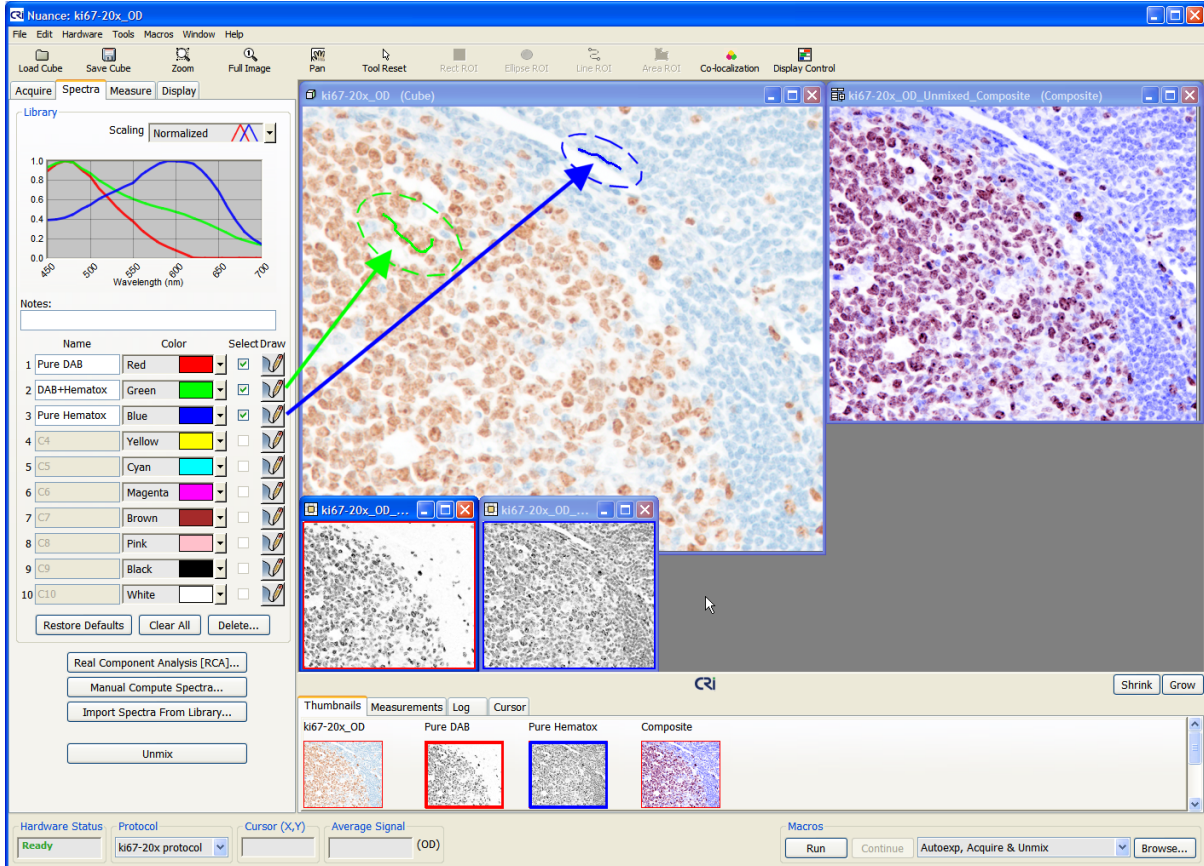

Figure 17: Unmixed Fluorescence Signals

5 Brightfield and Fluorescence Imaging

This topic explains how to set up your microscope for brightfield and fluorescence imaging and then acquire images using the Nuance system's image acquisition functions. During this topic, it is presumed that you are familiar with the general procedures of brightfield imaging and fluorescence imaging in microscopy. The workstation computer and imaging module should be powered up and ready as described in Launching the Nuance Software 22.

5.1 Setting Up Your Microscope for Brightfield Imaging

Accurate imaging depends on proper alignment and operation of the microscope. Because multispectral imaging acquires a cube of images over the visible spectrum, the quality of the objective lens is important. The eye is most sensitive to green light, which is usually chosen, and is the Nuance default, for focusing a live image. The live image is always in gray scale, not color, because the Nuance camera collects gray scale images at each of the wavelengths selected by the user.

Notes: Apochromatic lenses on microscopes are corrected for multiple colors and provide essentially constant focus throughout the visible spectrum. If at all possible, apochromatic lenses should be used for multispectral imaging.

Achromatic lenses, on the other hand, are not as highly corrected and therefore the focus is not perfect over the entire spectral range. If one focuses an achromatic lens in the green wavelength, the same image will be slightly out of focus at blue and red wavelengths. The result being that any image collected with an achromat lens will display color fringes around every object in the image. While this may not be too apparent without close scrutiny, it will affect the ability to collect accurate data (particularly with respect to areas) from such images. Achromatic lenses should not be used for multispectral imaging.

The microscope illumination system should be carefully aligned for Koehler illumination 117. This ensures that the light available from the illuminator fully illuminates the objective being used. While Koehler illumination will achieve the best performance available for a given condenser and objective combination, it does not ensure even illumination of the field of view. If the microscope lamp has controls for centering and focus of the illumination source, these should be carefully adjusted to provide the most even illumination possible in the field of view.

One way to accomplish this lamp adjustment is to capture images of a blank field after finding a focus plane using a stained slide and adjusting for Koehler illumination. This blank-field image can then be examined using the mouse pointer in Nuance, and the intensity of white pixels in the center of the screen can be compared to those at the edges and corners of the screen. It will be difficult or impossible to achieve identical illumination over the entire field of view. However, you should adjust the lamp to achieve the most even illumination as possible. Any remaining variations in intensity will be corrected by the flat fielding algorithm, which is part of the brightfield acquire procedure in Nuance.

Koehler illumination is specific for each objective used on the microscope. In other words, every time the magnification is changed, the microscope should be checked for proper Koehler alignment. Also, each time you change the magnification, you must also collect a new "reference cube" in order to ensure correct flat fielding of the image. This provides a baseline for performing an optical density (OD) conversion of the image data. Be certain to adjust the condenser diaphragm correctly for optimum image quality. Closing the condenser diaphragm too far will produce exaggerated contrast in the image, and will degrade image quality.

5.2 Setting Up Your Microscope for Fluorescence Imaging

Setting up a microscope for fluorescence is highly variable, and depends on the brand and model of the microscope being used. Almost all fluorescent microscopes in use today, both upright and inverted, use a version of the Ploem or vertical illuminator system. The critical portion of this unit is the "filter cube," which is made up of two narrow band filters and a dichroic mirror. The filter cube turns the excitation light through 90 degrees, in order to direct light from the lamp to the objective of the microscope.

In the vertical illuminator arrangement, the objective being used for examination is also used for illumination. This ensures that if the specimen is in focus, the illumination is also in focus at that point on the specimen. Another feature of this illumination system is that only the part of the specimen that is visible through the objective is being illuminated, and this assists with issues such as fluorophore fading with exposure to excitation illumination.

The first narrow band filter in the filter cube is an excitation filter. This filter passes light from the lamp that excites the particular fluorophore in use. It is chosen to pass a very narrow range of excitation wavelengths.

The dichroic mirror is used to reflect the chosen excitation wavelength of light to the specimen. A unique property of dichroic mirrors is that the mirror reflects only the wavelength desired for excitation, and passes other wavelengths that have passed through the excitation filter out of the optical path of illumination.

The result is that the combination of the excitation filter and the dichroic mirror produces an even narrower band of excitation than could be generated by either the excitation or the dichroic mirror alone.

The final filter in the filter cube is the emission filter. This filter is a narrow band filter that selects the emission wavelength of interest. It also works in combination with the dichroic mirror to provide a very narrow band of emission wavelengths. It should be appreciated that the entire filter cube and vertical illuminator arrangement is designed to select a very narrow band of excitation and a very narrow band of emission.

Since current fluorescence microscopes are designed for very narrow wavelengths of excitation and emission, they may complicate the use of multispectral imaging, which is designed for evaluation over extended wavelength ranges. In order to overcome these limitations, the filter cube should be reconfigured by replacing the emission filter with a longpass filter. The longpass filter should pass all wavelengths starting just above the wavelength being used for excitation.

When multiple fluorophores are used on a single specimen, these fluorophores should be chosen to excite with similar excitation wavelengths. If the fluorophores require widely differing excitation wavelengths, then two or more excitation/emission filter sets may be required to collect the desired data in a single image cube. The Nuance software's Multi Filter Support feature lets you acquire an image cube using multiple excitation/emission filters sets.

Fluorescence microscopes are generally illuminated by lamps providing wavelengths that are "bluer" than normal incandescent (filament) bulbs. These are often of the arc lamp type, and provide small point sources of illumination. As small points of high intensity, lamp alignment in fluorescent microscopes is critical. Various microscopes have different tools to assist with lamp alignment, and the manufacturers instructions should be carefully followed. The objective is to provide the most even illumination possible at the specimen. This is made more difficult as the eye cannot see the actual illumination, unless there is a fluorophore present. And to check the entire field, one needs a specimen that provides an identical response over the entire image area.

One type of specimen that meets this requirement is the various types of plastic slides available from providers of fluorophores. These slides fluoresce when exposed to excitation light, and by acquiring an image of such a slide, one can check for even field excitation. Using such a field standard, the lamp

should be adjusted to provide as even illumination as possible.

Note: When performing fluorescence imaging using the Nuance system, be certain that the wavelength selected for the "live" image used to focus is set to the expected **emission** wavelength of the fluorophore being imaged. If the live wavelength is set to the **excitation** wavelength, you will not see an image, and it will be impossible to correctly focus.

In all fluorescence work, it is important to minimize exposure to the florescence excitation, and for quantitative work, one should avoid collecting images from areas of the specimen already examined. If multiple image cubes are required, take care to minimize total exposure time to the excitation light.

5.3   About Optical Density Images

Optical Density (OD) is used to measure the interaction of light with absorbing materials. The science of absorbing spectroscopy is based on the Beer-Lambert law, which states that if the molar extinction of a true absorbing material is known, and the absorber is homogeneously distributed, then the amount of material present is the negative logarithm of the percent transmission. Percent transmission is defined as the amount of light present without the specimen (100%) as compared to the amount of light present with the specimen in the light path. Since the molar extinction is constant for a given material, we can assume that this is a non-varying term, and ignore it for all practical purposes.

When absorbing images collected in brightfield are converted to OD images, the information contained in each pixel is quantitative, as to the amount of absorbing material present. In the case of brightfield microscopy, the absorbing material is an applied dye of some type. If we know the manner of attachment of the dye to sites within the specimen, we have a quantitative measure of a particular component within the specimen. In the case of many histochemical stains, this can result in an accurate measure of a particular chemical constituent within the specimen.

▬ Automatic OD Conversion

The Nuance system automatically generates optical density values in brightfield images. To do this, you must first collect a reference cube, which provides the 100% light level needed for the OD computation. If you open an image cube that has been collected without a reference cube, the Nuance system can still generate OD images based on the white (100% transmission) values within the image cube.

In either case, accurate OD images result, and the resulting OD values reflect the actual amount of absorbing material present. Therefore OD conversion should always be employed if quantitative information regarding the amount of dye is desired.

▬ Non-OD Converted Images

The Nuance system can unmix brightfield images that have not been converted to optical density. In this case, the resulting unmixed images are not quantitative. The reason to perform such an analysis might be simply to produce unmixed image results or composite images that have exaggerated visual contrast.

In certain cases, one might find such images advantageous for certain types of image segmentation, but it should be understood that these images are accurate only in terms of spatial areas, and no longer contain quantitative data with respect to amount of chromogen present. PerkinElmer does not recommend using non-OD converted images.

5.4 Tips for Obtaining Quantitative Results

➢ Whenever possible, acquisition of image cubes should be done using previously established Nuance protocols that have been saved and validated with your Nuance imaging system for each of your specimens/experiments. Protocols are recipes for collecting and measuring data. Protocols are discussed further in Saving Protocols 35.

➢ Set up all hardware identically each time you acquire images during the course of an experiment.

➢ Orient all samples similarly. Use the Live Image Overlay 84 feature.

➢ Obtain a spectral library from known control samples.

➢ Use one spectral library for unmixing all datasets.

➢ Use the same gain, binning, spectral ranges, and exposure times for all measurements.

➢ Save resulting images as "Unscaled Data" so they can be exported to other quantitation software, if desired.

5.5 Acquiring Images Using a Saved Nuance Protocol

Nuance protocols are recipes for collecting and measuring data. By using a saved protocol to acquire images of similar specimens throughout an experiment, you don't have to specify acquisition settings every time. This helps ensure consistency throughout the experiment.

If a Nuance protocol has already been saved, tested, and validated for the type of specimen/cube you want to acquire, load the protocol first, then acquire the cube using the settings from the protocol.

Note: Be aware that using a protocol from one specimen to acquire an image of a specimen that contains different fluorophores will lead to incorrect results.

▬ Opening a Protocol

Tip: The **Protocol** drop down box on the status bar lists all protocols that were opened during the current Nuance session. If the desired protocol was opened earlier, select it from this list. Otherwise, use the following method.

1. Select **File > Open Protocol**.

2. Nuance may ask if you want to save the current protocol if one is open and changes have been made.

3. In the **Choose Protocol** dialog box, select the protocol you want to open and click **Open**.

4. Nuance now contains all the settings of the selected protocol including the last saved exposure and wavelength.

Tip: You can also open a Nuance protocol by double-clicking the protocol (*.pro) file in a My Computer directory window. If Nuance is not already open, this action will open Nuance with the selected protocol. You can also drag a protocol file and drop it into an open Nuance window to open the protocol.

5.6 Viewing a Live Image Stream

The frame rate of the live image varies according to the set exposure time. If you see an all black image, the exposure setting is probably too low. Saturated pixels appear red in the live image. If some or all of the image is solid red, the image is saturated. The exposure setting may be too high.

1. Use the eyepieces and the stage/focal controls to position/focus the specimen for viewing. Then divert all of the light to the camera and make sure the Nuance slider is in the MSI position, not the MONO position.

2. For brightfield imaging, switch to the **Brightfield** acquire panel; for fluorescence imaging, switch to the **Fluorescence** acquire panel. The **Live Stream** window opens automatically. (If you closed the **Live Stream** window, click the **Live** button.)

3. Click the **AutoExpose Mono** button to automatically adjust the exposure time to the best setting for the current wavelength.

4. Fine-tune the focus if necessary.

Applying a Live Image Overlay

When quantitative results are needed, use the **Live Image Overlay** feature to help you orient the current specimen as when it was previously imaged. Open the image you want to use as an overlay. Then right-click the image and select **Set As Live Image Overlay**. See also <u>Live Overlay</u> 84.

Freezing the Live Image

Click the **Live** button a second time. The image in the **Live Stream** window remains frozen until you click the **Live** button again.

5.7 Selecting Camera Binning and a Region of Interest

Binning combines multiple pixels into a single pixel. Higher binning reduces exposure time and image size but results in a lower resolution image. There are three camera binning options in the **Binning** selector. For brightfield images, this selector defaults to no (1x1) binning. For fluorescence imaging, the default is 2-pixel (2x2) binning.

*Figure 18: Region Of Interest And Binning
Selectors*

Select one of the following:

➢ If you want no binning, for maximum image resolution and size, select the 1x1 binning option.

➢ 2x2 binning combines each 2x2 square of pixels into one pixel. This reduces the resolution in each direction by a factor of 2 and requires 1/4th the exposure time.

➢ 4x4 binning combines each 4x4 square of pixels into one pixel. This reduces the resolution in each direction by a factor of 4 and requires 1/16th the exposure time.

The **ROI** (Region of Interest) selector sets the imaging area, which may be set to include all or just a portion of the entire field of view. The active area is always centered, and there are five ROI options in the **ROI** selector. Four options are predefined, and one is user-definable. The default setting is Full-frame.

▬ Selecting a Region of Interest

Do one of the following:

➢ To capture the Full-frame, 2/3-frame, 1/2-frame, or 1/3-frame, select from the predefined options.

➢ To select a custom region of interest, select "Custom" and use the mouse pointer to draw an ROI box within the **Live Stream** window. You can also create a custom region of interest by selecting **Hardware > Set Custom Camera ROI**.

5.8   Specifying the Current Wavelength and Exposure

The **Wavelength And Exposure** boxes are used to manually adjust the current **Wavelength** in nanometers (nm) and the **Exposure** in milliseconds (ms).

▬ Adjusting the Current Wavelength

Depending on the expected wavelength of the emission light, you may need to adjust the current wavelength for the filter being used. The **Wavelength (nm)** box is used to set the wavelength in nanometers for manual control of the live camera view.

To manually set the current wavelength:

Do one of the following:

➢ Drag the slider to the desired wavelength. The slider snaps to the wavelength intervals set in the acquisition settings. As shown in this figure, the wavelength slider indicates the current filter range. The highlighted region on the slider represents the acquisition wavelength range. The colors on the slider approximate how we would see the wavelengths within the range.

➢ Type a value or use the up/down scrollers in the **Wavelength** text box. When typing a value, you can set the wavelength in 1 nm increments. The up/down scrollers snap to 10 nm intervals.
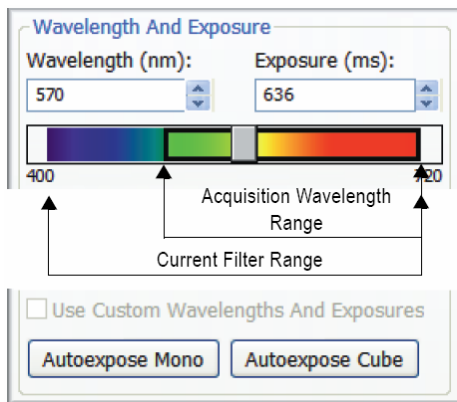

Figure 19: Wavelength and Exposure

▬ Adjusting the Exposure Time (Autoexpose)

Use the autoexposure buttons to automatically determine the correct exposure setting, or manually enter a value (in milliseconds) in the **Exposure** entry field. You can also use the **Custom Wavelengths And Exposures** option. This option is enabled only if custom collection settings have already been specified. (See Specifying Custom Wavelength and Exposure Settings 46).

To autoexpose at the current wavelength:

Click the **Autoexpose Mono** button. Nuance will calculate the best exposure time for the current wavelength and display the value in the **Exposure** text box.

To autoexpose a cube:

1. Before you acquire an image cube, click the **Autoexpose Cube** button.

2. If you are using the multiple filter feature (see Acquisition Setup Using Multiple Filters 50), the system will prompt you to install the correct filter set. Install the requested filter set before continuing.

3. Nuance will calculate the exposure time(s) that should be used to acquire an image cube of the specimen. The value at the current wavelength will display in the **Exposure** text box. You may also notice that the **Use Custom Wavelengths and Exposures** check box automatically becomes selected. This occurs when different exposure times are required for individual cube wavelengths.

To manually enter an exposure time:

Type an exposure value into the **Exposure (ms)** text box or use the scrollers to increase or decrease the current value in increments of one millisecond. As you change the exposure setting, you will see the live image become brighter or darker.

Note: Be careful not to overexpose or underexpose your specimen. Doing so may result in loss of valuable data, which will affect spectral classification or unmixing operations later.

If you are using the multiple filter feature (see Acquisition Setup Using Multiple Filters 50), you can enter a different exposure time for each filter in the sequence. Select the first filter letter ("A") in the **Multi Acq** drop down box and enter an exposure time. Repeat for each filter in the filter sequence.

To use custom wavelengths and exposures:

If you have specified the custom settings, select the **Custom Wavelengths And Exposures** check box. You may now acquire the cube using the custom collection settings.

▬ Specifying Custom Wavelength and Exposure Settings

Custom wavelength and exposure settings let you use unevenly spaced wavelength settings and varying exposure times to acquire an image cube. You can specify new settings and save them, or you can load a saved wavelength and exposure table.

To specify custom wavelength and exposure settings:

1. Select **Hardware > Edit Hardware Settings** (or press Ctrl+H) to reveal its dialog box.

2. Click the **Brightfield Custom** tab or the **Fluorescence Custom** tab.

3. The **Wavelength/Exposure** table defaults to the acquisition settings of the currently selected presets.

4. To change the defaults, specify new **Start/End Wavelengths** and **Step** interval using the drop down boxes in the **Fill Wavelengths** area below the table.

5. Specify the exposure **Time (milliseconds)** in the **Fill Exposures** box.

6. Return to the **Wavelength/Exposure** table and edit individual settings as necessary.

7. Click **Save** to save the settings and close the dialog box.

8. On the **Brightfield** or **Fluorescence** panel, select the **Use Custom Wavelengths And Exposures** option to use the custom collection settings to acquire the image cube. (All fields in the **Cube Wavelength Selection** group become disabled.)

To save custom collection settings as a table to load again later:

1. After specifying custom collection settings, click the **Save** button. The **Choose Custom Table** dialog box opens.

2. Specify a directory and filename and click **Save** to save the new wavelength and exposure table.

To load a wavelength and exposure table:

1. Click the Load button on the Brightfield Custom or Fluorescence Custom tab, respectively. The Choose Custom Table dialog box opens.

2. Locate the table you want to load and click Open. The collection settings appear in the Wavelength/Exposure table. Click OK to use the custom settings.

5.9 Acquiring Cubes in Brightfield

Selecting Cube Wavelengths

The controls within the **Filter/Wavelength Selection** group control the acquisition wavelength settings for acquiring spectral cubes.
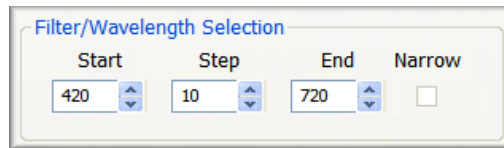

Figure 20: Spectral Cube Acquisition Controls

Enter the start, step, and end values to be used to acquire the spectral cube. The values shown in this figure represent the default values for cube acquisition in brightfield.

Narrow Bandwidth Acquisition (FX & EX models only)

Narrow mode can help you obtain better resolution of closely spaced and overlapping emission spectra. It can also increase the number of chromogens or fluorophores in your sample by using more overlapping spectra. (See also Nuance FLEX Multispectral Imaging 15 .)

If your Nuance camera is equipped with a Flex filter, and you want to use its narrow bandwidth capability, select the **Narrow** check box on the **Brightfield** panel.

Acquiring a Raw Cube (No Post Processing)

When the Nuance system takes a spectral cube in brightfield, it automatically flat-fields the images and converts them to optical density. In order for this to occur, you need to acquire a reference image before you can acquire a cube. If you do not want this automatic conversion to occur (and you want to skip acquiring a reference image), then clear the **Convert to OD** check box. Cubes will now be taken without automatic conversion to optical density. (See also, Taking a Reference Image for Flat Fielding 51 .)

Acquiring a Brightfield Cube

1. Click the **Autoexpose Cube** button to start an autoexposure sequence for the cube. (See also, Adjusting the Exposure Time (Autoexpose) 46 .

2. It is highly recommended that you leave the **Convert to OD** option checked when acquiring cubes. If you turn off this conversion feature, you may notice that the outer edges of the image are slightly darker than the rest of the image. (See also, Taking a Reference Image for Flat Fielding 51 .)

3. If you selected the **Convert to OD** option, acquire a reference image: Move the specimen out of the live view so that nothing but empty white background is visible (typically still on the glass slide, but off the tissue specimen).

4. Click the **Acquire Reference** button.

Note: If you change the binning or field of view, the current reference image becomes invalid. You will have to take a new reference image before acquiring cubes with a different binning or field of view.

5. After acquiring the reference image, click the **Acquire Cube** button.

6. When the Nuance system has completed taking the image cube, a color representation of the cube now in memory displays in the image gallery.

7. If you intend to save this cube, save it now as described in Saving Images and Image Cubes 54 .

5.10 Acquiring RGB (Color) Images

The **RGB** acquisition functions are found on the **Brightfield** acquisition panel.

1. Select a region of interest and binning. See also Selecting Camera Binning and a Region of

2. Select the wavelength and exposure. See also Specifying the Current Wavelength and Exposure

3. Click the **Autoexpose RGB** button to start an autoexposure sequence to determine exposure values that the imaging module believes to be optimal for the RGB image.

4. Click the **Acquire RGB** button to acquire a single color picture of the live image.

Note: PerkinElmer does not recommend the use of this function. Better color fidelity will be obtained using OD-converted MSI (multi-spectral imaging) data.

5.11 Acquiring Fluorescence Cubes

▬ Selecting Cube Wavelengths and a Filter

Filter settings define the wavelengths for which the filter is tuned when acquiring an image cube. The **Cube Wavelength Selection** group has preset acquisition settings as well as entry fields for manually entering cube wavelengths.
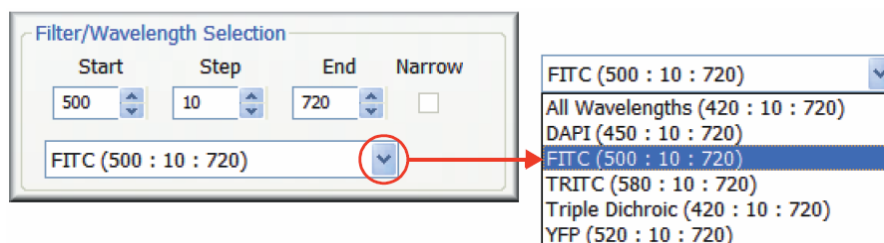
Figure 21: Filter/Wavelength Selection

▬ Using Preset Filter Settings (fluorescence imaging only)

The predefined filter and wavelength settings on the **Fluorescence** panel store fixed filter names with their corresponding acquisition wavelengths. For example, "FITC (500 : 10 : 720)" means the starting wavelength is 500 nm, the ending wavelength is 720 nm, and images are acquired in steps of 10 nm.

Select an option from the drop down box. The wavelength setting for the Live image autoadjusts to within the wavelength range of the current selection.

▬ Manually Entering Acquisition Settings

If you don't see the filter settings you want in the presets drop down box (for fluorescence imaging), you can edit the current values in the **Start**, **Step**, and **End** wavelength text boxes. When you edit any of these values, a new "Unnamed" filter setting is automatically created using the new manual settings.

Narrow Bandwidth Acquisition (FX & EX models only)

Narrow mode can help you obtain better resolution of closely spaced and overlapping emission spectra. It can also increase the number of chromogens or fluorophores in your sample by using more overlapping spectra. (See also, Nuance FLEX Multispectral Imaging [15].)

If your Nuance camera is equipped with a Flex filter, and you want to use its narrow bandwidth capability, select the **Narrow** check box.

Customizing the Preset Filter Settings

You can edit and save changes to the preset filter settings for fluorescence imaging:

1. Select **Hardware > Edit Hardware Settings** (or press Ctrl+H) to reveal its dialog box. Click the **Filters** tab. (Filter settings that you enter and save here become available for use in your Nuance acquisition protocols.)

2. Edit the settings as needed:

   ➢ To edit existing settings, select the filter setting name in the list and edit its tunable filter settings. Click the **OK** button to save.

   ➢ To create a new filter setting name with a new set of tunable filter settings, click the **New** button. Enter a label name and click **OK** to add the new filter setting name to the list. Then specify its tunable filter settings and click the **OK** button to save.

   ➢ To delete a currently selected filter setting name, click the **Delete** button.

   ➢ To restore all preset filter settings to their factory defaults, click the **Factory Defaults** button. This will also delete all new filter setting names you have saved.

Acquisition Setup Using Multiple Filters

The Nuance system offers a **Multi Filter Support** feature for fluorescence imaging that lets you acquire a cube while employing two or more excitation/emission filter sets. For example, if multiple fluorophores are used on a single specimen, and these fluorophores require widely differing excitation wavelengths, then more than one excitation/emission filter set may be required to collect the desired image data.

Note: There is no need to select and use the **Multi Filter Support** option when acquiring image cubes that require a single filter set.

While different exposure times can be assigned to each filter set in a multi-filter acquisition, the region of interest, binning, bit depth, and gain must be the same for all filter sets. Any changes you make to these parameters apply to all the filter sets you have selected.

To configure multiple filter sets:

1. Click the **Add** button in the **Multi Filter Support** group. When the **Add Filter** dialog box appears, select a filter set to add. Click **OK** to add the filter set to the list of acquisition filters.
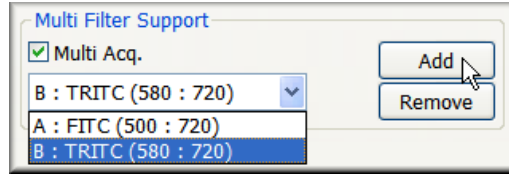
Figure 22: Multiple Filter Support

2. If you want to employ a third filter setting, click the **Add** button again and select the third filter setting to add to the acquisition list. Repeat this process for each filter you want to use for the multi filter acquisition.

   ➢ If you want to remove a filter setting from the list, select it in the **Multi Acq** drop down box and click the **Remove** button. The remaining filters will move up in the sequence.

   ➢ If you want to autoexpose or acquire a cube using just one of the filter settings in your multi acquisition list, select the letter of the filter you want to use ("A," "B,", etc.) and clear the **Multi Acq** check box. The selected filter will be used. (See also Saving Many Filter Protocols in One Nuance Protocol 51 .)

3. Save this protocol if it will be used again later (see Saving Protocols 35).

4. When you are ready to autoexpose or acquire the cube using multiple filters, select the **Multi Acq** check box. During multi-filter cube autoexposure and acquisition, the Nuance system pauses during the operation and prompts you to install the next filter set in the sequence according to the filter line-up you created.

Saving Many Filter Protocols in One Nuance Protocol

You can use the **Multi Filter Support** feature to save multiple filter protocols in a single Nuance protocol. This is useful when you need to take multiple cubes of a specimen using different filter sets. For example, you may want to take separate cubes of the same mouse using blue, yellow, and red filter sets. (Remember, each filter set can have a different exposure time, but all sets will have the same camera ROI, binning, bit depth, and gain.)

Follow the instructions earlier in this section to configure the filter settings (blue, yellow, and red, for example) in the multi acquisition list, and then save the protocol. When you are ready to take multiple cubes of a specimen, load the saved multi-filter protocol that contains the appropriate filter sets. Choose the filter set you want to use to acquire the cube, and then clear the **Multi Acq** check box so only the selected filter set will be used.

Taking a Reference Image for Flat Fielding

Flat Fielding makes it possible for the Nuance software to create better, more evenly bright mono images and image cubes in fluorescence. If you acquire fluorescence images or cubes without Flat Fielding, you may notice that the outer edges of acquired images are slightly darker than the rest of the image.

Before acquiring a mono image or spectral cube, acquire a reference—or background—image. This requires a fluorescence sample slide that is evenly bright across the field of view. Once you have taken a reference image, it will be saved with the current Nuance protocol, if you save the protocol.

Note that if you change the binning or region of interest (ROI), the current reference image becomes invalid. You will have to take a new reference image before acquiring an image or cube with new binning or ROI.

1. Select the **Flat Field** check box. This activates the **Acquire Ref. Image** button and disables the **Acquire Mono** and **Acquire Cube** buttons until you acquire the reference image.
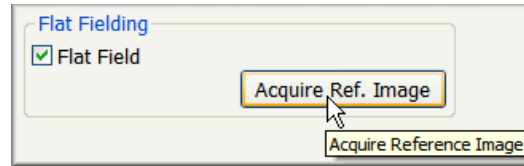

Figure 23: Flat Fielding

2. Replace the specimen with a plastic fluorescent slide (not included).

3. Click the **Acquire Ref. Image** button. (If you have activated multi-filter support, you will be prompted to install the correct filter set(s) before continuing.)

4. After acquiring the reference image, move the specimen back into position.

Acquiring a Fluorescence Image Cube

1. Click the **Autoexpose Cube** button to autoexpose the cube (see <u>Adjusting the Exposure Time (Autoexpose)</u> 46).

2. Click the **Acquire Cube** button.

3. When the Nuance system has completed taking the image cube, a color representation of the cube now in memory displays in the image gallery.

4. If you intend to save this cube, save it now as described in <u>Saving Images and Image Cubes</u> 54.

Tip: When you are finished taking image cubes, remember to close the microscope's fluorescence shutter to avoid photo-bleaching the specimen.

5.12 Acquiring a Mono Image (Snapshot)

To take a snapshot of the current live image at the current wavelength and exposure, click the **Acquire Mono** button. This function acquires a single picture, not an image cube.

5.13 Assigning Sample IDs and Notes

Assigning a Sample ID to an Image Cube

Use the **Sample ID** text box to assign an ID to the current cube. When you save the cube, it will be saved with its sample ID. All measurements derived from the cube's component images will be saved with the cube's sample ID.

Cube IDs display in the **Cube ID** column of the **Measurements** page near the bottom of the Nuance work area.

Use the **Notes** text box to save notes with the current cube. Whenever the cube is opened, any notes previously saved with the cube will display in the **Notes** box.

5.14 Acquiring Timed Sequences of Image Cubes

The Nuance system can acquire multiple cubes of a specimen using a timed acquisition interval that you specify. This is useful for observing changes in a specimen over a specified time span, generally no longer than thirty minutes to one hour.

1. Select **Tools > Acquire Time Sequence**. The **Acquire Time Sequence** dialog box opens.

2. Enter the amount of time (in seconds) that you want to elapse between the <u>start</u> of each cube acquisition. For example, if you enter 60 seconds and it takes 15 seconds to acquire the first cube, there will be a 45 second time lapse until the start of the next cube acquisition. Be sure to enter a time that is greater than the amount of time it will take to acquire each cube.
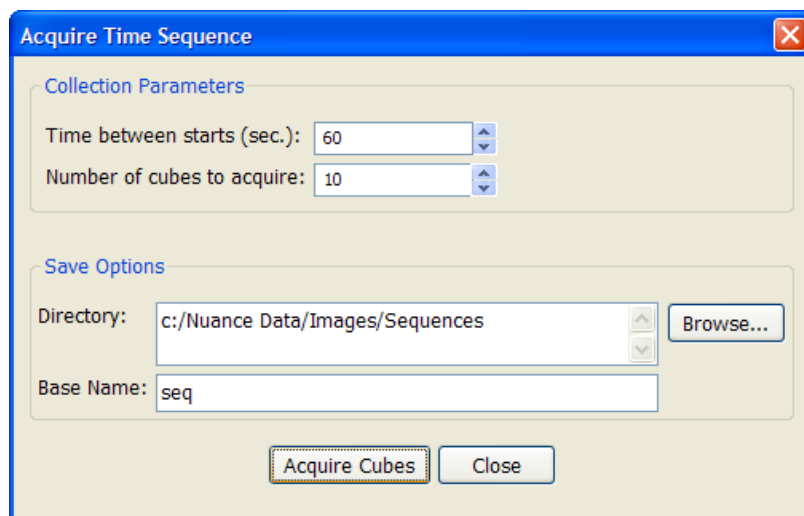
Figure 24: Acquire Time Sequence

3. Enter the number of cubes you want to acquire during this acquisition sequence.

4. Click the **Browse** button to select a destination directory where you want the Nuance software to save all the cubes acquired during the sequence.

5. Enter a base filename for all the saved cubes.

6. Click the **Acquire Cubes** button to begin the acquisition sequence. A progress indicator displays the amount of time until the next exposure (cube), the current exposure (cube) number, and the total time remaining until the acquisition sequence is complete.

7. The Nuance software automatically saves each cube in the specified directory.

8. When the acquisition sequence is complete, you can locate the cubes at the destination directory and open them in Nuance. As seen in the figure below, the cube filenames begin with the base name you specified and include the date and time of acquisition.
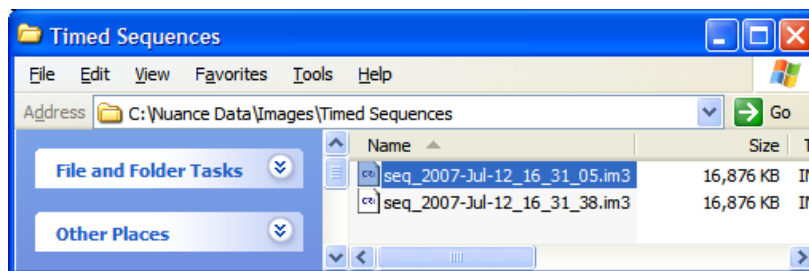
Figure 25: Cubes acquired during timed acquisition

5.15 Saving Images and Image Cubes

Devise a consistent naming convention for your images and image cubes. Use file and folder names that incorporate specimen names as well as dates and/or times and acquisition parameters such as filter cube, binning and exposure.

Saving an Image Cube

A PerkinElmer cube can include hardware settings, spectral libraries, user comments, and display settings. (PerkinElmer format image cubes cannot be opened using third-party tools.)

1. To save the current image(s) and associated data, click the **Save Cube** button on the toolbar, or select **File > Save Cube**. The **Choose Cube** dialog box opens.

2. Navigate to where you want to save the cube.

3. In the **Save as type** box, select a cube type option:

   ➢ Select "Image Cubes" if you want to save the cube as a PerkinElmer image cube. A PerkinElmer cube includes hardware settings, spectral libraries, user comments, and display settings. Cubes saved in the PerkinElmer format cannot be opened in third-party tools.

   ➢ Select "Image Cubes (with Lossless Compression)" if you want to save the cube as a compressed or "zipped" file. Lossless compression saves disk space but also takes longer to save the cube.

   ➢ Select "TIFF Cubes" if you want to save the cube as a series of TIFF images. Nuance saves a TIFF image in the specified location for each of the wavelengths used to acquire the cube. When saving a cube that was acquired using multiple filters, filename_A_* will be used for the first filter set, filename_B_* will be used for the second filter set, and so on. TIFF Cubes contain only images; none of the associated data or settings is saved with this format.

4. Enter a file name for the cube and click the **Save** button.

Saving Images

1. Select **File > Save Image** and select a save option to save the image(s):

   ➢ **Save (As Displayed)** prompts you to enter a name for the currently selected image, and saves the image as a TIFF or JPEG image.

   ➢ **Save All (As Displayed)** prompts you to enter a name for each of the current images in the

image gallery, and saves the images as TIFF or JPEG images.

> Note: When you save images "as displayed," the Nuance software saves the images as bitmap files. You cannot subsequently perform any sophisticated image processing operations on the bitmap files, since the underlying data is no longer present. Saved (as displayed) processed images can be opened in other third-party programs for morphology operations such as determining area, shapes, or for counting objects. As an alternative, you could export data as a series of TIFF files and open them in a program that can handle wavelength sequential image files.

  - ➢ **Save Image (As Unscaled Data)** prompts you to enter a name for the currently selected component image, and saves unscaled image data as a TIFF component with the data values multiplied by 10.

  - ➢ **Save All Images (As Unscaled Data)** prompts you to enter a name for each of the component images, and saves all unscaled image data as TIFF components with the data values multiplied by 10.

2. When the dialog box opens, navigate to where you want to save the image(s). (The default location is in *C:\Nuance Data*.)

3. Enter a file name and click the **Save** button.

Saving Image Cubes Automatically

The Nuance system can save your image cubes automatically.

1. Select **Hardware > Edit Hardware Settings** (or press Ctrl+H). Click the **Autosave** tab.

2. Select the **Autosave Image Cubes** option to enable the autosave fields.

3. To specify a default directory, click the **Browse** button and select from the browse dialog box.

4. Type a base name in the **Base Name** field, if desired.

5. In the **Auto-naming Options** group, specify a naming option. A sample appears in the **File Name** box.

6. Click the **OK** button to save the changes and close the dialog box.

5.16 Viewing Cube Information

Cube information includes its size in pixels, the number of planes in the cube, a timestamp, any comments associated with the cube, and the wavelengths and exposure times used to generate the cube.

Right-click on the image in the **Cube** window and select **Cube Info** from the pop-up menu. The **Cube Info** window opens.
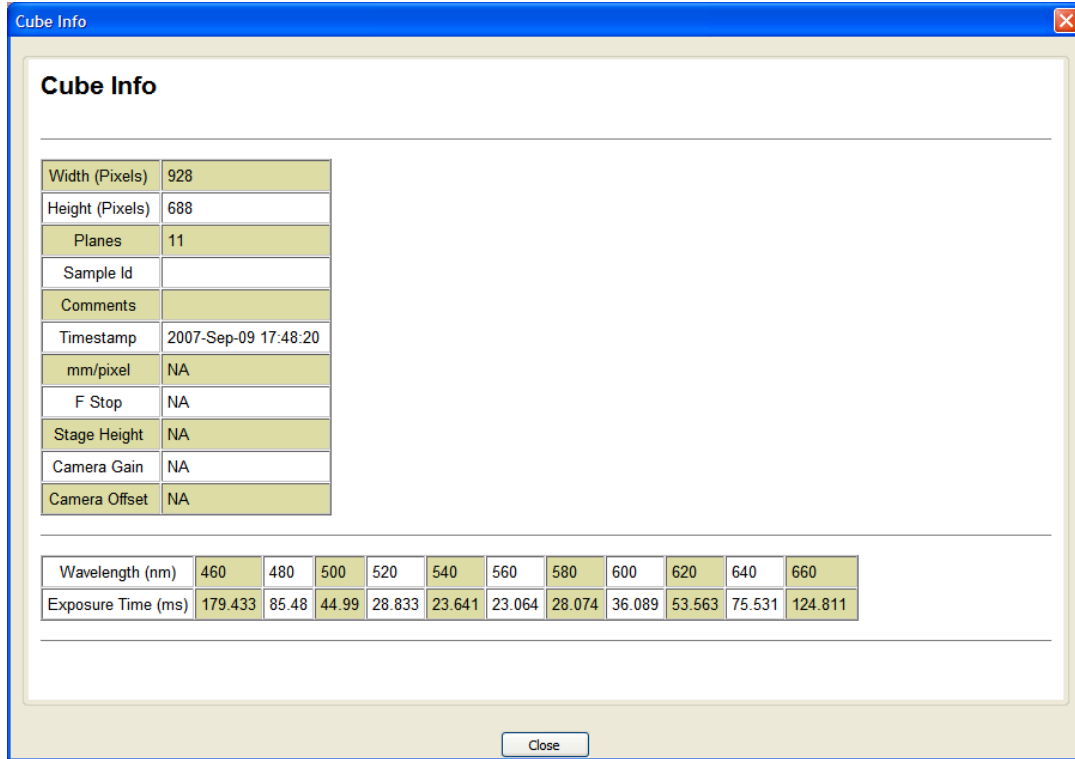
Figure 26: Cube Information

5.17 Extracting an Image Plane from a Cube

You can extract and view individual wavelength images from a cube.

1. Right-click on the image in the **Cube** window and select **Extract Channel** from the pop-up menu.

2. Select the channel you want to extract by clicking a wavelength value in the list. A monochrome image of the selected wavelength opens in a new window.
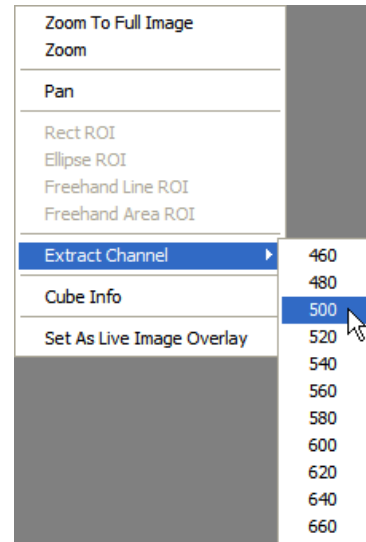

Figure 27: Extract Channel

6 Unmixing Spectral Images

Whenever possible, spectral processing—or unmixing—of cubes should be done using established Nuance protocols. These are protocols that you have saved and validated with your Nuance multispectral imaging system for each of your specimen/experiment types. In most cases, these protocols contain spectral libraries that will be used when unmixing images (as explained in the Method Development [31] topic).

This section explains how to create a spectral library and unmix an image cube using the spectral processing functions on the **Spectra** panel. Once you have created your spectral library, save the protocol and library for repeated use throughout the experiment with the same or similar specimens.

6.1 Tips for Accurate Unmixing

> Don't assume that the spectra you acquired from purified fluorescent dyes are the same as those present in your sample. Case in point: A researcher recently measured quantum dot (q-dot) spectra by smearing samples on a clean glass slide. When the spectra were measured for the same q-dot species in-situ (i.e., on the sample after having gone through processing), the spectra had shifted about 10 nanometers towards the blue part of the spectrum for at least 3 different species. So, if at all possible, use in-situ spectra acquired from the specimen itself for the unmixing.

> Don't try to unmix more than 3 or 4 similar things simultaneously, since the unmixing algorithm is sensitive to the presence of noise, and more end-members lead to more inaccuracy.

> Always design experiments to validate the unmixing procedure. Do single, double, and triple labels and take quantitative measurements. As you add more species, the chance for error goes up dramatically in all noisy, imperfect, situations. Use appropriate controls (irrelevant antibodies, unstained specimens) and approach the system critically. Multispectral capabilities are valuable, but can be misused if applied uncritically.

> When unmixing the fluorescence signals of specimens that have multiple strong and different autofluorescent species in addition to the signals of interest, it may be necessary to use more than one autofluorescence spectrum. For example, you may need to add more than one autofluorescence signal to the spectral library if—in addition to general tissue autofluorescence—there is also specific collagen spectra, to prevent the collagen signal from inappropriately being allocated to another channel.

> Do not use the same general autofluorescence spectrum for different tissues, species of organism, sex of organism, or any other variable that may influence the local biochemistry of the specimen without checking its appropriateness. All of these factors will alter the autofluorescence spectra. The best approach is to derive autofluorescence spectra from appropriate unstained controls.

6.2 Opening a Spectral Library

If a spectral library has already been saved and validated for the type of specimen/cube you want to unmix, use that library to unmix the cube. Or if an appropriate Nuance protocol is available, which should include the spectral library, then load the protocol instead (see Acquiring Images Using a Saved Nuance Protocol [43]).

By using a saved protocol and/or spectral library on similar specimens to unmix and perform analyses throughout an experiment, you don't have to recreate a spectral library every time. This helps ensure

consistency throughout the experiment. Protocols and spectral libraries can be opened before or after loading an image cube.

> **Note:** Be aware that using a spectral library from one specimen on a specimen that contains different fluorophores will lead to incorrect results.

1. Select **File > Open Spectral Library** (or press Ctrl+L). Select the location and filename of the library and click **Open**. One of the following will occur:

   ➢ If the current library is empty, the library you selected will open.

   ➢ If a library is open already, the **Import Spectra From Library** dialog box opens for you to select the spectra you want to import (see Importing Spectra Into a Library 36).

2. The color palette on the **Spectra** panel will be populated with the new spectra.

> **Tip:** You can also open a Nuance spectral library by double-clicking the library (*.csl) file in a My Computer directory window. If Nuance is not already open, this action will open Nuance with the selected library. Or you can drag and drop the library file into an open Nuance window to open the library.

6.3   Opening an Image Cube

1. Click the **Load Cube** button on the toolbar, or select **File > Open Cube** (or press Ctrl+O).

2. In the **Choose Cube** dialog box, browse to the location where the image cube is saved.

   ➢ If opening a PerkinElmer format (.im3) image cube, double-click the file to open it.

   ➢ If opening a TIFF image cube, open the folder containing the images and double-click any one of the TIFF image files to open the cube.

3. The cube will open, and a color representation of it opens in the Nuance image gallery.

> **Tip:** You can also open an image cube by double-clicking the cube (*.im3) file in a My Computer directory window. If Nuance is not already open, this action will open Nuance with the selected cube. Or you can drag and drop the cube (or multiple cubes) into an open Nuance window to open the cube(s).

6.4   Computing and Unmixing Spectra Automatically

This section explains how to use the **Real Component Analysis (RCA)** feature to unmix a fluorescence image cube. This feature is intended for fluorescence unmixing operations and may not give consistent or reliable results with brightfield datasets.

The RCA feature lets you explore how many different spectral features are present, extract spectra for further analysis, and calculate "pure" spectra even if autofluorescence is present.

In this section we will open and analyze a fluorescence image cube (included with the Nuance software) of a kappa lambda tonsil specimen using the RCA feature. The green regions in the raw data represent the signal from 605-nm quantum dots and the red regions represent the signal from 655-nm quantum dots. There is a lot of green autofluorescence present, obscuring the pure signals.

Open the demonstration kappa lambda image cube (see Opening an Image Cube 58). The cube is located in the Nuance sample data folder: *C:\Nuance Data\Images\ Sample Data*. Double-click the *"kappa lambda tonsil 605 655.im3"* file to open the cube.

Computing Spectra Using Real Component Analysis

1. Select the **Spectra** tab and click the **Real Component Analysis (RCA)** button.

2. The **RCA** dialog box opens (see figure below).

3. If you want to select a smaller region of the image on which to perform the RCA computation, click the **Select Area** button. Then use the mouse to draw a rectangular region on the Cube image. If you don't draw a region, the whole image will be used. Clicking the **Clear Area** button clears any user selected area from the image.

   In the kappa lambda example, use the entire image in the computation (do not draw a box).

4. If you want to provide an initial guess of the background (autofluorescence) signal, click the **Select Area** button. Then use the mouse pointer to specify the background signal in the image cube.

   ➢ In the kappa lambda example, leave this option un-checked.

   ➢ This option also lets you import a known background spectrum from the current library by selecting from the drop down box in the **Starting Spectrum** group. Use this option if there is no region in the current sample that contains only autofluorescence.

   ➢ These settings can be discarded by un-checking the **Use Selected Background** box.

5. Click the **Find Component Images** button to find the purest spectral features in the image. A set of monochrome images of spectrally distinct signals will soon appear at the right of the cube display window (see figure below). The number of images reflects the spectral complexity of the sample.

6. Select component images for use in unmixing by clicking on them. Click an image once for "Signal" and twice for "Background."

   ➢ If autofluorescence (or other background signal) is present, designate the autofluorescence image (typically the top-left) as "Background" by clicking on it twice. Only one image can be designated as background.

   In the kappa lambda example, select the image in the top left as the background and set its color to Black to hide the background signal in the unmixed image. (If you want to see the background autofluorescence after unmixing, you can choose a pastel color such as Pink or White, but this will cause a lack of contrast in the unmixed composite image.)

   Note: If you were unmixing a brightfield image cube and you wanted to hide a signal (such as the background) from the unmixed composite image, unmix into white the signal that you want to hide.

   ➢ Look for other images that appear to contain "significant" signals (a sample-specific judgement) by comparing the RCA images to the original image. Specific images should match specific signals in the original image. Click once on images to designate them as "Signals," and select a color to represent each signal.

   In the kappa lambda example, select the image that matches the 605 nm quantum dots in the original image and set its color to Red. Repeat for the image that matches the 655 nm quantum dots and set its color to Green. You may ignore the other images.
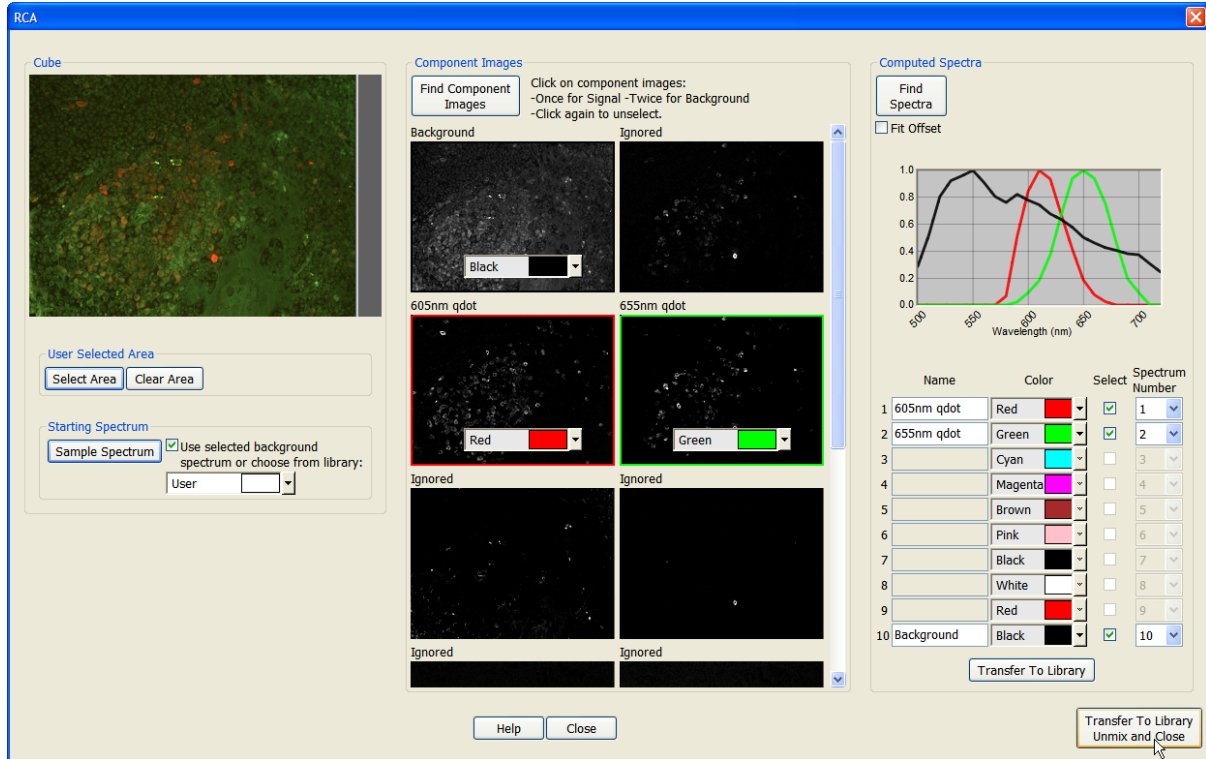
Figure 28: Real Component Analysis

7. Click the **Find Spectra** button in the **Computed Spectra** group.

   ➢ If no Background component was selected, then the "purest" spectra present in the cube will be found.

   ➢ If a Background component was selected, then all other signals will be used as inputs into a computation that removes the presence of the background spectrum to give the spectrally "pure" components.

8. The computed spectra should look like simple gaussian curves, which is the shape of accurate quantum dot spectra. Occasionally, for weak signals, there may be too much baseline offset in the data. Try selecting the **Fit Offset** option and clicking the **Find Spectra** button again and see if the results improve.

9. You may now use the controls in the **Computed Spectra** group to change a spectrum's name, color, and where it should reside in the spectral library.

10. When finished, click the **Transfer to Library** button to add the spectra to the main spectral library. If a library is open already, you may be asked if you want to overwrite the existing spectra.

11. Click the **Close** button to close the **RCA** dialog box.

12. When you are ready to unmix the image, first verify that the spectra you want to unmix is selected in the library, then click the **Unmix** button.

    Alternately, you may press the **Transfer to Library, Unmix and Close** button to perform all these actions with a single click.

13. If the spectral library you have created will be used again for future unmixing operations, save the protocol and/or spectral library.
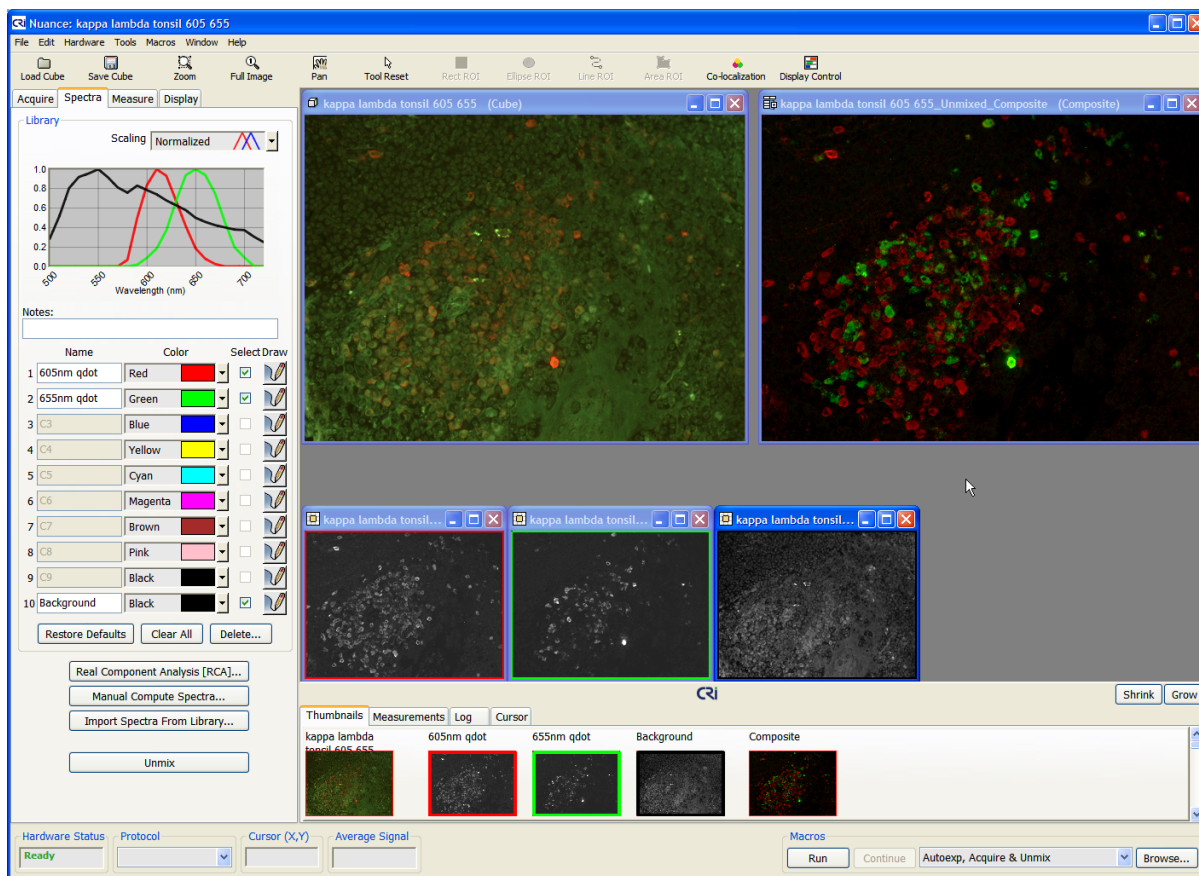
Unmixed Signals

6.5   Computing and Unmixing Spectra Manually

You can compute pure spectra without using RCA by sampling the known and mixed spectra manually. Use the color palette on the Spectra panel to build your spectral library. Select colors for separating mixed signals into pure signals.

If the cube you want to unmix was acquired using multiple filters and overlapping acquisition wavelengths (see Acquisition Setup Using Multiple Filters 50), you will notice that there are two or more overlapping curves in the spectral graph for each of the spectra in cube. Although this may look unusual, the overlapping curves are used together as one spectrum for all unmixing.

There are ten rows available that you can use to build a spectral library. Be careful not to use the same color for more than one signal.
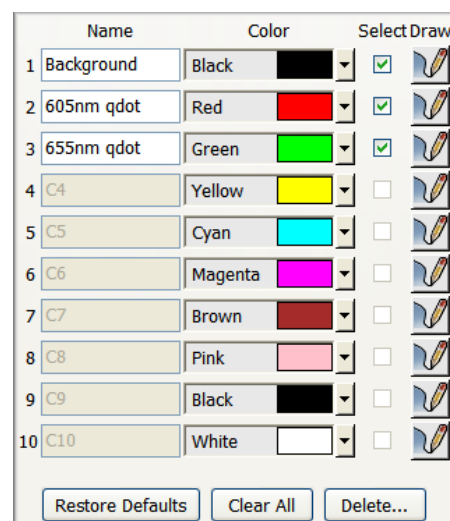
Figure 29: Color Palette

To sample spectra:

1. Open or acquire a cube and select the **Spectra** tab.

2. Click the **Draw** button within the row where you want to place the sampled spectrum.

3. Click and draw a line on the part of the cube you want to sample. (Holding down the Control or Shift key will add additional regions to the sample.) When you release the mouse button, a check-mark appears in the **Select** column.

Tip: The Control and Shift keys also let you sample multiple cubes: Sample the current cube, then select another cube from the thumbnails and hold down the Shift or Control key while sampling to obtain an average of the spectra from the cubes.)

4. The default line sampling tool can be changed by selecting another region of interest shape from the toolbar (see Selecting a Region of Interest Shape 62).

5. Assign a helpful name to each spectrum in the **Name** column.

6. To change the color assigned to a spectrum, select from its drop down color menu.

To hide a signal (e.g., background) in an unmixed image:

Unmix the signal you want to hide (such as the background) into black.

To clear the spectral library:

Click the **Clear All** button. This clears all spectra from the library, clears all spectral curves from the Spectral Scale, and restores the default colors.

To restore default or saved colors:

Click the **Restore Defaults** button. This restores the color selection for each row in the library to the default or last saved color.

To add notes to the current spectral library:

Enter notes for the current library in the **Notes** box. Notes will be saved with the spectral library when you save the library.

━ Selecting a Region of Interest Shape

1. Click the **Draw** button for the row where you want to place the sampled spectrum.

2. Click the **ROI** button on the toolbar for the shape you want to use.

3. Click and drag over the part of the cube you want to sample.

Manually Computing Spectra

1. Open or acquire a cube and select the **Spectra** tab.

2. After loading or building a spectral library, press the **Manual Compute Spectra** button to display the dialog box.

3. In the **Known Spectrum** group, select the known spectrum—the spectrum of the autofluorescence. Multiple known spectra may be selected by holding down the Control key while making selections.

4. In the **Mixed Spectrum** group select the spectrum that represents the selected known spectra plus any additional spectrum. For example, autofluorescence plus a label.

5. The **Computed Spectrum** will be displayed in the color specified at the bottom of the dialog box.

6. If necessary, use the **Scale** functions to fine-tune the fit adjustment. See Using Fit Offset 64 and Error Scaling When Manually Computing Spectra 63 for instructions.

7. Select the row, name, and color for this spectrum and press the **Transfer To Library** button.

8. Repeat this process for the other mixed spectra.

9. When you are ready to unmix the cube, close this dialog box and click the **Unmix** button on the **Spectra** tab.

10. Save the new spectral library as described in the Saving Spectral Libraries 35 topic.
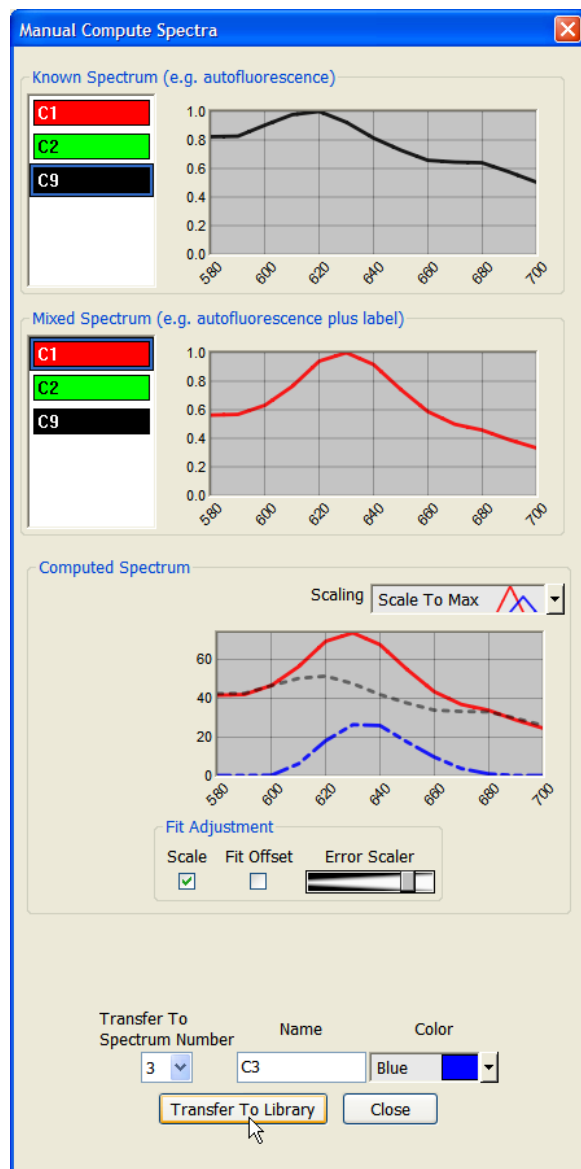
Figure 30: Manual Compute Spectra

Error Scaling When Manually Computing Spectra

To obtain an accurate pure label spectrum (which is required for an accurate unmixing), the correct amount of autofluorescence must be subtracted from the mixed spectrum. To do this, it is important

that the non-overlapping regions between the "pure" fluorophore and the autofluorescence line up closely.

The Nuance software attempts to determine the amount of autofluorescence to subtract automatically. When there is a bright fluorophore signal (relative to the autofluorescence), scaling is usually not needed. In that case, clear the **Scaling** check box so that Nuance will subtract the known spectra from the mixed spectra without any scaling.

When there is a weaker signal, you can scale the amount of autofluorescence that will get subtracted from it to ensure that the best "pure" spectrum is found. This is done by checking the **Scale** check box and sliding the **Error Scaler** slider.

1. In the **Computed Spectrum** group, select "Scale To Max" in the **Scaling** drop down box. This provides the best visual representation of the spectra.

2. The mixed spectrum and computed spectrum are shown in their respective colors. The dotted "best fit" line is a scaled representation of the known autofluorescence signal (see figure above).

3. With the **Scaling** box checked, try sliding the **Error Scaler** to the right to increase the scaling (or to the left to decrease it) until the non-overlapping portions of the known and mixed spectra line up as closely as possible (as shown in the figure above).

4. When you are satisfied with the computed spectrum, select a Spectrum Number, Name, and Color and click the **Transfer to Library** button.

5. When you are finished adding spectra to the library, remember to save it as described in Saving Spectral Libraries 35.

Using Fit Offset

Occasionally, for weak signals, there may be too much baseline offset in the data. If you did not get the desired unmixing results, select the **Fit Offset** option in the **Manual Compute Spectra** dialog box and see if the unmixing results improve. The computed spectra (the plot in the Spectral Graph) should have a simple gaussian curve.

When you are satisfied with the computed spectrum, select a Spectrum Number, Name, and Color and click the **Transfer to Library** button. When you are finished adding spectra to the library, remember to save it as described in Saving Spectral Libraries 35.

This feature is also available when using Real Component Analysis, as explained in Computing and Unmixing Spectra Automatically 58.

Changing the Spectral Graph Scale

The Spectral Graph displays the spectral curves of selected pixels, as well as computed values for unmixed signals. When you move the mouse pointer over the cube, the spectra associated with each pixel appear as a white line on the Spectral Graph.

There are two places where you can select a scale for the Spectral Graph: at the top of the Spectra panel and on the **Manual Compute Spectra** dialog box.

1. Select an option from the **Scaling** drop down box above the spectral graph:

➢ Select the "Normalized" option to view the spectral curves as normalized. The Y-axis in the spectral graph is scaled 0 to 1. Each spectral curve is independently scaled to 1 at its maximum height.

➢ Select the "Scale To Max" option to scale the Y-axis in the spectral graph from 0 to maximum height of the highest spectral curve in the image cube.

➢ Select the "Un-Normalized" option to view the spectral curves as un-normalized. The Y-axis in the spectral graph is scaled 0 to 255 (8-bit) or 0 to 4095 (12-bit).
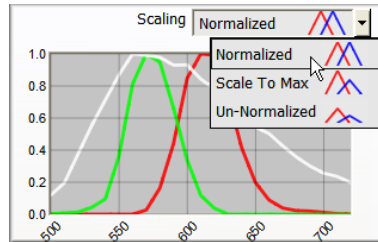
Figure 31: Spectral Graph
Scaling Selector

2. Move the cursor over the image to see how the white curve changes shape. You are seeing the spectral curve associated with each pixel as you move the mouse pointer.

6.6   Saving an Unmixed Result Set

After unmixing an image, you can save all unmixing results without having to save each unmixed image independently. The **Save Result Set** feature saves the entire workspace (excluding the cube) in a single file. This file will contain the component and composite image, the file name of the cube (but not the cube itself), an RGB representation of the cube, and other data such as measurements and regions, display parameters, user notes, and the spectral library.

You can then reload the saved result set later to review the unmixed results and conduct further analysis/quantitation. You cannot do additional unmixing, however, because there is no cube associated with a saved result set, unless you import the associated cube into the result set (see Importing a Cube Into a Result Set 66).

1. With the unmixed images on your screen, select **File > Save Result Set.**

2. The **Choose Results Set** dialog box opens.

3. Enter a name for the new result set and click **Save**. Result set files are saved with a ".umx" file name extension.

6.7   Working With Saved Result Sets

If unmixed result sets have been saved, you can open them for further analysis and quantitation. (See Saving An Unmixed Result Set 65 for instructions on saving unmixed result sets.)

Opening a Result Set

1. Select **File > Open Result Set**.

2. In the **Choose Results Set** dialog box, locate and select the "result_set.umx" file you want to open, and click **Open**.

Tip: You can also open a Nuance result set by double-clicking the result set (.umx) file in a My Computer directory window. If Nuance is not already open, this action will open Nuance with the selected result set. You can also drag and drop a result set into an open Nuance window.

3. The unmixed images will open in the image gallery. If the Nuance software can locate the cube associated with the result set (it must be a PerkinElmer format ".im3" Image Cube in its original location on the computer), the cube will open along with its unmixed images. If the Nuance software cannot find the cube or it is a TIFF Cube, an RGB representation of the cube (labeled "RGB - Cube Is Not Loaded") will open.

4. If the cube did not load automatically, you can import the cube into the result set. See Importing a Cube Into a Result Set 66 for instructions.

Importing a Cube Into a Result Set

When a result set is saved, the original image cube is not saved as part of the result set (.umx) file. An RGB representation of the cube is saved instead. When you open a result set, the Nuance software attempts to open the associated cube as well. If the associated cube cannot be found, the RGB proxy opens instead. You can replace the RGB proxy with the actual cube.

1. Right-click on the RGB image and select **Import Cube** from the pop-up menu.

2. The **Choose Cube** dialog box opens.

3. Select the cube (an Image Cube or TIFF Cube) you want to import and click **Open**.

6.8 Checking Your Spectral Library

Viewing Error Images

The Nuance software unmixes a cube's spectral signals based on the spectra in your current library. The **Error Images** utility in Nuance saves and can display any signals that are not accounted for in the component images. Those signals are thus omitted from the composite image. In other words, the Error Images contain all signals that would be left over if a cube could be recreated from the unmixed component images.

After unmixing, select **Tools > Show Error Images**. Two new images (Error Cube and Error Magnitudes) appear. The Error Cube contains the spectral variations of the error, while the Error Magnitudes image contains the error energy (not actually energy) not present in the component images. These images should contain no bright signals, but only "background noise" in the region(s) of interest. If bright signals are present, this indicates that the cube was not optimally unmixed.

Use this utility to validate your spectral library and identify spectral features that are not being accounted for in your unmixing. If you missed a spectral feature that needs to be in your library, the Error Images show you where to look for that spectral feature. Add it to your library using Compute Pure Spectra. Unmix the cube again using your improved spectral library.

6.9   Subtracting Spectra from a Cube

The Baseline Subtract utility can be used to subtract a spectrum from a cube. For example:

➢ In fluorescence, if there is stray light (background) in a cube, which contributes to a baseline offset, use this utility to subtract the background from the cube and improve contrast. This will ensure that the background is near zero (little or no offset).

➢ In OD-converted brightfield images, use baseline subtraction to remove unwanted signals such as excess chromogen or counterstain.

Baseline subtraction is done numerically (without scaling or other changes in value), wavelength-by-wavelength, from every pixel of the cube. This alters the spectral shape of remaining signals and their overall intensity. The magnitude of these changes depends on the intensity of the spectral signal being subtracted. Typically, when background spectra are small compared to the signals, spectral library entries derived from unsubtracted datasets can still be used, but this should be validated when quantitative results are required.

1. Select **Tools > Baseline Subtract**. The **Baseline Subtract** dialog box opens.
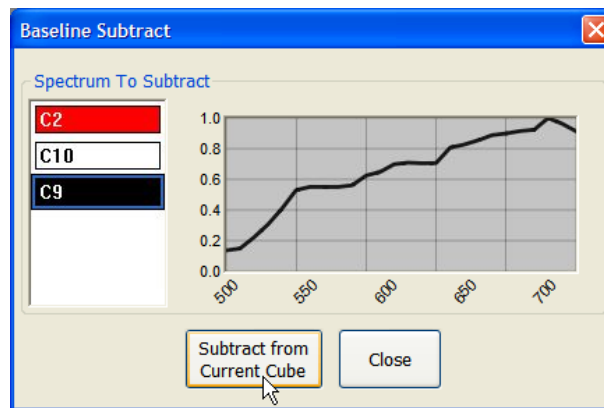
Figure 32: Baseline Subtract

2. This dialog box displays the spectra from the current spectral library.

3. Select spectra you want to subtract and click the **Subtract from Current Cube** button.

4. The Nuance software subtracts the selected spectrum from the cube and closes the dialog box. The cube's filename is also appended with "_subt" to remind you that a spectrum has been subtracted from the cube.

6.10 Using Line Profiles to Analyze Signals

The Line Profiles tool can be used to analyze any single-channel (monochrome) image, including component images and live images in Nuance. (Cubes and composite images cannot be analyzed with the Line Profiles tool). To use this tool, draw lines on the component images. The pixels sampled by the line show up as a plot of intensity versus pixel count in the Spectral Graph of the **Line Profiles** dialog box.

1. Select or open the monochrome image (or images) in the Nuance image gallery.

2. Select **Tools > Line Profiles**. The **Line Profiles** dialog box opens.

➢ Ten rows in the color library allow you to draw up to ten profile lines on multiple monochrome images.

➢ Each line profile appears as a plot with corresponding pen color in the Spectral Graph.

➢ You can change the Spectral Graph Scale to either "Normalized" or "Scale To Max."

➢ The **Clear All** button is used to remove all profile lines from the image(s) and clear the Spectral Graph.

3. To draw a profile line, click the **Draw** 🖊 button on the first row (Red) of the color library, and then use the mouse pointer to click and draw a line across the area of interest in the image. As you draw a line within the image, the Spectral Graph displays the intensity of the selected pixels.

4. This figure shows two profile lines drawn on a component image. These lines show the variation in the amount of 655 nm Qdot™ present in the cells sampled by the lines.
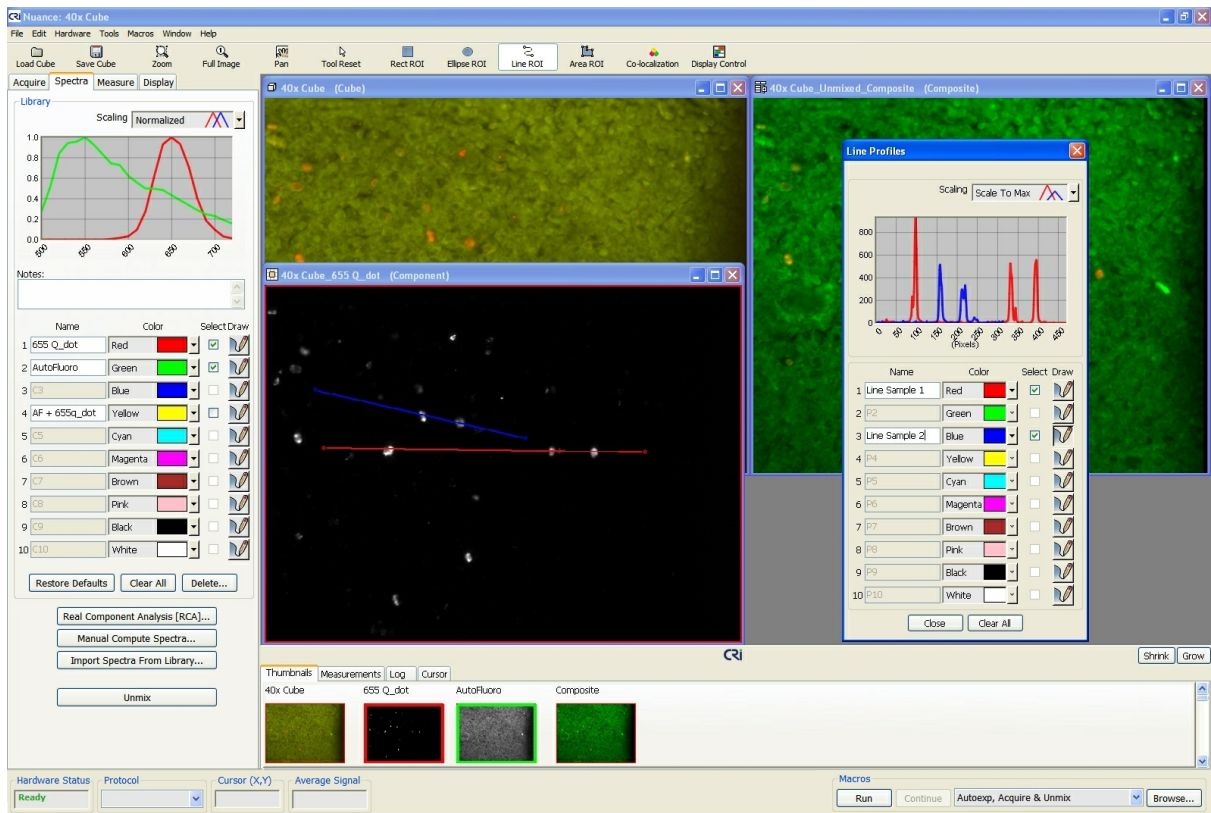
Figure 33: Line Profiles drawn on component images

Move the mouse pointer across the Spectral Graph in the **Line Profiles** dialog box. You will notice a black dot appear on each line. These dots correspond with the dots on the profile lines of the images. They can help identify specific pixel intensities on the line and in the spectral graph.

The Line Profiles tool is for data comparison purposes only. The ability to export profile data has not yet been enabled.

▬ Working with Line Profiles

After drawing line profiles on an image, you can copy, clone, move, and delete lines by right-clicking on them.

To copy line profiles to the clipboard:

1. Right-click on the image that contains the lines you want to copy, and then select **Copy Line Profiles To Clipboard** from the pop-up menu. This feature copies all profile lines in the image onto the clipboard. If you want to copy a single line to another image, simply drag and drop the line onto the other image. (Be sure to deselect the **Draw** 🖊 tool before attempting to drag and drop the line.)

   Note: When dragging a copy of a line to another image, the line will not move while you drag the mouse pointer. However, the copy will appear on the new image, at the same spatial coordinates as the original copy, when you release the mouse button at the destination.

2. Right-click inside the image to which you want to paste the line profiles and select **Paste Line Profiles From Clipboard**.

3. All line profiles on the clipboard are pasted into the current image. Each new line profile is assigned the next row/color in the color library, and all lines are plotted in the Spectral Graph.

To clone a line profile:

1. Right-click on the line you want to clone and select **Clone Line Profile** from the pop-up menu. (Cloning creates an exact copy of the original that you can move to another area within the current image. You cannot move a clone to another image.)

2. The mouse pointer changes to a hand with a floating clone of the original line attached to it. Move the clone and click to release it at the desired location.

To move a line profile:

1. Right-click on the line you want to move and select **Move Line Profile** from the pop-up menu.

2. The mouse pointer changes to a hand with the floating line attached to it. Move the line and click to release it at the desired location.

To delete a line profile:

1. Right-click on the line you want to delete and select **Delete Line Profile** from the pop-up menu.

2. The line is removed from the image, the selection is removed from the color library, and the profile plot is removed from the Spectral Graph.

6.11 Comparing Multiple Images

The Compare Images tool lets you compare greyscale images from a single cube or from cubes collected over time so that you can more easily visualize differences in intensity in the component images. Images compared with this tool do not need to have the same scaling or image display parameters. Compare Images will display images on the same scale, accounting for differences in intensity due to bit-depth, exposure time and binning.

The datasets in the following example illustrate dynamic range variability of different signals and how they can be compared using the Compare Images Tool.

1. Open and unmix the relevant cubes so that their component thumbnails are listed in the **Thumbnails** list at the bottom of the window.

2. Select **Tools > Compare Images**. The **Compare Images** dialog box opens (see figure below).

3. From the image gallery or the **Thumbnails** list, drag the greyscale component images to be compared onto the display boxes in the dialog box. (You may need to scroll the Thumbnails list to locate all your greyscale images.)

4. By default, the dialog box has room to compare 16 images. You can use the scroll boxes in the **Grid Dimensions** group to change the display grid. While it is possible to compare more than 16 images, this is limited by the available amount of computer memory. Try using the **Close all windows (including thumbs)** command in Nuance, and close other applications that may be running before trying to open additional images.

5. All images are scaled based on the image with the brightest signal. As you can see in the figure below, the **Display Key** for the upper-left image, which contains autofluorescence, has the brightest signal with 786 counts. The red outline in each image **Display Key** indicates the display range of the individual image.

6. The other images contain the two other fluorophores in the same sample. Both fluorophores are much weaker than the autofluorescence.
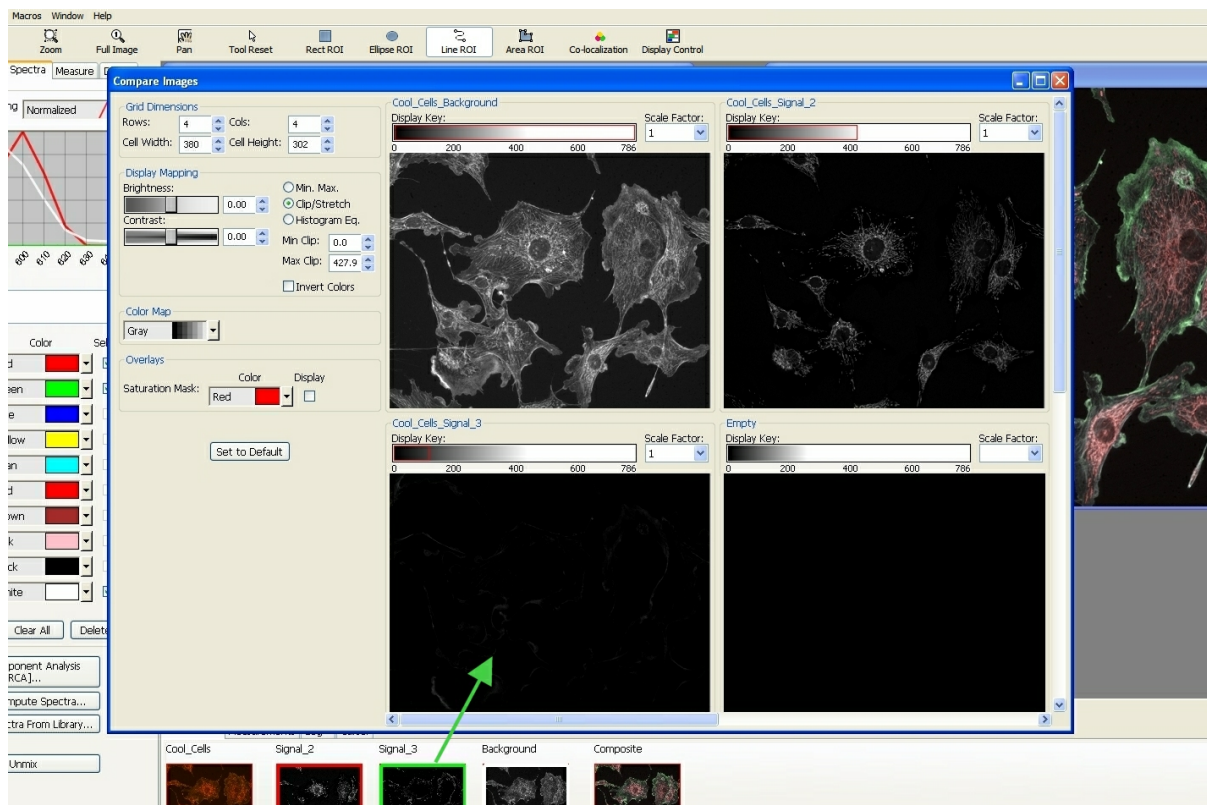
Figure 34: Compare Images

7. If an image you are trying to compare is either too dark or too bright to display any visible detail at the display range of the other images, use its **Scale Factor** multiplier to multiply its pixel intensity.

   ➢ In the above figure, the upper-right image is only half as bright (~ 400 counts) as the autofluorescence. Increasing its Scale Factor to 2x (see figure below) makes the image brighter (notice also the change in its Display Key).

   ➢ The lower-left image is completely dark. It is only one-eighth (~ 100 counts) as bright as the autofluorescence image. Increasing its **Scale Factor** to 10x makes the image brighter to

match the other images
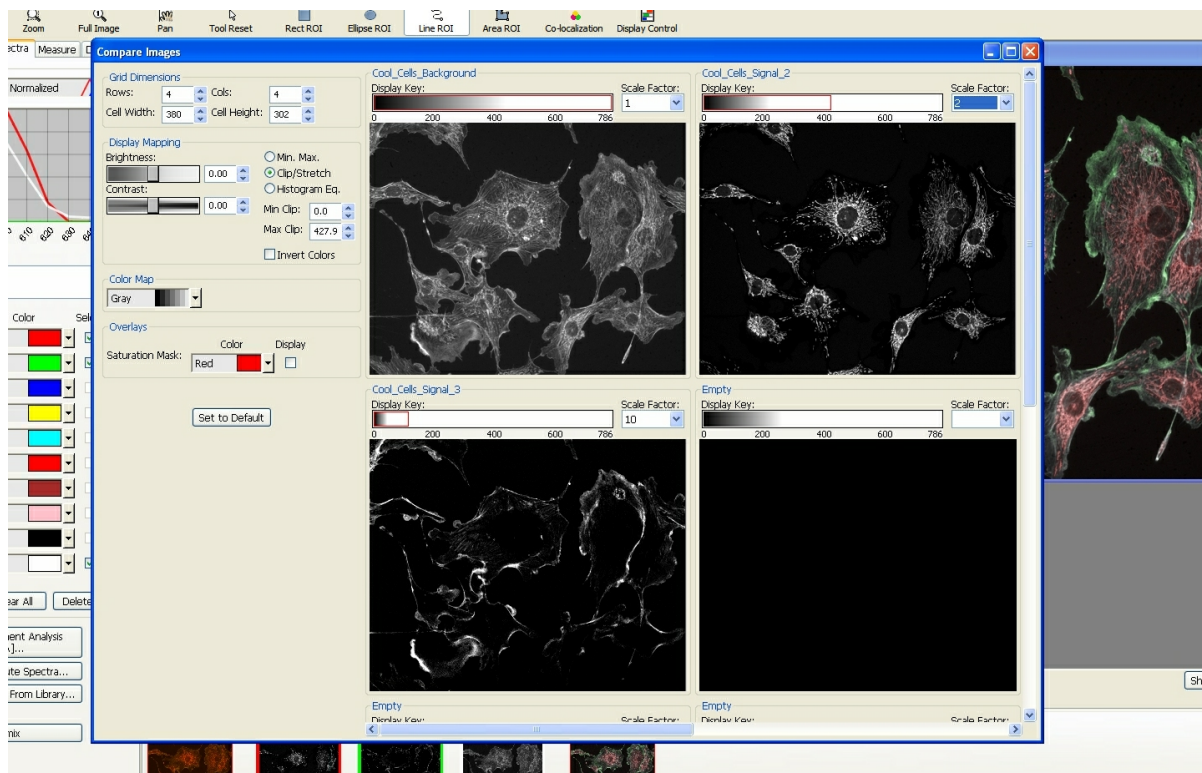
Figure 35: Compare Images using different scaling factors and the "Jet" color map

Modifying Display Settings

➤ The **Display Mapping** options are used to adjust the brightness and contrast of all the images together.

➤ The **Color Map** drop down box lets you map the display to a variety of pseudo colors. The example in this figure is shown using the "Jet" color map.

➤ The **Saturation Mask** option allows you to mask saturated pixels if desired.

Zooming and Panning Images

➤ Right-click on any of the images and select **Zoom** from the pop-up menu. The pointer changes to a magnifying glass. Use the magnifying glass to draw a box around the area you want to zoom.

➤ To pan zoomed images, right-click on any image and select **Pan**. You can now drag the image to the region you want to view.

➤ When you want to return to the full image, right-click any image and select **Zoom To Full Image**.

➖ Saving Displayed Images

To save a copy of an image with its current display settings, right-click on the image and select an option from the pop-up menu:

➢ **Save Image As Displayed** opens a **Choose RGB File** dialog box where you can save the image (as it is displayed) in TIF or JPG format.

➢ **Copy Whole Image To Clipboard** copies the entire image, regardless of the current zoom level, to the clipboard. This can then be pasted into a third party software program.

➢ **Copy Displayed Image To Clipboard** copies the current viewable region of the image to the clipboard. This can then be pasted into a third party software program.

6.12 Processing a Batch of Cubes

In some instances, you might prefer to acquire a large number of cubes before analyzing them, or you might want to re-analyze a set of images with new analysis settings. The Batch Data Processing feature is useful for unmixing and measuring multiple cubes or a large set of images all at once. It can also be used to perform co-localization staining detection on a batch of cubes.

1. Select **Tools > Batch** from the main menu. The **Batch Data Processing** dialog box opens.
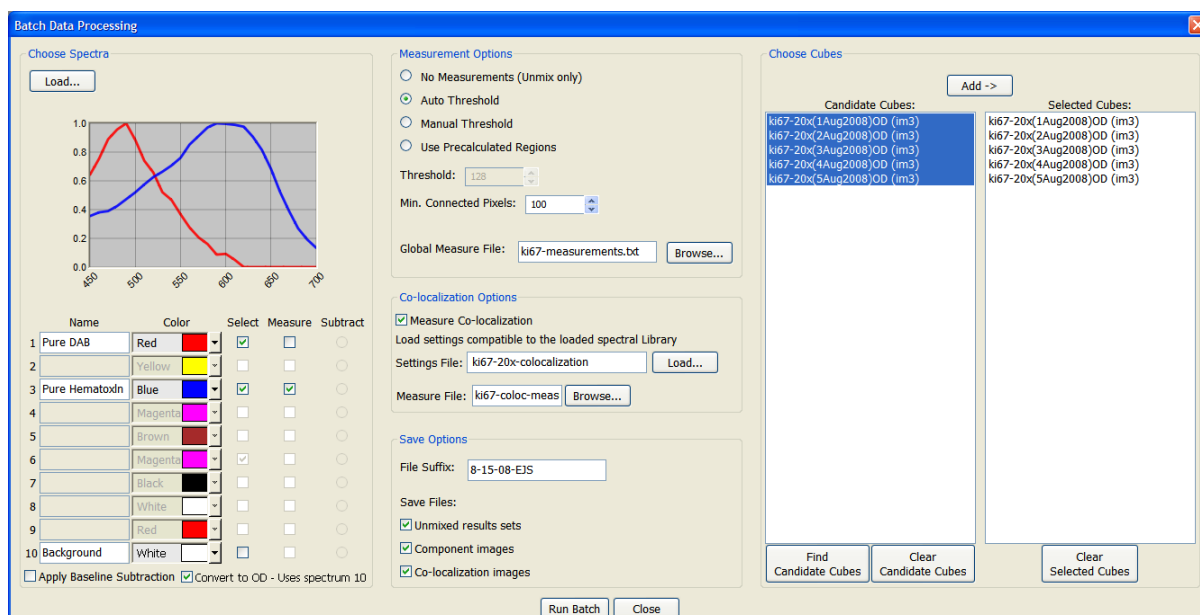
Figure 36: Batch Data Processing

2. If the spectral library you want to use is open already, its spectra will display in the color library. You can add spectra to the library or open a new one by clicking the **Load** button.

3. Use the check box(es) in the **Select** column to select the spectra you want to use for unmixing. Use the **Measure** column to select the unmixed component images that you want to measure during batch processing.

4. The **Apply Baseline Subtraction** check box offers the same functionality as the Baseline Subtraction 67 feature. If there is stray light (which contributes to a baseline offset) it can be

subtracted from the batch of cubes using this option. Select this check box, then indicate which spectrum to subtract from all cubes.

5. The **Convert to OD** check box lets you convert all of the cubes in the batch to Optical Density. This function automatically uses spectrum 10 to convert all cubes to OD, so your background spectra must be assigned to spectrum 10.

6. The **Choose Cubes** group lets you select the image cubes you want to include for batch processing.

    a. Click the **Find Candidate Cubes** button below the list.

    b. Select a folder from the dialog box and click **OK**. The Nuance software will search this folder and its subfolders for cubes that are compatible with the current spectral library and display them in the **Candidate Cubes** list.

    c. Select the cubes you want to process, and click the **Add** button. This will add them to the **Selected Cubes** list on the right.

7. The **Measurement Options** group lets you select the measurement parameters that will be used in the batch processing. Use the radio buttons to select one of the following options:

    ➢ **No measurements (Unmix only)** – This option unmixes the selected cubes.

    ➢ **Auto Threshold** – If you select this option, then specify the **Min. Connected Pixels** parameter and choose at least one component image to measure. (Select from the **Measure** column in the spectral library.) Also, browse to specify a **Global Measure File** to contain all of the exported measurement results.

    ➢ **Manual Threshold** – If you select this option, then specify both the **Threshold** and **Min. Connected Pixels** parameters. Next, choose at least one component image to measure. Browse to specify a **Global Measure File** to contain all of the exported measurement results.

    ➢ **Use Precalculated regions** – If you select this option, select a Regions (.roi) file and/or a Measurements (.txt) file that you want to use to measure the unmixed results.

8. If you want to run co-localization detection on a batch of cubes, check the **Measure Co-localization** check box. Notice the prompt to *"Load settings compatible to the loaded spectral library."* The spectral library used to unmix the candidate cubes must match the library referenced in the co-localization settings file.

    a. Click **Load** to load a co-localization settings (.col) file that was created and saved earlier using one of the candidate cubes. See also Saving and Loading Co-localization Settings 95 .

    b. Browse to specify a **Measure File** to contain all of the exported co-localization measurement results.

9. The parameters in the **Save Options** group control which file types are saved and how the files are named. Results are saved in the same directory as the cube.

    ➢ The **File Suffix** parameter defines the suffix that you want added (such as the date, operator's initials, etc.) to the file name of each saved file. The unmixed results will be named *"originalcubename_filesuffix.umx"*. If you do not specify a suffix, none will be added to result set file names. Measurements files are saved with a *date_timestamp_measures.txt* filename.

    ➢ Under **Save Files**, select any or all of the following:

- o **Unmixed result sets** saves each unmixed result set as a result set (.umx) file.

- o **Component images** saves a separate data file (.tif) for each unmixed component image.

- o **Co-localization images** saves a co-localization image (.jpg) for each cube.

10. Click the **Run Batch** button. This will iterate through the selected cubes, unmix them, measure any components selected for measurement, and run co-localization detection if selected. A **Batch Mode Progress** box displays the estimated time remaining until completion.

11. When the batch process is complete, you can open an explorer window of the cube directory to view the exported files. For example, the ki67-20x(1Aug2008)OD candidate cube shown in the figure above resulted in the following exported files:

- ➢ The exported Co-localization image file was saved as

  ki67-20x(1Aug2008)OD_coloc_8-15-08-EJS.jpg

- ➢ the exported Co-localization measurements file was saved as

  ki67-coloc-measurements.txt

- ➢ The exported Measurements file was saved as

  8-15-08-EJS_2008-Aug-15__09_39_43_measures.txt

- ➢ The exported Component image (*.tif) files were saved as

  ki67-20x(1Aug2008)OD_Pure DAB_8-15-08-EJS.tif_Data_x10000

  ki67-20x(1Aug2008)OD_Pure HematoxIn_8-15-08-EJS.tif_Data_x10000

- ➢ The exported Result set was saved as

  ki67-20x(1Aug2008)OD_8-15-08-EJS.umx

7   Quantifying Results

The **Measure** panel is used to draw measurement regions and set measurement parameters. The **Measurements** page at the bottom of the Nuance work area displays the measurements of regions drawn on component images. This section explains each of the tools used for measuring regions on component images.

7.1   Measuring Regions

⊟ Finding Regions Automatically Using Threshold Segmentation

1. Select the component (or any other single-plane) image to be measured and make sure the **Measure** panel is visible.

2. In the **Threshold Segmentation** group, click the **Auto Calculate Threshold** button. The Nuance software will attempt to find a threshold level that is appropriate for the image.
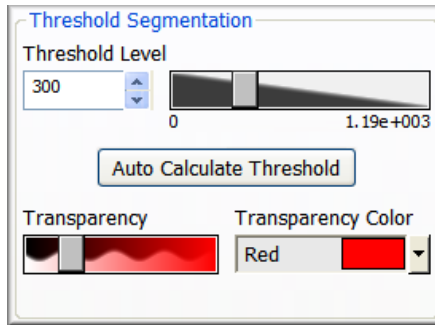
Figure 37: Threshold Segmentation

3. Pixels with intensity values below the specified threshold are ignored. If you are not satisfied with the auto calculated threshold level, you can enter a new value in the **Threshold Level** field by using the slider or manually typing in a value. You will see the region(s) change as you edit the threshold level.

4. After a threshold is set, the image will have an overlay showing the regions that were measured, as shown in this figure.
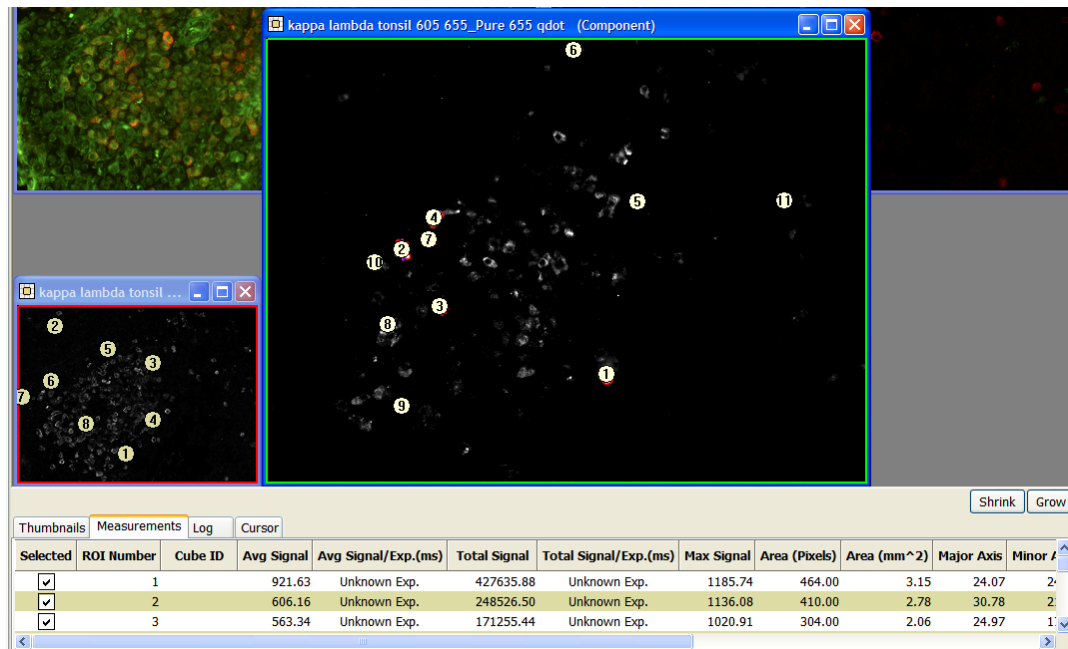
Figure 38: Measured Threshold Regions

5. If there are too many or too few measurement regions, adjust the **Threshold Level** or **Minimum Connected Pixels** value (see Ignoring Smaller Regions⟨80⟩).

6. You can adjust the region's transparency or change its color (see Adjusting Region Transparency and Color⟨80⟩).

Manually Drawing and Modifying Regions

The Nuance software provides tools for manually drawing and modifying measurement regions. These tools are located in the **Manual Draw Regions** group on the **Measure** panel:

1. Click the **Draw** button on the **Measure** panel.

2. Select the draw tool shape by selecting from the toolbar or right-clicking the image and selecting from the pop-up menu (rectangle-, ellipse-, line-, or area-ROI).

3. Use the mouse pointer to draw new regions of the image to measure.

4. Each region is assigned an ROI number and is listed in the **Measurements** page at the bottom of the screen. Measurement regions are numbered and sorted according to their size, with the largest region first, and the smallest last.
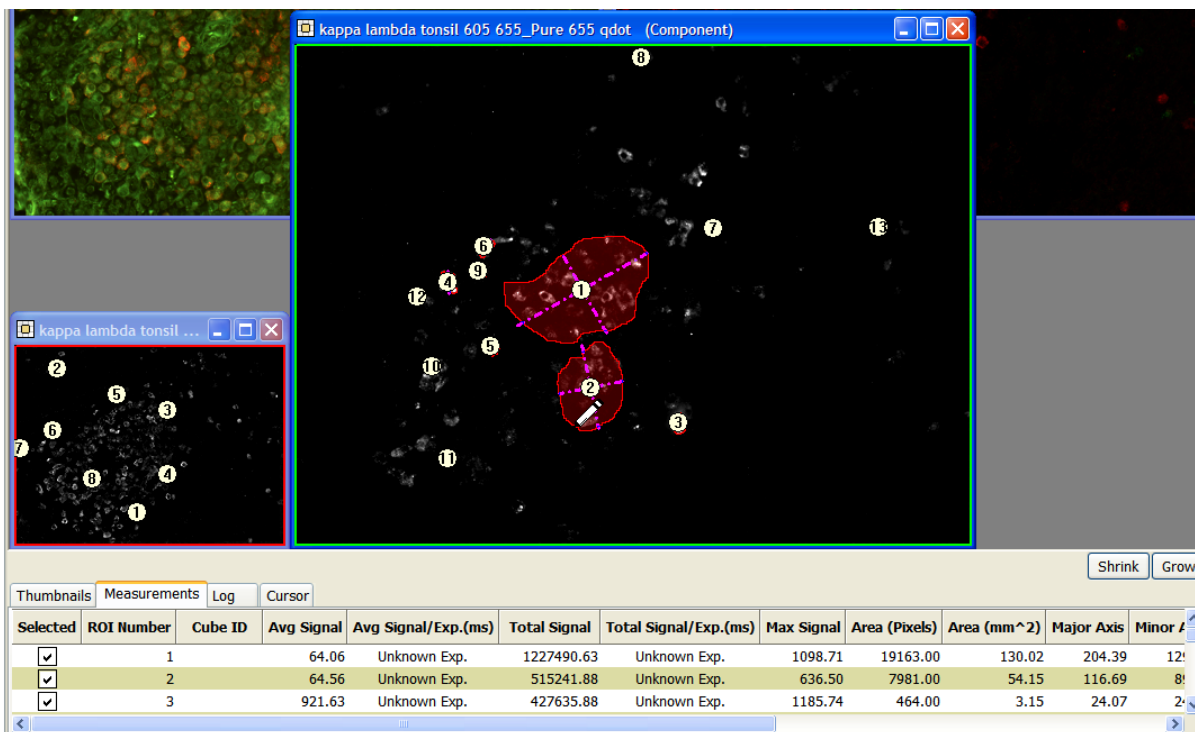
Figure 39: Manually Drawing a Region

5. The dashed lines drawn on top of each region show the region's major and minor axis drawn through the center of gravity.

To erase part of a region:

1. Click the **Erase** button on the **Measure** panel.

2. Select the erase tool shape by selecting from the toolbar or right-clicking the image and selecting from the pop-up menu (rectangle-, ellipse-, line-, or area-ROI).

3. Use the mouse pointer to remove pixels from existing regions.

To clear all regions:

Click the **Clear** button to remove all measurement regions.

To move or delete a region:

Right-click on the region you want to move or delete and select from the pop-up menu.

Cloning Regions

The Nuance software's ability to clone regions makes it easy to directly compare control regions and fluorophore regions of an image. Once you clone a region, you can then move the clone to another area of the image to compare the fluorescence signals.

1. Right-click on the region you want to clone and select **Clone Region** from the pop-up menu. A clone of the region is created.

2. Move the clone to the desired area of the image. The clone will become the next sequentially numbered measurement region.
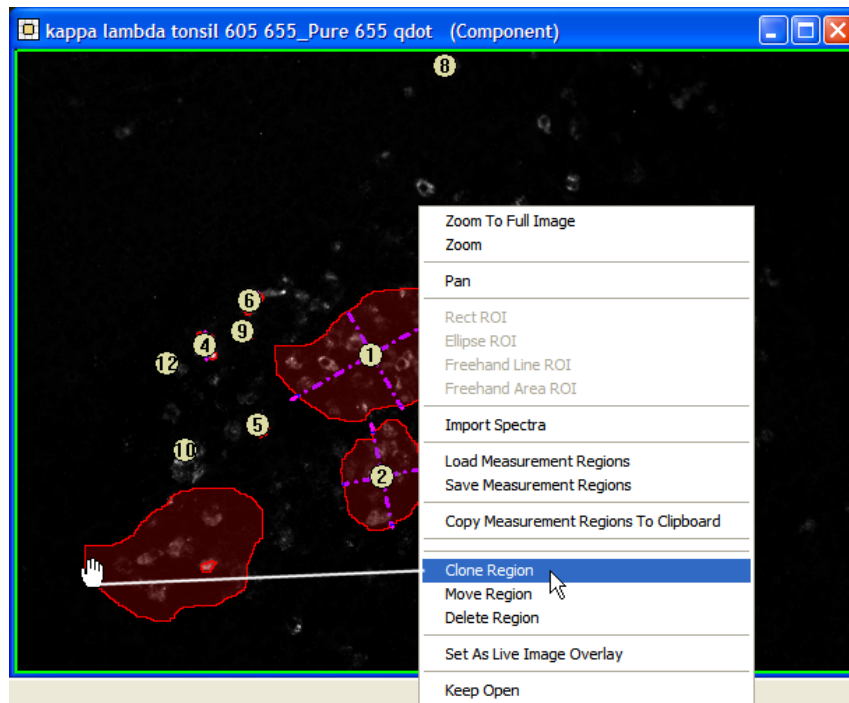
Figure 40: Cloning a Region

Dragging a Copy of a Region to Another Image

You can drag a copy of a region from one component image to another. When you drag a region to another image, the Nuance software automatically pastes the region at the exact same location in the new image. This feature is useful for comparing regions across two or more composite images.

1. Click on the region you want to copy, and while holding down the mouse button, drag it to the new image.

2. Release the mouse button anywhere in the new image. The copied region will automatically snap into position, corresponding to the position of the original.

Copying all Regions to the Clipboard

The Nuance software can copy all existing regions from an image to the clipboard. You can then paste the copied regions from the clipboard to other images. (All regions in the image are copied with this feature. See Dragging a Copy of a Region to Another Image 78 if you want to copy a single region.)

1. Right-click on the image that contains the regions you want to copy, and then select **Copy Measurement Regions To Clipboard** from the pop-up menu.

2. Right-click inside the image to which you want to paste the regions and select **Paste Measurement Regions From Clipboard**.

3. All measurement regions on the clipboard are pasted into the current image. Each new region is assigned a ROI number, and its measurement data displays in a new row of the **Measurements** page at the bottom of the Nuance window.

4. The ROI numbers can change from image to image depending on what other regions are present in each image.

Saving and Loading Regions

If you save your measurement regions, you can easily load them to other images. This is useful for comparing control regions and fluorophore regions across multiple images.

1. Right-click anywhere on the image (but not within a region) and select **Save Measurement Regions** from the pop-menu.

2. In the **Choose Measurement Regions** dialog box, enter a descriptive name, and click **Save** to save the measurement region(s).

To load a region to an image:

1. Right-click anywhere on the image (but not within a region) and select **Load Measurement Regions** from the pop-menu.

2. The region(s) will be loaded to their exact pixel location when they were saved. You can move the region(s) by right-clicking them individually and selecting **Move Region**.

Obtaining Accurate Measurement Data

The Nuance software calculates each region's area in pixels and in mm2 based on the current binning selection on the **Measure** panel. This selection must be correct for the mm2 measurement to be accurate.

For PerkinElmer format "Image Cubes," the Nuance software automatically displays the binning used to acquire the cube. This setting cannot be changed.

TIFF cubes do not save binning information. The binning value defaults to 1x1. For TIFF cubes, you can change this value in case it was set incorrectly during cube acquisition.
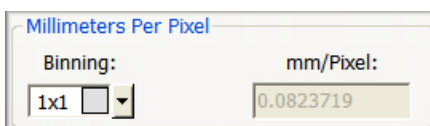
Figure 41: Millimeters Per Pixel

To change the binning:

Make a component image active, and then select from the **Binning** drop down boxes. (Binning can be changed for TIFF cubes only.) The Nuance software calculates and displays the new mm/Pixel value automatically, based on the calibrated pixel size (see Calibrating Pixel Size 29).

7.2 Ignoring Smaller Regions

The **Measure Objects** group on the **Measure** panel is used to specify a minimum region size based on its number of pixels. Only regions that are larger than the **Min. Connected Pixels** value are considered. The Nuance software ignores regions that have fewer than this number of pixels.
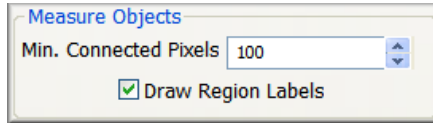
Figure 42: Minimum Connected Pixels

Use this control to change the size of the regions to filter out. This setting does not effect manual draw regions.

Hiding Region Labels

Numbered region labels correspond to the ROI Numbers on the **Measurements** page at the bottom of the Nuance work area. Sometimes a region label obscures important data or makes it difficult to see the region clearly. You can have the Nuance software draw or hide numbered labels on regions within an image.

A check-mark in the **Draw Region Labels** check box (see figure above) will show the region labels that are normally drawn on top of the region. Clearing the check-mark hides these labels.

7.3 Adjusting Region Transparency and Color

1. Use the **Transparency** slider (see Threshold Segmentation 75 image) on the **Measure** panel to change how much measurement regions block the image behind it. Moving this slider all the way to the left makes the region transparent (except for its outline). Moving the slider all the way to the left fills the region with the selected color.

2. If you want to change the transparency's color, select a color from the **Transparency Color** drop down box.

7.4 Understanding Region Measurements

Each region's measurements are displayed as rows on the **Measurements** data page near the bottom of the Nuance work area (see Measured Threshold Regions 75 figure). The following information is displayed:

➤ The **Selected** column indicates which measurement regions are displayed on the image. Clear the check box from a measurement row to remove its region from the image.

➤ The **ROI Number** for each measurement region corresponds to the number displayed in the center of the region drawn on the image.

➤ **Cube ID** is the ID entered in the **Sample ID** field on the **Fluorescence** panel when the cube was acquired.

➤ **Average Signal** is the average value of the pixels in the region.

➤ **Standard Deviation** represents the variation of the signal values.

➤ **Total Signal** is the sum of all the pixel values in the region.

➤ **Max Signal** is the maximum pixel value included in the region.

➤ **Area (Pixels)** is the number of pixels included in the region.

➤ **Area (µm^2)** is the size of the region in square microns. This measurement will only be displayed if the camera's pixel size has been calibrated (see [Calibrating Pixel Size](#) 29). Also, the accuracy of this measurement depends on setting the correct binning (see [Obtaining Accurate Measurement Data](#) 79).

➤ **Major Axis** is the length of the minimum area bounding box enclosing the region.

➤ **Minor Axis** is the width of the minimum area bounding box enclosing the region.

➤ **X Location** is the center of gravity's x coordinate.

➤ **Y Location** is the center of gravity's y coordinate.

➤ **Spectrum ID** is the ID of the spectrum used to unmix this image (entered from the **Spectra** panel).

➤ **Cube Time Stamp** represents the time the cube was created.

Hiding Measurement Columns

Right-click anywhere within the measurement rows. The pop-up menu lists all columns with check marks. Click any column to clear its check mark and hide the column from the display. Repeat for all columns you want to hide. You can hide any or all columns except for the **Selected** and **ROI Number** columns.

Copying Measurement Data to the Clipboard

1. Make sure the measurement regions you want to copy are selected on the **Measurements** data page (check marks in the **Selected** column).

2. Click the **Copy Measurements to Clipboard** button in the **Measure** panel. Any columns you have hidden will be omitted from the copied measurements.

3. You can now switch to a program such as **Microsoft Excel** and paste the measurements into a worksheet.

Saving Measurement Data as a Text File

1. Make sure the measurement regions you want to save are selected on the **Measurements** data page (check marks in the **Selected** column).

2. Select **File > Save Measurements**. The **Choose Measurement File** dialog box opens.

3. Select a location and enter a name for the text file. Click the **Save** button to save the new text file. Any columns you have hidden will be omitted from the saved measurements.

Appending Measurement Data

You can append new measurement data to end of an existing measurement data text file.

1. Make sure the measurement regions you want to append to a saved text file are selected on the **Measurements** data page.

2. Select **File > Append Measurements**. The **Choose Measurement File** dialog box opens.

3. Select the file to which you want to append the measurement data and click the **Save** button.

8 Customizing Spectral Displays

The topics in this section explain how to use the **Display** panel and the **Display Control** utility to adjust display parameters of a cube, component image, or composite image to obtain a clearer view of the image. The Display Control utility changes depending on the current image type.

8.1 Adjusting Brightness and Contrast Levels

The controls in the **Display Mapping** group on the **Display** panel are used to change brightness and contrast levels within images.

➢ Five sliders allow you to adjust the following:

  o Brightness

  o Contrast

  o Brightness for the individual red, green, and blue color channels. These sliders are enabled only when a cube or composite image is selected.
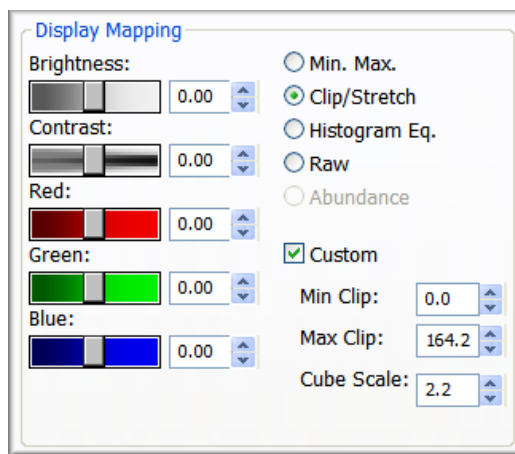
Figure 43: Display Mapping

➢ The **Min. Max.** option maps the minimum value in the image to 0, the maximum value to 255, and linearly interpolates in between those values. This stretches dark signals so they become visible.

➢ The **Clip/Stretch** option maps the lowest 0.01% of the pixels to 0, the highest 0.01% to 255, and linearly interpolates in between those values. This prevents a few bright or saturated pixels from skewing the display.

➢ The **Histogram Eq.** option maps the pixels so the histogram of the pixels have approximately the same number of pixels in each bin. This gives the best display of the whole dynamic range of dim and bright signals.

➢ The **Raw** option applies no scaling to the data.

➢ The **Abundance** option is enabled only when a composite image is selected. This option scales all the pixels in a composite by the ratio of 255 and the value of the single brightest pixel among all the components. This usually results in a brighter composite image.

Note: In fluorescence images, the autofluorescent background signal is often the source of the brightest pixels in the dataset. If this background signal was unmixed into black to make

it seem to disappear, selecting the **Abundance** option may result in an almost all black composite image. Unmix the background into white or other light color before applying **Abundance** scaling.

➢ The **Enhance Contrast** check box adjusts the display of the cube to compensate for the brightness and contrast of your monitor.

➢ The **Custom** check box lets you assign specific minimum and maximum clip values to the display of any image. This check box works with the **Min. Max.** and **Clip/Stretch** display options only. Increase the **Min Clip** value to exclude more of the lowest value pixels from the display. Decrease the **Max Clip** value to exclude more of the highest value pixels.

8.2   Applying Overlays

Saturation Mask

Saturated pixels in your images reduce the accuracy of your unmixing and quantitation results. The Nuance software considers a pixel in a cube "saturated" if the pixel is saturated at any of the cube's wavelengths. Pixels in a component image remain saturated if they were saturated in the cube used to create the component. In a composite image, a pixel will be saturated if it was saturated in the component image.

If saturation is present in your image, use the **Saturation Mask** controls on the **Display** panel to reduce or eliminate the effects of the saturated pixels.

1. Select the image to which you want to apply the saturation mask.

2. Select the color that matches the saturated pixel color.

3. Click the **Display** check box to apply the saturation mask to the image.

Live Overlay

The **Live Overlay** option lets you use a saved image as an overlay on top of the live view for positioning your specimen as when it was previously imaged. Open the image you want to use as an overlay. Right-click on the image and select **Set As Live Image Overlay** from the pop-up menu. The overlay is now visible in the live view. Use the **Display** check box on the **Display** panel to view or hide the overlay. Use the **Transparency** slider to increase or decrease the transparency of the overlay image.

8.3   Adjusting a Cube's RGB Mapping

Computers display color through varying intensities of red, green, and blue. To display an image cube, which has many more color planes, the Nuance software maps all colors in the cube to the red, green, and blue planes.

When a cube is the active image, the **Wavelength to RGB Map** group becomes visible in the **Display** panel. Wavelength ranges in the image cube are mapped to red, green, or blue to create the display image. The sample on the left displays for cubes that have not been converted to Optical Density (OD). The sample on the right displays for OD cubes.

(non-OD-Converted)   *(OD-Converted)*
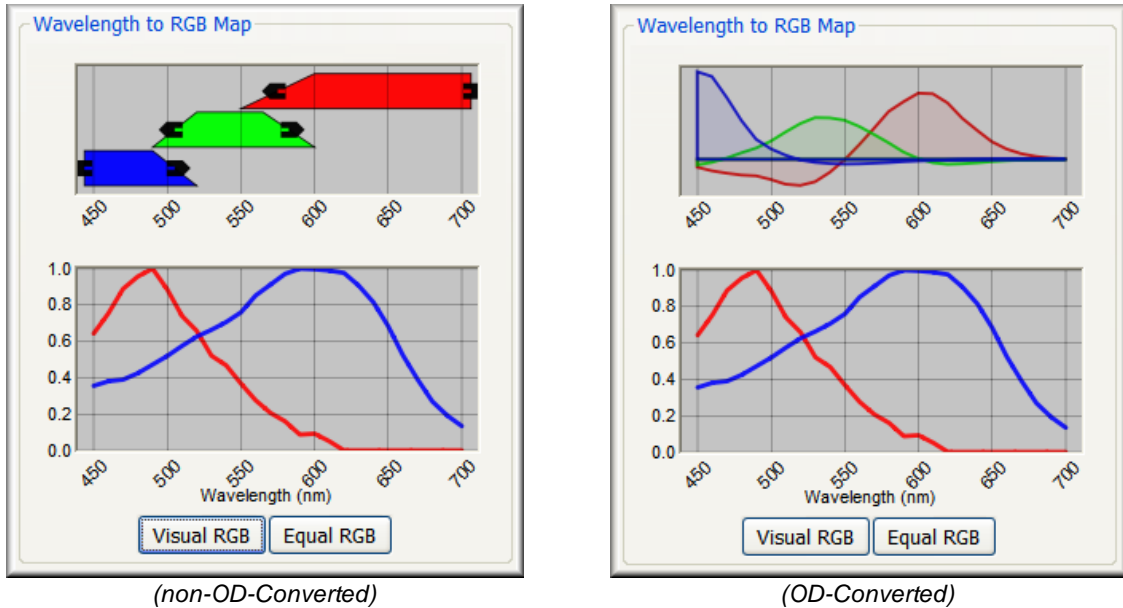
Figure 44: Wavelength to RGB Map

The non-OD sample (left) has red, green, and blue bars. The wavelengths in the red bar are mapped to the red plane in the display image (green to green, and blue to blue).

You can move these bars by clicking and dragging them. This will change the wavelength range that gets mapped to that color plane. The bars can also be resized to increase or decrease its wavelength range by dragging the handles on the ends of the bars.

You cannot change the wavelength mapping of OD cubes.

The plot below the bars shows the current spectral library. It also shows the spectrum underneath the cursor when it is moved over the display cube. The x-axis represents the wavelength range of the cube.

To View Visual RGB

Click the **Visual RGB** button to set the bars so they approximate the human eye.

To View Equal RGB

Click the **Equal RGB** button to set the bars so they divide the cube's wavelength range equally among the red, green, and blue planes.

8.4   Changing Components in a Composite Image

The Nuance software displays a composite image by combining the displays—not the raw data—of the component images. When a composite image is active, the **Component Selection** group becomes visible in the **Display** panel.

There is a row for each component image. Use the **Display** column to control which components are used to create the composite image. If you want to change the color used to display any of the component images, select a color from the component's **Color** drop down box.

This feature is very useful when you want to make the fluorophore signal of interest more clearly visible in a composite image. For example, you could exclude the Background component (thereby removing the autofluorescence) from the composite image. Doing this in combination with adjusting the **Brightness** and **Contrast** of the unmixed components (using the sliders in the **Display Mapping** group), can result in a clear bright image of the tumor, fluorophore, etc.
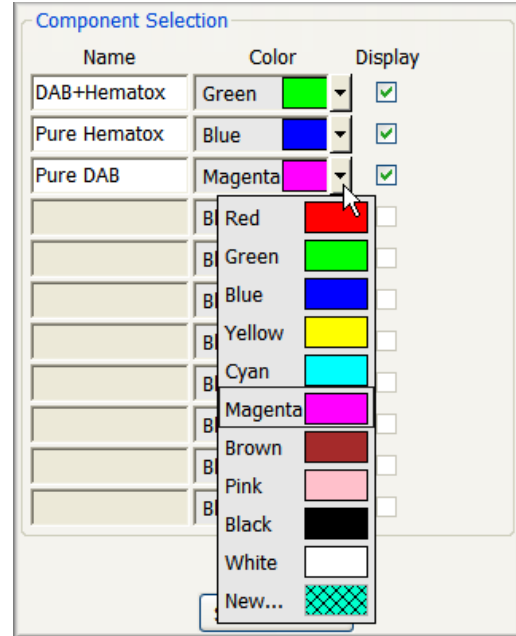
Figure 45: Component Selector

8.5   Advanced Display Controls

In addition to the tools available on the **Display** panel, the **Display Control** utility offers more advanced functions for adjusting how cubes, composites, and components are displayed. The functions that are available with this utility will vary depending on the kind of image that is currently active.

Opening the Display Control Utility

Click the **Display Control** button on the toolbar. The top half of this dialog box offers an enhanced version of the **Display Mapping** and **Scaling** selectors found on the **Display** panel (see Adjusting Brightness and Contrast Levels 83). You can adjust the brightness and contrast of the image.

Linking the Contrast and Brightness Sliders

Check the **Show Crosshairs** check box above the preview image. This lets you drag the intersection of these two sliders to any position on the image until the desired display is achieved.

Adjusting Composite Display Settings

Click on the composite to make it the active image. The bottom half of the **Display Control** utility expands with a list of the component images and other display options such as coloring style and blending style. These can be used to make the label signal of interest more clearly visible in the composite image.

For example, you could exclude the Background (thereby removing the autofluorescence) from the composite. Doing this and using the sliders to adjust the brightness and contrast of the label signal can result in a clear bright image of the specimen.

To include or exclude layers:

Click the layer's **View** 👁 button to include it, or its **Hide** 👁 button to exclude it from the composite.

To adjust a layer's brightness or contrast:

Click the layer's **Adjust** ✓ button. Then use the **Brightness** and **Contrast** sliders to adjust the display of the layer. (You can link these sliders by checking the **Show Crosshairs** check box.)

To view images in fluorescence or brightfield:

Use the **Composite Coloring Style** options. In general, you want to select the mode under which the image cube was acquired.
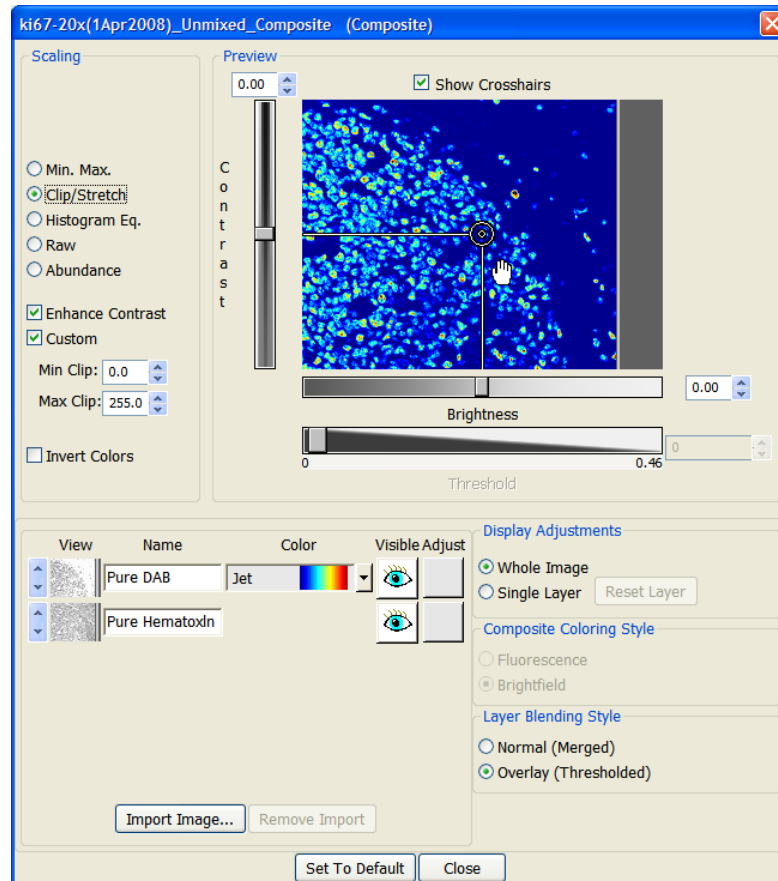
Figure 46: Display Control

To create a composite image by blending its component signals:

Select **Normal (Merged)** in the **Layer Blending Style** group. This will blend the signals from each of the components to create the composite.

To create a composite image by overlaying its component signals:

Select **Overlay (Thresholded)** in the **Layer Blending Style** group. This will overlay each component signal on top of the component signal(s) beneath it. When you select the **Overlay** option, this also enables the **Threshold Legend** right-click menu item for composite images (see Displaying the Intensity Legend 88).

The component at the bottom of the list is the base layer. You can change the order of any component in the list by clicking the up or down arrow next to its thumbnail.

If you want to import a new component image as the base layer, click the **Import Image** button and select the image you want to import. The imported image is added to the bottom of the components list. The Nuance software creates a new composite called a "Layered Image" and displays it in the image gallery.

Returning to the Default Display Setting

When viewing/editing a single layer, clicking the **Reset Layer** button returns all display settings for the selected layer to the default state. When viewing/editing the whole image, clicking the **Set To Default** button returns all values of all layers to their default state.

8.6 Displaying the Intensity Legend

For a composite image and any of its components, you can display a legend that represents minimum and maximum intensity values within the image. (Images can be from any multispectral cube.)

To apply the legend to a component image, right-click the component and select **Display Intensity Legend** from the pop-up menu. The legend displays in the lower right-hand corner of the image. For components, the legend is always in grayscale. For a composite image, first open the **Display Control** for the composite and select the **Overlay (Threshold)** layer blending style. Close the **Display Control** and right-click on the composite to select **Display Intensity Legend**. For composites, the legend always displays the color scheme selected for the first visible component listed in the **Display Control**.

The title of the legend—and the intensity values displayed—correspond with the **Measurement Pixel Units** that were selected at the time the cube was acquired. (This selection is made from the **Hardware > Edit Hardware Settings > Preferences** settings panel.) Intensity display possibilities include the following:

➢ "Raw Counts" (if the original cube was a TIFF stack), "Counts," "Scaled Counts/Sec." (see Selecting a Measurement Pixel Unit/Scaling 28 for definitions).

➢ If the title of the legend is "Optical Density," this means the cube has been converted to optical density.

9 Macros

A *macro* is a shortcut for a sequence of actions. You assign the macro a name and save it. Later, when you run the macro, the macro plays back as if you were performing the actions yourself. A macro can be simple (for example, it could autoexpose and acquire a cube) or it can be complex. For example, it might load a spectral library, pause for you to open a cube, unmix the cube, draw regions of interest, prompt you to adjust the location of the ROIs, save the result set and export the ROI data.

9.1 Overview of the Macros Dialog Box

Choose **Macros > Define Macro** from the main menu to reveal the **Macros** dialog box (see figure below). Each item in this dialog box is described below.

➢ The **Macros** drop down box lets you select a macro to run. When you select a macro, its steps display in the window. Saved macros cannot be modified. You can also select a macro to run from the **Macros** drop down box on the status bar, at the bottom-right of the screen.

➢ The **Run Macro** button runs the selected macro. This button is also available on the status bar at the bottom-right of the screen.

➢ The **Pause Recording** button places the macro recorder in pause mode. When the recorder is paused, it does not record any of your actions. To start recording again, click the Continue Macro button.

➢ The **Continue Macro** button continues running the macro after a message and/or pause in the macro that requests a user action. This button is also available on the status bar.

➢ The **Load** button is used to import a macro (.rcd) file that contains recorded commands.

➢ The **Save** button saves a new macro file of all the macros currently listed in the **Macros** drop down box. Macros that you create are automatically added to the **Macros** list; however, they are not permanently saved on the system until you click the **Save** button to save the new file. Any new macros that are not saved will be lost when you close the Nuance software.
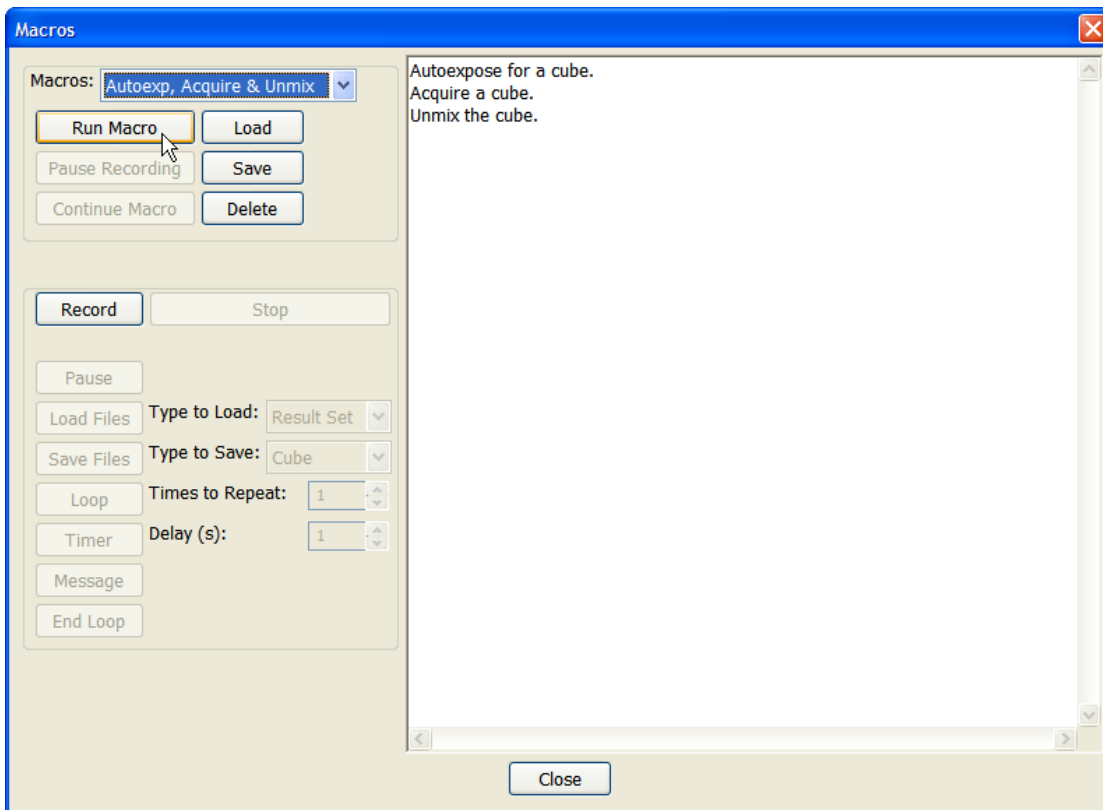
Figure 47: Macros Dialog Box

➢ The **Record** button starts the macro recorder. Once you click **Record**, most keystrokes mouse clicks, and menu selections can be recorded as commands in the new macro.

➢ The **Stop** button stops recording commands and ends the macro.

➢ The **Pause** button places a pause in the macro to allow the user to perform actions that may be required, such as creating a spectral library, acquiring a reference image, and so on. It is recommended to add a **Message** to the macro immediately before a **Pause** so that the user knows what action is required before continuing with the macro. The macro continues once the user clicks the **Continue Macro** button.

➢ The remaining buttons insert commands into the new macro:

➢ The **Load Files** button adds a prompt requesting that the user select a file to load that will be used in some way by subsequent macro commands. Before you click this button, use the **Type to Load** drop down box to select a file type (i.e., cube, result set, protocol, spectral library, image, or composite).

➢ The **Save Files** button adds a prompt for the user specify autosave options for files being generated or edited by the macro. Before clicking this button, use the **Type to Save** drop down box to select a file type (i.e., cube, result set, protocol, spectral library).

➢ The **Loop** button adds a loop command that controls the number of times to repeat the command that follows it. First select the number of **Times to Repeat**, and then click the Loop button. Then add the commands you want to repeat.

➢ The **Timer** button inserts a timed delay into the macro. First select the **Delay** time in seconds, and then click the **Timer** button.

➢ The **Message** button inserts a command to display a message to the user while running the macro. You can enter any text message or insert an HTML formatted file. The macro will present the message and wait for the user to acknowledge it before continuing the macro.

➢ The **End Loop** button terminates the loop cycle if you started a loop earlier in the macro.

9.2   Running Macros

1. Make sure the image is focused and centered in the Live window first, before running a macro that acquires a cube.

2. Select a macro from the **Macros** drop down box on the status bar. Or choose **Macros > Define Macros** from the main menu and select the macro to run.

3. Click the **Run Macro** button. Follow the prompts—if any were created as part of the macro—to proceed through the entire macro until it is finished.

9.3   Recording Macros

1. Choose **Macros > Define Macros** from the main menu to reveal the **Macros** dialog box.

2. Click the **Record** button to start recording commands. You can use the buttons in the dialog box to load files, add command loops, and so on (as described in the Overview of the Macros Dialog Box ⌐89⌐ topic). The recorder also records mouse clicks, menu selections, and other actions while the recorder is running.

3. When you are finished recording, click the **Stop** button. You can then play back the new macro.

9.4   Saving Macros

1. Macros that you create are not saved permanently until you save them. Click the **Save** button. Select a location and enter a filename for the new macro file.

2. The system automatically adds a ".rcd" extension to the filename.

10   Co-Localization Staining Detection

The Nuance system's Co-localization tool is used to find overlapping layers in a dataset and perform multi-analyte immunohistochemical analysis in brightfield and fluorescence. This tool can accurately evaluate nuclear or non-nuclear positivity for multiple antigens and can detect and quantitate co-localization of up to ten markers. It is designed for analyzing and quantitating molecular markers and can be used to determine the amounts of co-localization of multiple markers.

The Nuance system resolves the absorption pattern of co-localized chromogens and generates quantitative images of the individual analytes. The Nuance system also separates fluorophores from each other and from ubiquitous autofluorescence background, allowing more sensitive and quantitative studies.

10.1   Opening a Dataset for Co-localization Analysis

1. Open the cube or result set you want to analyze. (The dataset used in this example is included with the software and can be found at *C:\Nuance Data\Images\Sample Data\ER_PR Datasets\Breast Cancer_ER_PR_OD.im3*.)

2. If you opened a cube, then open a tested and reliable spectral library or protocol and unmix the cube into its component images. (The co-localization tool will only open a result set. It cannot open an image cube that has not been unmixed.)

3. Click the **Co-localization** button on the toolbar (or select **Tools > Co-localization**).

4. The result set opens in the Co-localization dialog box.

    a. The 'Unmixed_Composite' image from the result set displays at the upper-right. The image displays with the default settings, or with the display settings defined in the co-localization settings, if a settings file is loaded. (See Saving and Loading Co-localization Settings 95 .)

    b. The spectral **Components** of the result set are listed at the upper-left. The **Spectral Names** are taken directly from the library and cannot be changed here. The names in the **Marker** column are 'nicknames' for the components. Marker names derive from the spectral names and can be changed to something more descriptive. For example, you might change the 'Red' nickname to 'Fast Red.' Also, if a co-localization settings file is loaded, its name displays in the **Settings** box.

b. The spectral components of the result set are listed here.

c. The thresholding values for each component can be adjusted here.

d. The markers and counter-stains for co-localization can be selected here.

a. The 'Unmixed_Composite' image from the result set displays here.

e. Co-localization percentages for the selected markers and counterstains will display here.

f. Use these buttons to copy and export data and images.

Figure 48: Dataset in the Co-localization Tool

c. The **Settings** tab lists thresholding values for each marker. These are calculated automatically upon loading the result set. If you loaded a co-localization settings file containing saved threshold values, the thresholds from the settings file will be used instead. Use the **Statistics** tab to configure the display of the co-localization percentages, pixel counts, and component statistics. The **Display** tab is similar to the **Display Control** tool that lets you adjust image brightness, contrast, scaling, etc.

d. Use the **Co-localization** options at the lower-left to select which markers to use for co-localization detection and which to use for the counterstain. Visibility options let you adjust the co-localization mask.

e. As you select markers and counterstains, statistics are calculated and displayed in the **Statistics** area. Statistics include the percent of co-localization for the entire image as well as individual markers, the positivity of markers within other markers, and component statistics.

f. Use the **Copy** and/or **Export** buttons when you are ready to save co-localization images and statistics. Images can be exported as JPG or TIFF images, and data can be exported as TXT files. See also <u>Copying Images and Data</u> 99 .

10.2 Adjusting Threshold Mask Values

The **Co-localization** tool is pixel-based, and thresholding determines which pixels are considered positive for each component. Upon loading a result set, the tool autocalculates the optimum threshold value for each marker. However, if a co-localization settings file for the dataset is loaded, the tool will use the thresholds in the settings file.

Because thresholds must be set properly for accurate results, you should visually check the threshold of each marker, as follows:

1. Turn OFF **Visibility** for all but one of the components listed at the top-left. (For the example in the <u>Co-localization Tool</u> 92 figure, the **DAB** and **HEMA** components were turned OFF first.)

2. Now turn the threshold mask for the visible marker ON and OFF a few times to see the pixels that are represented by its mask.

3. Use the threshold slider or scroll box to adjust the threshold of the marker, such that all pixels of individual nuclei or membranes are represented by the mask.

Setting a threshold requires operator judgment, since this setting determines which intensities are included in the analysis. Each threshold mask should cover only the cells for its component, and individual nuclei or membranes should be identified in the mask as individual constituents. This ensures an accurate measure of the desired pixels in each channel. If threshold pixels are present between nuclei or between membranes, this usually means the threshold is set too low.

4. Repeat this procedure for the other marker(s), paying close attention to the cells and pixels that contain only the particular chromogen for that marker.

5. Also, note the **Minimum Pixels** value. This restricts the threshold mask to groups of pixels that are larger than this value. For this sample dataset, the default value of 20 was too large; there are smaller groups of pixels that should be included. Therefore, this value was changed to 10 for all three component markers.

10.3 Selecting Markers for Co-localization

The dataset's components are listed in two columns at the lower-left, in the **Co-localization** group. As you select markers and counterstains, you will see the **Statistics** area reflect the calculated percentages. See figure below.

1. In the **Markers for Co-localization** column on the left, select the markers for which you want to see overlap (co-localization) and their positivity percentages. (In this example, **Red** and **DAB** are selected as co-localization markers.)

2. In the **Denominator(Counterstain)** column, select the marker to use as the counterstain. If you want to see co-localization of markers throughout the entire dataset, and not just within a particular counterstain, then you would select **All Image Pixels**. (In this example, **Hema** was selected as the counterstain.)

3. The **Co-loc. Label** name defaults to 'Overlap' and can be changed if desired. This is the name given to the co-localization values when they are displayed in the component statistics and when the data is exported.

4. Clicking the **Show Co-loc.** button quickly turns off all threshold masks in the component image and turns on only the co-localization mask. Clicking the **Show All** button quickly turns on all threshold masks that participate in the overlap as well as the co-localization mask.

5. You can select any **Mask Color** from the pull down color menu.

6. The **Visibility** eye turns the co-localization mask visibility on or off. In the figure below, all threshold masks (except 'Autofluor') and co-localization masks are turned on.
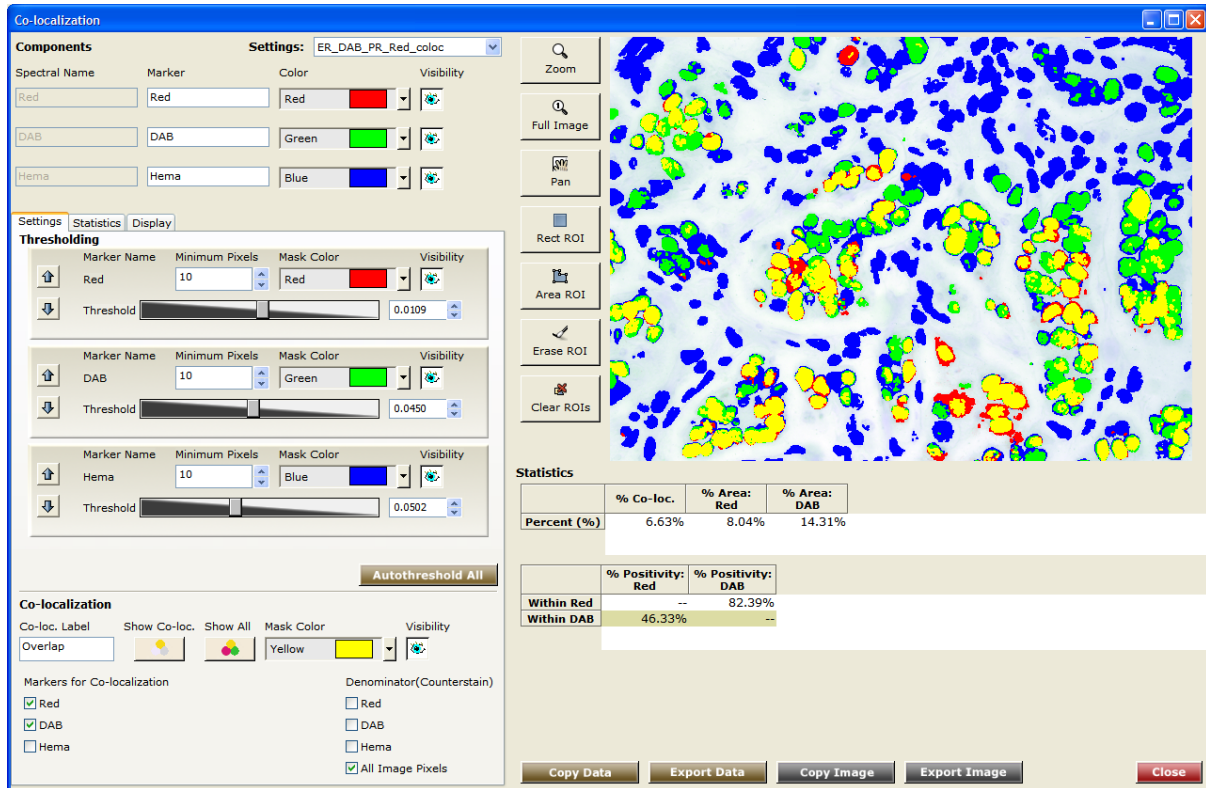
Figure 49: Co-localization & positivity percentages

10.4 Saving and Loading Co-localization Settings

When you want to save the current co-localization display and configuration settings, save a Co-localization Settings file. This file will include Component settings, Thresholding settings and values, Co-localization Marker selections, and Statistics and Display options. If a settings file is loaded already, you can overwrite the existing file or save a new one. (Co-localization settings are not saved in Nuance Protocols.)

1. Leave the **Co-localization** window open and select **File > Save Co-localization** from the main menu.

2. Name the settings file and click **Save**. You can now open and run co-localization detection on any number of compatible result sets using this settings file.

Loading Co-localization Settings

You can load a saved settings file before or after opening a dataset in the Co-localization tool.

1. Select **File > Open Co-localization** from the main menu.

2. Select the settings file you want to load and click **Open**.

3. The selected settings file displays in the **Settings** box at the top of the Co-localization tool.

Co-localization settings must be compatible:

Before the Co-localization tool loads a settings file, it checks to see if the settings file is compatible with the result set (if one is loaded). Both must have the same number of spectra.

If the result set does not have the same number of spectra as the settings file, you will be notified that *"The current result set is incompatible with the selected co-localization settings."* Load a matching settings file or result set, or create a new settings file for the current result set.

However, if the *number* of spectra in the result set matches the number of spectra in the settings file you want to load, the tool is able to load the file even if its spectra are not identical. In this case, you will have to use the spectra in the result set. This will reset the component names to the spectra names, but their nicknames will remain unchanged.

10.5 Interpreting the Statistics

Note the following in the **Statistics** area:

Figure 50: Co-localization Statistics Display

a. The percent of Red and DAB Co-localization in this dataset is 20.64%. This means that out of all the Hematoxylin counterstain signal (selected as the Denominator), 20.64% of those pixels have Red and DAB co-localization. The number of pixels within the Hematoxylin counterstain that are also co-localized with the Red and DAB markers is 93,535.

b. The percent positivity of Red labeled pixels within the Hematoxylin counterstain is 23.47% (106,362 pixels).

c. The percent positivity of DAB labeled pixels within the Hematoxylin counterstain is 44.01% (199,413 pixels).

d. Of the 203,943 DAB labeled pixels, 46.33% of them are also Red positive.

e. Of the 114,673 Red labeled pixels, 82.39% of them are also DAB positive

f. The **Component Statistics** rows display for all components that you selected on the **Statistics** tab (at the left in the **Co-localization** dialog box).

- ➢ Every component has an **Overlap** row that displays the area and signal statistics of the overlap (co-localization) for each component.

- ➢ There is also a **Full Image** ROI for every component. If there are no ROIs drawn on the image, the default ROI is the full image. If you draw ROIs (see Drawing Regions of Interest 97), a new row will be added to the **Component Statistics** area for each ROI. The system automatically numbers and labels ROI's to distinguish them in the image window and in the statistics table.

10.6 Customizing the Statistics Display

Click the **Statistics** tab. Here you can turn on or off the **Percentage** and **Pixel Count** displays for each statistical category. You can also select to display or hide the Component Statistics. This figure shows all of the statistics options turned on.
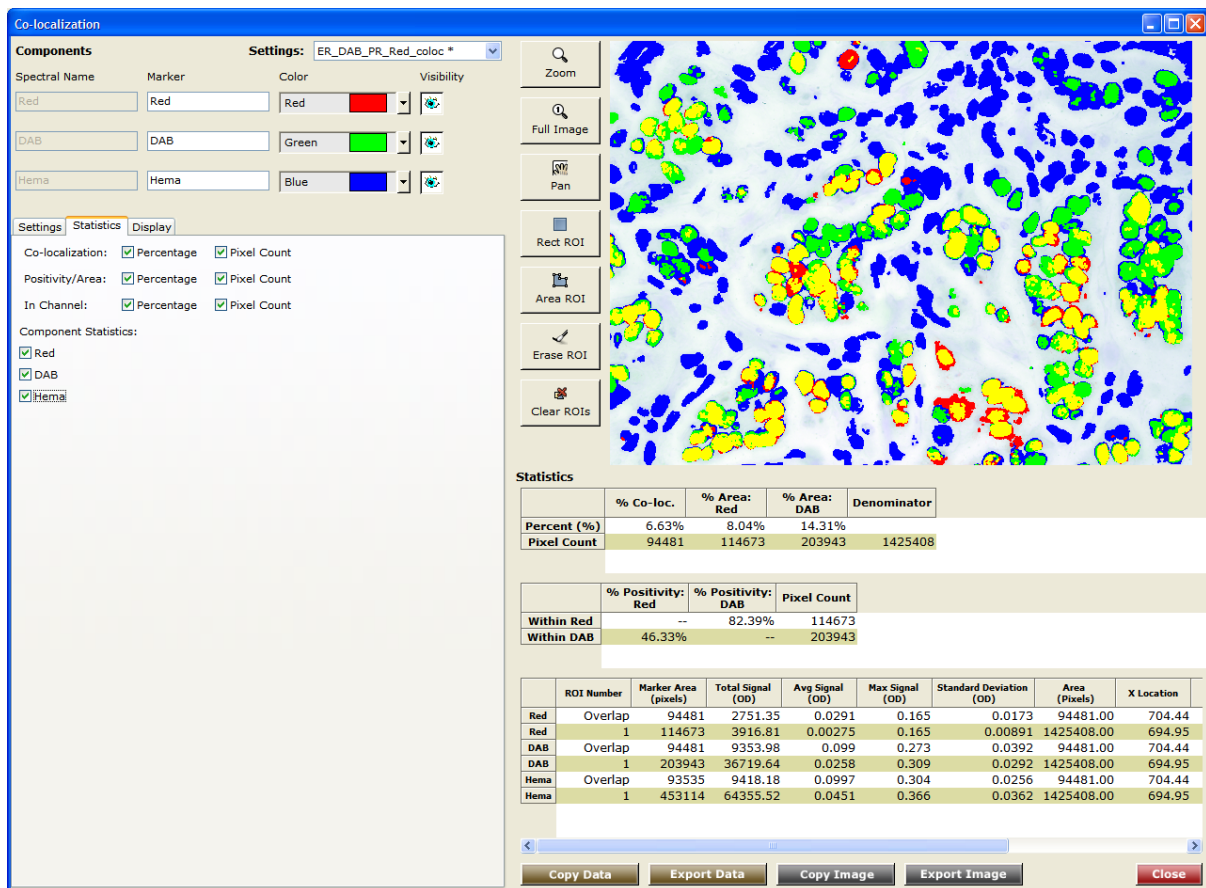
Figure 51: Co-localization Statistics

10.7 Drawing Regions of Interest

If you want to select a specific area of the image to process, click an **ROI** button at the left of the image and use the mouse to click-draw region(s) of interest. Note that once an ROI is added to the image, the **All Image Pixels** option in the **Denominator (Counterstain)** list becomes **All ROI Pixels**. The statistics are updated automatically as you draw and modify ROIs. Additional buttons let you erase an ROI and clear all of the ROIs. You can also zoom and pan the image.

Cloning, Moving, Deleting ROIs

Right-click on the region within the image and select the desired action from the drop down menu.

Applying Regions from a Saved ROI File

You can apply regions from a saved ROI file to the composite image. (Working with regions is described in more detail in the [Quantifying Results](#) [75] topic.) Right-click on the image and choose **Load User Regions** from the drop down menu. Select an ROI file from the dialog box.

Saving Regions

To save the current regions, right-click on the composite image and choose **Save User Regions**. Enter an ROI file name, select a location, and click **Save**.

10.8 Customizing the Composite Image Display

Click the **Display** tab to view the display controls:

➢ To view images in fluorescence or brightfield, use the **Composite Coloring Style** options. In general, you want to select the mode under which the original image cube was acquired.

➢ Use the **Display Adjustments** and **Component** selectors to choose whether you want to adjust the display of the whole image or a selected layer.

➢ Use the **Brightness** and **Contrast** sliders to adjust the brightness and contrast of the image, or of the selected layer if **Single Layer** was selected.

➢ The **Min. Max.** option maps the minimum value in the image to 0, the maximum value to 255, and linearly interpolates in between those values. This stretches dark signals so they become visible.

➢ The **Clip/Stretch** option is the default display scale. It maps the lowest 0.01% of the pixels to 0, the highest 0.01% to 255, and linearly interpolates in between those values. This prevents a few bright or saturated pixels from skewing the display.

➢ The **Histogram Eq.** option maps the pixels so the histogram of the pixels have approximately the same number of pixels in each bin. This gives the best display of the whole dynamic range of dim and bright signals.

➢ The **Raw** option applies no scaling to the data.

➢ The **Abundance** option scales all pixels in the composite image by the ratio of 255 and the value of the single brightest pixel among all the components. This usually results in a brighter composite image.

➢ Regions are numbered according to size, from largest to smallest. Check **Show Region Labels** if you want region numbers to appear inside each region.

➢ The **Show Markers and Co-localization Masks** option lets you quickly turn on or off the mask settings you selected on the **Settings** panel.

10.9 Copying Images and Data

Copying Images

To copy the current image *as displayed* to the Windows clipboard, click the **Copy Image** button. Paste the image into any software program capable of displaying graphic images.

Copying Data

To copy all of the displayed data *in tab delimited format* to the Windows clipboard, click the **Copy Data** button. Paste the data into any spreadsheet program such as Microsoft Excel.

10.10 Exporting Images and Data

Exporting Images

To export the current image *as displayed* as a JPG or TIFF RGB file, click the **Export Image** button. In the **Choose RGB File** dialog box, enter a file name and location for the image and click **Save**.

Exporting Data

To export all displayed data *in tab delimited format* as a TXT file, click the **Export Data** button. In the **Choose Measurement File** dialog box, enter a file name and location for the file and click **Save**.

11 FAQs

What is the CCD sensor active area dimensions and the individual pixel size?

The active light sensitive area of the CCD is 1392 pixels in the horizontal direction and 1040 pixels in the vertical direction. Each pixel is 6.45 µm square.

What is a C-mount adapter and why do I need a 1x version for my microscope?

Most microscopes can be equipped with an industry standard 1x (no magnification or demagnification) camera adapter with a male C-mount on one side and a microscope camera-tube connector (usually called a 'dovetail' because the cross-section of the connector is beveled) on the other side.

Since the Nuance imaging module contains a 0.65x demagnifying optical coupler and 2/3-inch CCD, a 1x adapter is needed on the microscope camera port in order to prevent vignetting-darkening or loss of part of the image in the corners and sides.

Nuance systems are designed with a female C-mount to attach ONLY to a male C-mount microscope camera port. If you have an older microscope with an F-mount or ENG mount, contact your microscope reseller to see if your microscope can be upgraded to accept a C-mount camera. Because of the Nuance imaging module's internal 0.65x optical system, you must utilize a 1x C-mount camera adapter on your microscope.

Can I use non-apochromatic or achromatic lenses for brightfield microscopy using the Nuance system?

When using relatively inexpensive achromatic objectives, images acquired below 450 nm (the deep blue part of the visible spectrum) may suffer from a loss of sharpness and/or a shift in focus. We have not found that there is a dramatic improvement in brightfield unmixing results when using expensive apochromatic objectives. We recommend trying a variety of objectives that you own, and determining your own level of satisfaction with the processed images.

Can I use my own C-mount lenses on a Nuance system for macro-imaging applications?

The Nuance multispectral imaging system performs best with its own high-quality lenses. While there are many C-mount lenses on the market, you must beware of those designed for simple color (RGB) imaging. They are often not designed for high-quality multispectral imaging. Contact PerkinElmer or your authorized PerkinElmer distributor to discuss lens selection for your wavelength range of interest.

PerkinElmer sells a Nikon 60 mm micro-Nikkor lens with an optimized F-to-C mount adapter (with field-flattening lens element), as well as PerkinElmer-labeled lenses for visible imaging and PerkinElmer-labeled lenses for NIR imaging. Other lenses may also be usable. We have not found other third-party lenses that produce an undistorted image with respect to flatness of field, and also function appropriately in the spectral realm. PerkinElmer recommends trying a variety of lenses to determine which is acceptable in your particular application.

Will high numerical aperture (NA) lenses (e.g. 40x 1.4 NA) work better on a Nuance system?

Yes, especially if the light level is low and the lens is fully color-corrected (apochromatic). The numerical aperture of a lens defines its ability to capture light (i.e., the higher the NA, the greater the ability to capture light). For dimly emitting fluorescence, one goal is to capture the maximum

amount of light. When more light is able to travel through the lens, shorter exposure times and faster acquisition times can be achieved.

The ideal combination is a high N.A. lens, at the lowest magnification possible for the task at hand. The rationale for lower magnification is due to the decreased brightness per object as the magnification increases.

⊟ What can you tell me about the polarization sensitivity of the Nuance optical system?

The Nuance optical system incorporates a linear polarizer in the light path when the tunable wavelength element is in place (the "MSI" setting; not the "Mono" setting), which can conflict with other polarizing elements in the microscope or lens system, greatly reducing the light throughput.

Do not use a linear polarizer with the Nuance system unless necessary. If you must use one (for DIC imaging, for example), make sure that the last polarizer in the microscope is aligned with that of the Nuance for maximum light throughput.

⊟ Why do I need to use a longpass emission filter with the Nuance system to do fluorescence imaging?

Modern fluorescence microscopes incorporate two narrow filters, as well as a dichroic mirror. This combination is selected to provide narrow wavelength excitation light, and then exclude the majority of the excitation light from the fluorescence emission. The dichroic mirror is matched to both the excitation filter and the emission filter to improve the performance of each.

For the Nuance system, it is desirable to see as much of the emission spectrum as possible, not just what might be the peak transmission wavelength. So, it is advantageous if the narrowband emission filter is replaced with a longpass filter.

This effectively suppresses the excitation light, but passes a much larger percentage of the emitted light, including the autofluorescence. The Nuance system then can not only remove the autofluorescence, but also identify multiple fluorescence emissions. If a longpass emission filter is not used, the excitation light may bleed through the Nuance tunable filter system and cause artifacts and poor performance.

⊟ Why can't I just use my standard (emission) filters, collect the images at each of the peak transmission wavelengths, and then run the images through your software? Won't I get more light, especially at wavelengths were I am dealing with dim samples?

The Nuance system doesn't just take images at the so-called peak emission wavelengths. The predicted (or published) peak emission wavelengths may vary according to other variables, including chemistry. In many situations, such as autofluorescence, the peaks of two signals may be very close to one another, but their spectral curves may differ in shape. Simply having more light with a limited number of wavelengths, in the case of overlapping signals, won't help.

⊟ I am about to install an upgrade to the Nuance software onto my computer. Should I take any precautions in case I want to go back to the previous version of the software?

In general, updating the Nuance software can be accomplished by simply running the installer for the new version. In some cases, usually with legacy or Beta (test) software, you must remove the old version of the software before running the installer for the new version of the software. To install the new version of the Nuance software, see the separate *"System Setup & Installation"* guide.

12   Troubleshooting

☐ **At high magnifications, I sometimes see a texture in my images, similar to what one might see looking through a shower curtain. This appearance could also be described as an "orange peel" texture. What is this phenomenon?**

At 60x or higher magnifications, shadows from the multi-layer liquid crystal tunable filter (LCTF) in the light path may become visible. You can effectively eliminate it by white-cube correcting in brightfield imaging. PerkinElmer presently is working on methods to automatically correct for this effect.

☐ **How can I verify that the Nuance Imaging System is working correctly?**

To verify that the Nuance imaging system is working correctly, take some images of a brightfield stained microscope slide and a fluorescence microscope slide. Dual-stained brightfield microscope slides and quantum dots show up well. You can use these acquired images to test the Nuance software's unmixing feature.

☐ **When doing brightfield microscopy, the background appears yellowish. How do I get white backgrounds?**

Turn up the light all the way and use a Neutral Density (ND) filter to bring exposure times to around 30 to 50 ms. A blue-green correction filter, as used for color photography, is recommended for tungsten-halogen illumination systems. This filter corrects for the red-biased light that our eyes—but not digital imaging systems— automatically correct for.

☐ **When unmixing a cube, how can I get the background signal to disappear in the unmixed composite image?**

For brightfield image cubes, unmix into white any signal (such as the background) you want to hide. For fluorescence images, unmix into black the signal you want to hide.

☐ **How do I go back to a previous version of the software?**

If you created a Restore Point before you installed a new version of the Nuance software, returning to the old version of the software merely involves restoring your computer to the earlier time, before you installed the latest version of the software. If you did not create a Restore Point, you should remove the new version of the Nuance software using "Add/Remove Programs" and then install the old software from the original CD-ROM or downloaded installation file.

☐ **What should I do if the camera or hardware is not detected?**

Power OFF the computer and disconnect the Nuance module's power cable. Next, unplug the Nuance's USB cable from the computer. Reboot the computer, and then reconnect the Nuance module's power cable. After both systems finish rebooting, plug the USB cable back into a USB port, preferably a different port than last time.

If this does not resolve the problem, please contact PerkinElmer at global. techsupport@perkinelmer.com.

☐ **Why does the image look different on the screen than through the eyepiece?**

The Enhance Contrast option may be turned on in the **Display Control** tab, which will affect the

view of the image cube. Also, the **Wavelength to RGB Map** may be switched from the default *Visual RGB* to *Equal RGB*. Visual RGB maps the cube wavelengths to mimic the wavelength response of the human eye; Equal RGB maps the wavelengths equally. Refer to Adjusting a Cube's RGB Mapping 84 .

13   System Specifications & Dimensions

The specifications given in the following topics represent typical Nuance multispectral imaging systems as of September 2010 and are subject to change.

Liquid Crystal Tunable Filter

Parameter	Specification
Wavelength tuning range	**VX/FX:** 420 - 720 nm **EX:** 450 - 950 nm
Bandwidth flexibility, FWHM	**VX:** 7, 10, or 20 nm **FX:** ~20 and ~40 nm (software selectable) **EX:** ~20 and ~40 nm (software selectable)
Wavelength accuracy	Bandwidth/8
Typical acquisition time	Approximately 5 seconds

> **Note:** Acquisition time depends on a number of factors, including the bandwidth of the Nuance system and the amount of light emitted, reflecting off, or transmitted through the specimen at each wavelength of interest. The Nuance system is designed to dwell at individual wavelengths if it determines that more light is needed to produce images with adequate spatial and spectral detail. The system is not limited by a need to acquire images using identical exposure times at each wavelength. The benchmark performance standard is the acquisition of a 16-wavelength stack from a typical brightfield specimen within 5 seconds using 8-bit mode and 2x2 pixel binning, light permitting.

CCD Camera

Parameter	Specification
Cooling	TE cooled to 8 °C
Image sensor format	2/3" diagonal
Effective pixels	1392 x 1040 pixels
Image sensor pixel count	1.4 megapixels
Optical interface	C-mount (female) 1X
Mounting method	US ¼-20 tripod mount
Optical input (maximum)	500 mW/cm^2

Imaging Module

Parameter	Specification
Dimensions	3 ½" high x 5 ¼" wide x 8 ¾" long
Weight[2]	4 lbs 10 oz (2 kg)
Center of C-mount	1½" from center of C-mount to lower surface with standard tripod mount
Filter sets[3]	See the Filter Selection Guide 107

[2] Does not include workstation computer.

[3] Filter sets must be purchased separately. Contact PerkinElmer or your authorized local distributor for pricing and availability.

Environmental

Parameter	Specification
Operating environment	Indoor
Operating temperature	15 °C to 40 °C
Operating humidity	65% maximum, non-condensing
Operating altitude (maximum)	2000 meters
Storage temperature	-10 °C to 55 °C
Storage humidity	100% maximum, non-condensing
Pollution degree	2

Utilities

Parameter	Specification
Electrical power	+5V Universal[4]

[4] A set of wall outlet plugs may be provided for common US and International wall outlet types. The electrical supply itself is a switching-type for 100-230 VAC.

Computer System Requirements

Parameter	Specification
Processor	3.0 GHz or higher Intel™ Pentium™ 4 class or Intel Centrino™
RAM (minimum)	2 GB
Hard disk drive (minimum)	160 GB
CD-ROM drive	writable CD-ROM drive
Operating system	Included: Microsoft® Windows 7 (English Language Version) 32-bit operating system (64-bit OS is supported as well; the Nuance 3 software will run as a 32-bit application) Supported: Windows® XP Professional (US)
Display (minimum)	24-bit color, 1280 by 1024 pixels
Computer interface	One available USB 2.0 interface

Using Third-Party Computers with the Nuance Software

If using your own computer (one not purchased from PerkinElmer) to run the Nuance system or to process Nuance data offline, consider the following parameters for optimum performance:

➢ The faster the computer CPU, the faster certain operations in software will be accomplished.

➢ More hard drive space allows more and larger datasets to be acquired and stored.

➢ At least 2 GB of RAM is recommended for data processing.

➢ A USB 2.0 port is required (if you want to connect to the Nuance system).

➢ The US English versions of Windows® 7 and Windows XP Professional are the only supported operating systems.

14 Filter Selection Guide

Color denotes usable longpass filters, **X** denotes the recommended longpass filter.

Fluorophore			Nuance Longpass Emission Filters				
Name	Excitation (nm)	Emission (nm)	420 LP	475 LP	515 LP	590 LP	610 LP
525 nm QDot®	<500	525	(purple)	**X**			
545 nm QDot®	<520	545	(purple)	**X**	(blue)		
565 nm QDot®	<540	565	(purple)	(light blue)	**X**		
605 nm QDot®	<580	605	(purple)	(light blue)	**X**		
655 mn QDot®	<620	655	(purple)	(light blue)	(blue)	**X**	
705 nm QDot®	<650	705	(purple)	(light blue)	(blue)	(green)	**X**
800 nm QDot®	<750	800	(purple)	(light blue)	(blue)	(green)	**X**
Alexa Fluor® 350	347	442	**X**				
Alexa Fluor® 488	492	520			(blue)		
Alexa Fluor® 500	500	530			(blue)		
Alexa Fluor® 514	514	545			(blue)		
Alexa Fluor® 532	532	554			(blue)		
Alexa Fluor® 546	556	573			(blue)	**X**	
Alexa Fluor® 555	555	580			(blue)	**X**	
Alexa Fluor® 568	577	603				**X**	(yellow)
Alexa Fluor® 594	590	618				(green)	**X**
Alexa Fluor® 610	612	628					**X**
Alexa Fluor® 633	632	650					**X**
Alexa Fluor® 647	647	666					**X**
DAPI	358	460	**X**	(light blue)			
Hoecsht	352	450	**X**	(light blue)			
AMCA	350	430	**X**				
Cy 2™	498	506		**X**	(blue)		
Cy 3™	554	568			**X**		
Cy 5™	649	666					**X**
Cy 5.5™	675	695					**X**
GFP	475	509		(light blue)	(blue)		
FITC	490	525			**X**		
eGFP	498	515			**X**		
YFP	520	532			**X**		
Rhodamine	550	573				**X**	
TRITC	555	580				**X**	
RFP (dsRed)	558	583				**X**	
Texas Red	595	615				(green)	**X**
SYTOX® Blue	445	470		**X**			
SYTOX® Green	505	532			**X**		
SYTOX® Orange	570	547				**X**	

QDot, Alexa Fluor, and SYTOX are registered trademarks of Invitrogen, Inc.
Cyanine dye (Cy 2, Cy 3, Cy 5 and Cy 5.5) is a trademark of Amersham Biosciences.

15 MetaMorph Support

Support for MetaMorph in Nuance lets MetaMorph users perform their scripted work using PerkinElmer's Nuance camera with or without the Nuance software. Note the following examples:

1. Use MetaMorph alone to operate the microscope and the PerkinElmer Nuance camera (Nuance software is not running).

   ➢ When not using the Nuance software, the camera's tunable filter can be moved out of the optical path (by sliding the thumbswitch on the front of the camera to the **MONO** position).

   ➢ The PerkinElmer Nuance camera is used just like other scientific digital cameras. You can select 8-bit or 12-bit acquisition mode by choosing **Acquire > Select Acquisition Channel** from the menu in MetaMorph. For most fluorescence imaging applications, the 12-bit acquisition mode will generate better quality images.

   ➢ The MetaMorph software controls the microscope (can move the stage position, autofocus the image, etc.) and uses the Nuance camera to acquire fluorescence images.

   ➢ You can adjust the camera gain, offset, and cooler function from within MetaMorph. Choose **Acquire > Settings** from the menu. Click on the **Special** tab. Adjust the **Gain** and **Offset** values as needed for your application. The gain and offset values selected here are specific to MetaMorph; they will not affect the Nuance software/camera interaction. Note also that the **Cooler** can be turned off, but this can degrade image quality. Leave the cooler on.

2. Use MetaMorph and Nuance software interactively. Both applications share the PerkinElmer Nuance camera.

   ➢ Mostly used for multispectral imaging and unmixing using MetaMorph journals.

   ➢ MetaMorph software has access to the camera via the journal system. For example, MetaMorph can start a live image stream, move the microscope stage to the desired location, autofocus the image, and freeze the live stream. MetaMorph can then give control of the camera to the Nuance software to acquire an image or a multispectral image cube.

   ➢ Acquired images can be processed in MetaMorph, and cubes can be unmixed in Nuance prior to further processing in MetaMorph.

   ➢ The Nuance journal taskbar is accessed by choosing **Journal > Taskbar > Load Taskbar** from the menu. The figure below shows an example of the Nuance taskbar. Instructions on how to install the journals are provided later in this topic.

   ➢ Use the journals on the Nuance taskbar to perform actions such as **Start Live** (start the live Nuance image), **Load Protocol** (load a Nuance protocol), and **Take Cube** (acquire a multispectral image cube). You can also use the Nuance taskbar journals as individual commands in your own MetaMorph journals: Choose **Journal > Start Recording** and click the taskbar commands in the order you want them to occur. (Remember to stop recording when finished.) Finally, you can open and examine the Nuance journals from within the **Journal Editor** to help you customize your own MetaMorph journals.
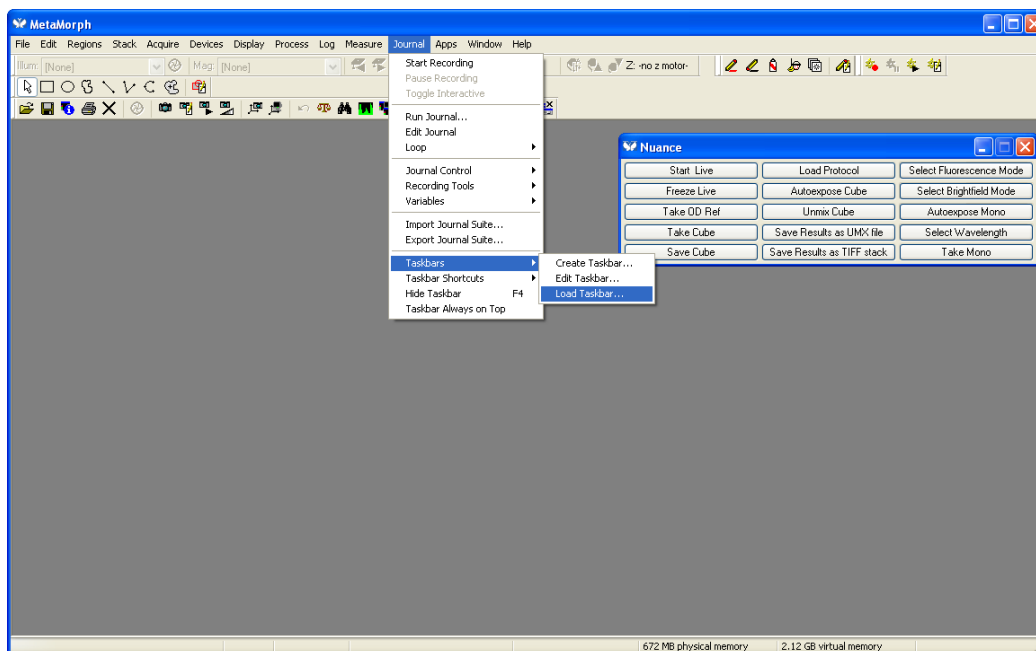
Figure 52: Nuance Journal Taskbar

3. Use Nuance software as usual (MetaMorph software is not running). Installation of *MetaMorph Support* in the Nuance software does not effect its independent operation of the PerkinElmer Nuance camera.

15.1 Installing and Configuring MetaMorph

Software Requirements

➢ Requires MetaMorph 7.5.4 or later

➢ Requires Nuance 2.8 or later

Both Nuance and Metamorph must be run as an administrator.

Purchasing the Software

➢ PerkinElmer is an authorized distributor of MetaMorph software: Nuance customers who would like to purchase the MetaMorph software with Nuance support can do so directly through PerkinElmer.

➢ Existing MetaMorph customers who would like to upgrade to Nuance support may contact either Molecular Devices or PerkinElmer to purchase the required Nuance drivers.

Installing and Configuring MetaMorph

1. Before installing the Nuance software, make sure the MetaMorph software is installed. If an earlier version of MetaMorph is installed, obtain and install the necessary updates to achieve version 7.5.4.

2. When installation (and any necessary updates) is complete, open the **Meta Imaging Series Administrator** tool from the **MetaMorph** application group on the Windows desktop.

3. Click the **Drop-ins/Toolbars** button.
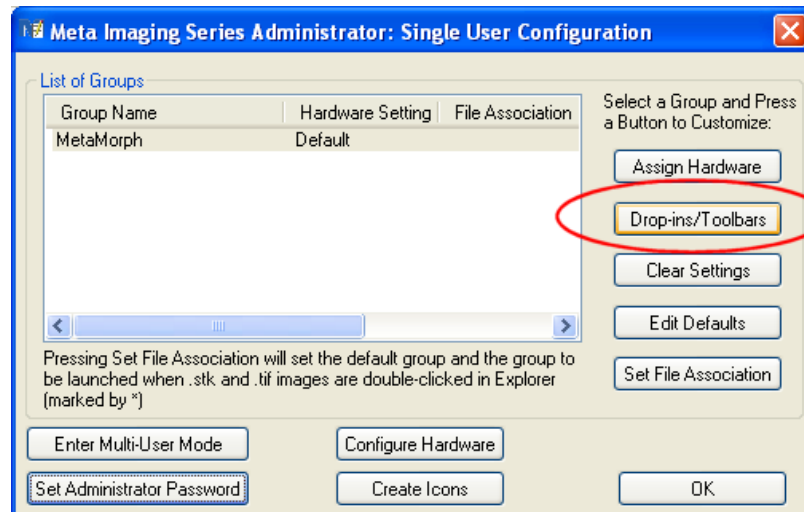
Figure 53: Meta Imaging Series Administrator

4. Make sure a check mark appears next to the **acquire** and **applink** drop-ins.
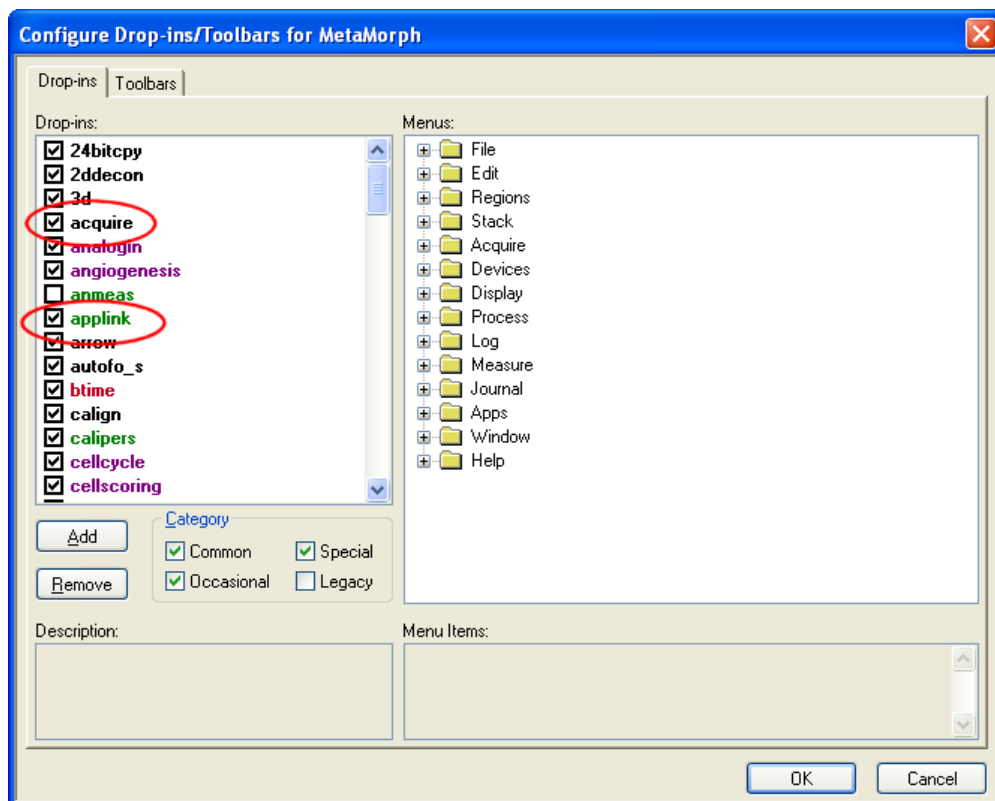
Figure 54: Drop-ins/Toolbars

5. Click **OK** to save the changes.

6. Now click the **Configure Hardware** button.
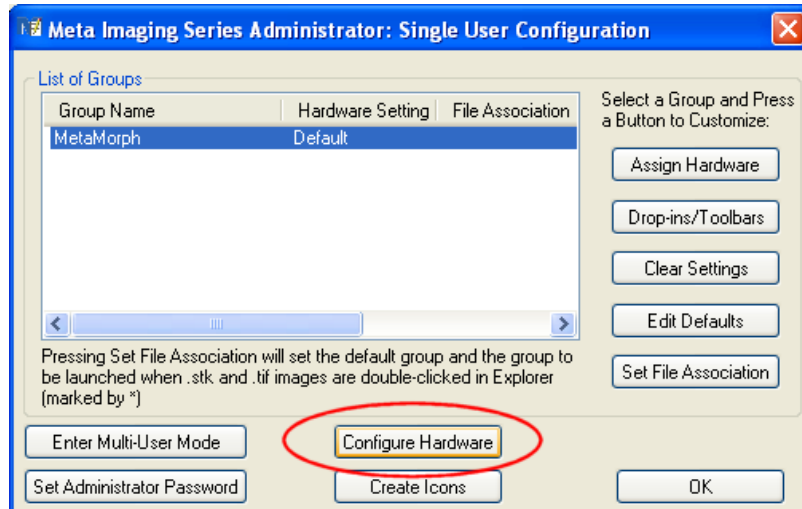
Figure 55: Meta Imaging Series Administrator

7. Click the **Configure Acquisition** button.
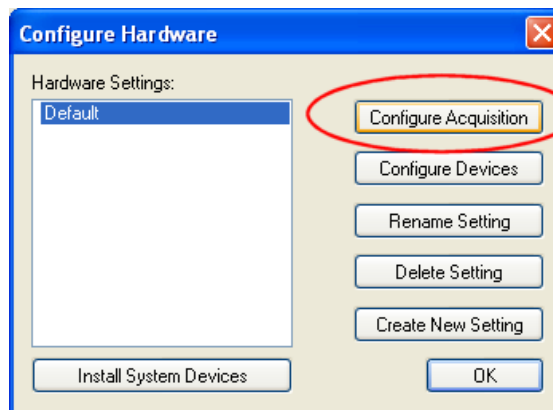
Figure 56: Configure Hardware

8. The Nuance driver must be moved to the list of installed drivers in order to enable the MetaMorph support option in the Nuance software installation wizard. Proceed as follows: In the **Configure Acquisition** dialog box, select **CRi Nuance Demonstration** in the **Available Drivers** list and **Add>>** them to the **Installed Drivers** list.
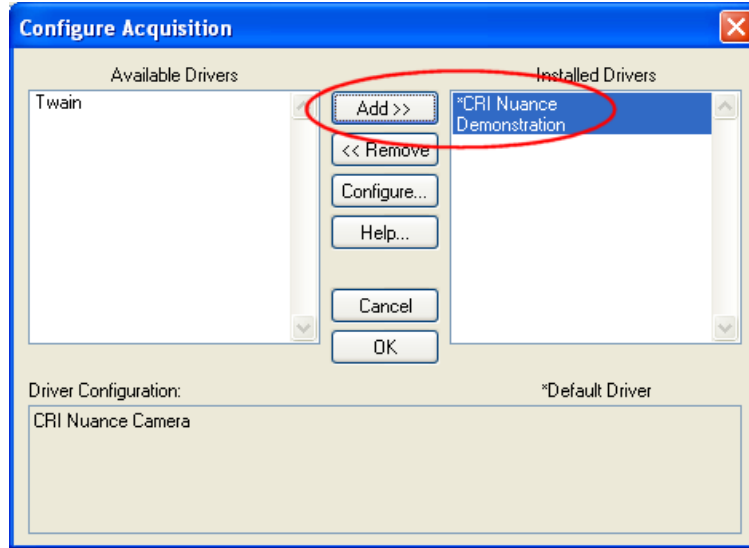
Figure 57: Configure Acquisition

9. Click **OK** to save the changes.

15.2 Installing Nuance with MetaMorph Support

1. Make sure the Nuance camera is turned off and disconnected from the computer. Also, remove the MetaMorph software license key from the computer's USB port.

2. Insert the Nuance software install CD. If the Installation Wizard does not start automatically, open a **My Computer** window and explore the CD drive. Double-click the **Install.exe** file to start the wizard.

3. Follow the wizard prompts to install the software. At the **MetaMorph Support** dialog, select *"I wish to install MetaMorph support"*.
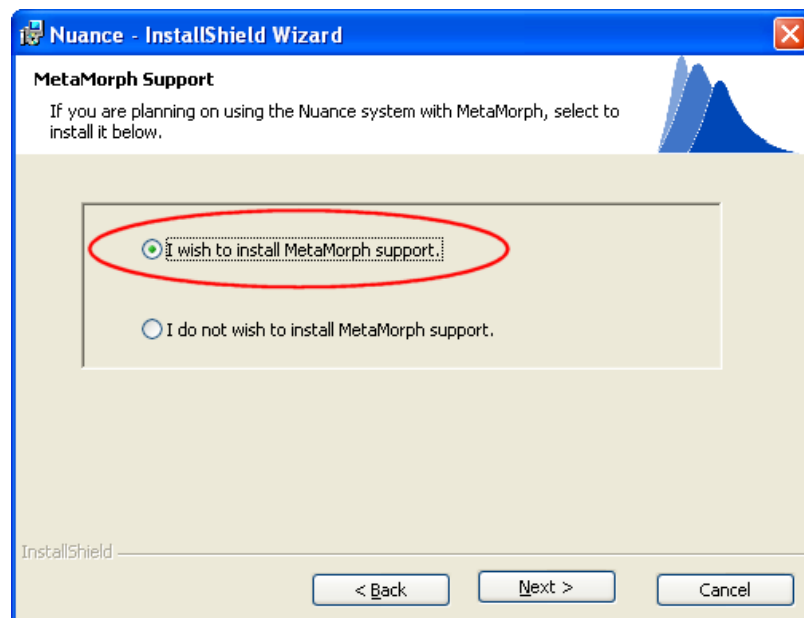
Figure 58: CRi Nuance Installer – MetaMorph Support

4. Click **Next** to proceed through the rest of the installer.

5. After installing the software, reconnect the Nuance camera to a USB port. Then re-connect the camera to power.

6. Open the Nuance software. Verify that the camera has initialized properly by checking for a live image in the **Live Image** window.

7. Close the Nuance software, but leave the camera ON.

15.3 Import the Journal Suite for MetaMorph

1. Plug the MetaMorph license key into a USB port on the computer. Launch the MetaMorph software.

2. If no camera is connected, MetaMorph will alert you that the software may not be fully functional. Accept these messages to continue with launching the software anyway.

3. Select **Journal > Import Journal Suite** from the MetaMorph main menu.

4. Click the **Select Journal Suite** button.
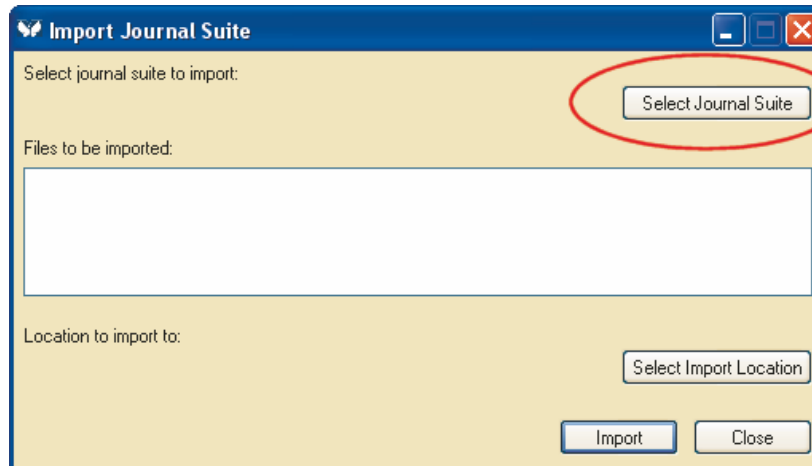
Figure 59: Select Journal Suite to Import

5. Navigate to **C:\MM\CRi\Nuance**. Look for the **NuanceSupport.jzp** file. If the file is not found, look in **C:\Program Files\PerkinElmer\Nuance x.y.z\MetaMorph support** (where **x.y.z.** is the latest installation of Nuance).

6. Select the **NuanceSupport.jzp** file and click **Open**.
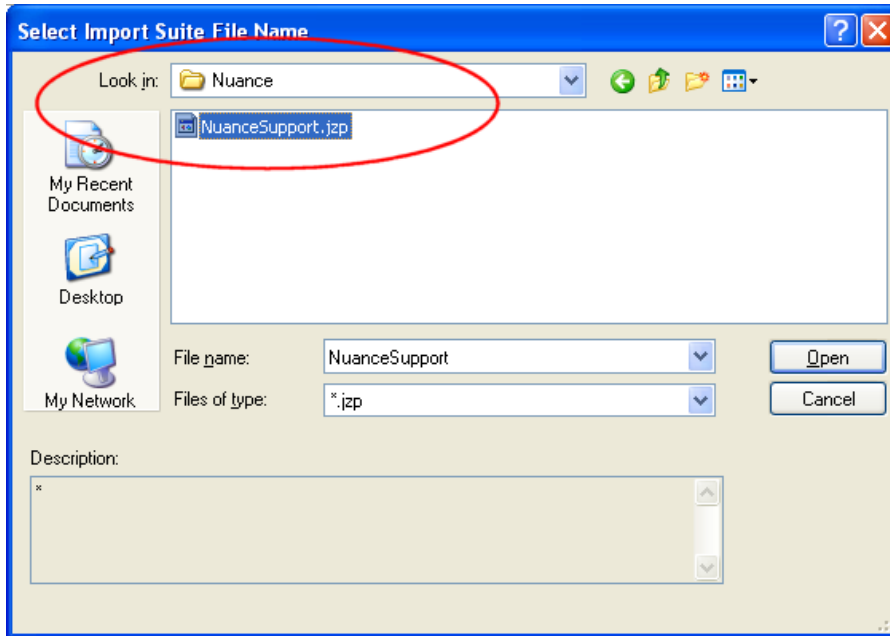
Figure 60: Select Import Suite File

7. **NuanceSupport.jzp** should appear in the **Files to be imported** list. Click the **Select Import Location** button.
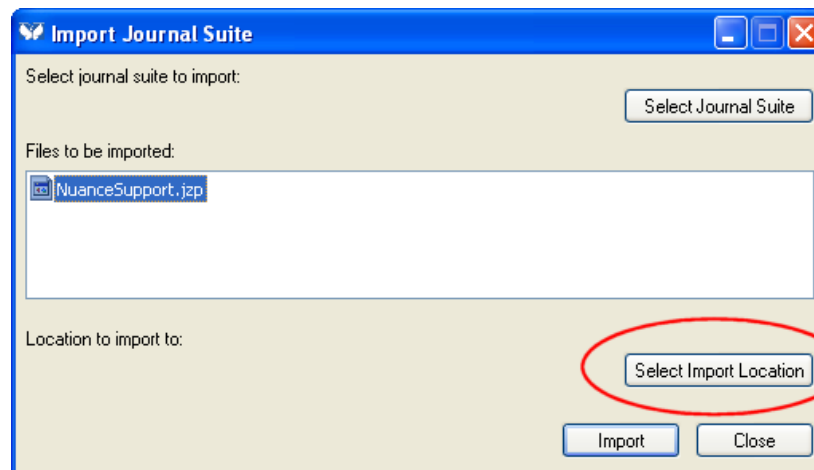
Figure 61: Select Import Location

8. Navigate to the location where your MetaMorph journal files are stored. Suggestion: **...\app\mmproc** within your MetaMorph folder. Select the **journals** folder.
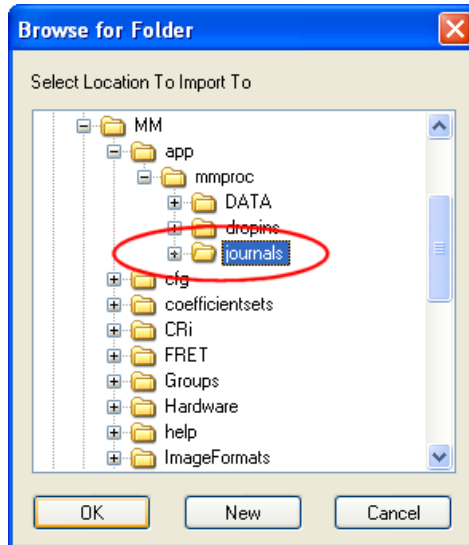
Figure 62: Browse for a Folder

9. Click **OK** to save and return to the **Import Journal Suite** dialog.
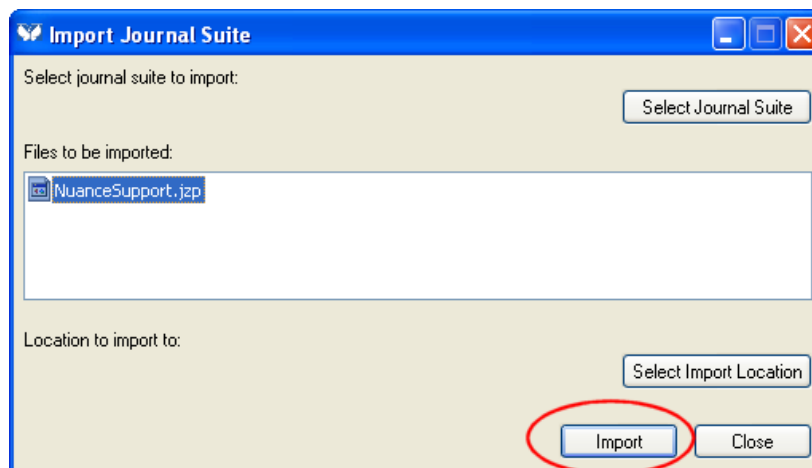
Figure 63: Import Journal Suite

10. Click the **Import** button to import and extract the Nua**nceSupport.jzp** file.

11. The individual journal files will be extracted to the selected folder.
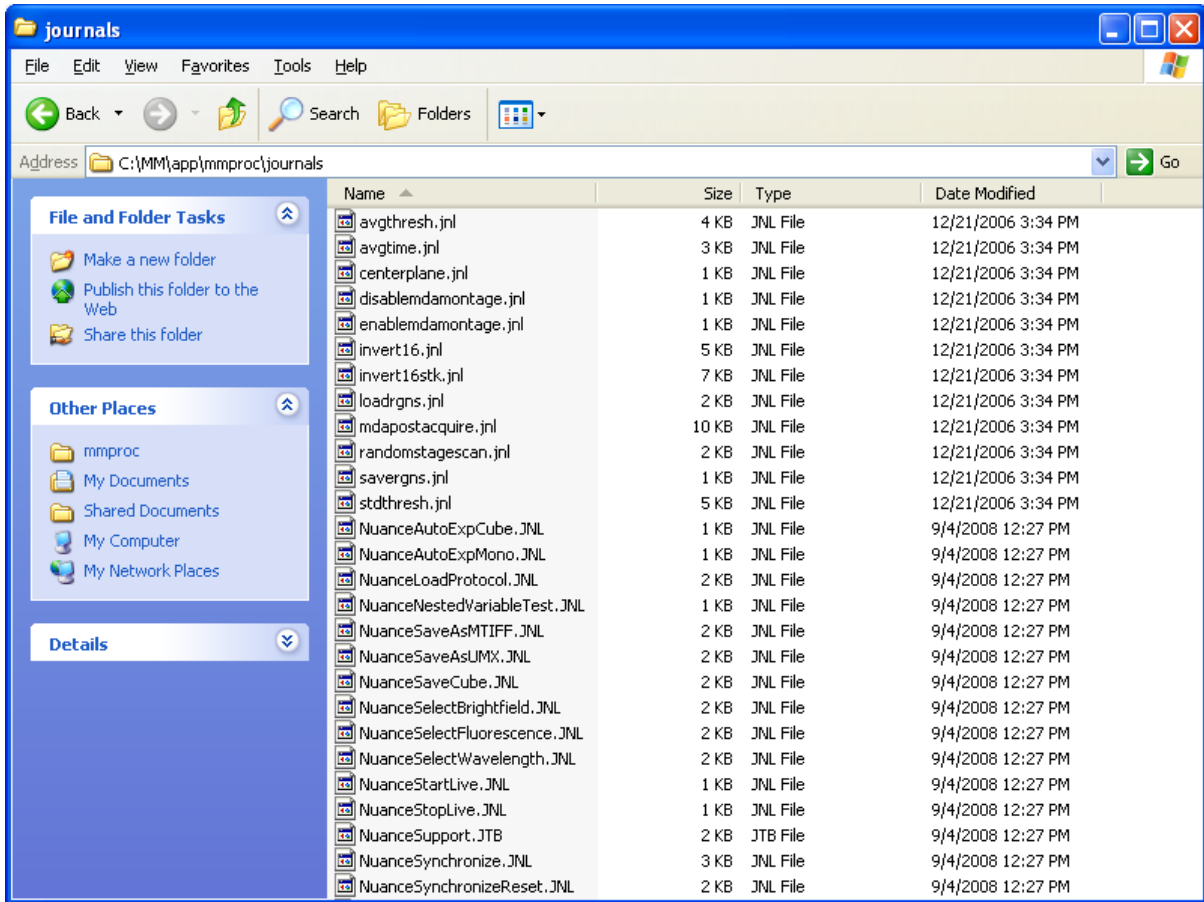
Figure 64: Extracted Journal Files

15.4 Troubleshooting
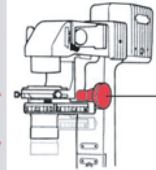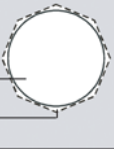
Although MetaMorph offers support for other PerkinElmer products such as the Varispec™ tunable filter, do not enable this feature. Doing so will cause conflicts between the Metamorph software and the Nuance software. If this is enabled already, you should disconnect MetaMorph's Varispec support.

16 Koehler Aligning the Microscope

Professor August Koehler developed a method in 1893, now commonly referred to as "Koehler" illumination, to precisely control the light path and the illuminating beam upon a microscope specimen. You can get the best possible image from nearly all types of optical microscopy, whether brightfield, fluorescence, DIC, phase contrast, or polarization microscopy by using Koehler illumination.

Even a slightly misaligned microscope can exhibit symptoms such as uneven illumination, glare, or internal reflections. Decreased spectral resolution often results because of color fringing and poor contrast. Aligning your microscope is important for obtaining the best results from the Nuance system.

Follow the directions below to achieve Koehler illumination and the best possible image quality from your microscope.

Step 1. Turn on the light source and adjust the intensity to a comfortable level.

Step 2. Select a 5x or 10x low-magnification objective. Center and focus the specimen in your field of view.

Step 3. Switch to a 20x or 40x objective.

Step 4. Adjust the Condenser Diaphragm so that the edges of the diaphragm form a polygonal shape well within your field of view. On the Nikon TE300, the Condenser Diaphragm is a controlled by a black lever located on the top right side of the illumination pillar. The lever, labeled "F", is behind the Supplementary Lens Pocket and in front of the Filter Sliders.

Step 5. Adjust the condenser height until the edges of the diaphragm are sharply in focus. If you are using an HMC™ condenser, you may use the Condenser Height Tool that is usually supplied with the condenser to find the correct height.

Step 6. Center the diaphragm opening in your field of view through the eyepieces. The Nikon TE300 has two knurled chrome Condenser Centering Screws located just above the condenser turret.

Step 7. Open the Condenser Diaphragm until the edges disappear from your field of view.

Congratulations! Your microscope is now set up for Koehler illumination.

17 About Legacy Hardware

This topic discusses operation-specific aspects of legacy Nuance multispectral imaging system hardware.

Nuance Models with a FireWire Interface

Previous models of the Nuance system incorporated a different enclosure design and used the FireWire™ interface, as well as an RS-232 serial connector, instead of the present USB interface. The power transformers also differ between the present and legacy hardware and ARE NOT INTERCHANGEABLE between present and legacy systems. Contact PerkinElmer if you need a copy of the previous User's Manual specific to the hardware of these older models.

At the time of the release of this User's Manual, all current versions of Nuance software will properly operate legacy hardware. Therefore, you may refer to this User's Manual for basic instructions on using the most recent software with legacy hardware.

Setup and Operation

See the Nuance User's Manual, revision 1.1 for instructions on setting up Nuance systems hardware with the FireWire™ interface.

USB Copy Protection Keys supplied with version 1.30 of the Nuance software or later will run older versions of the Nuance hardware. USB Copy Protection Keys supplied for software versions 1.2.x or earlier will only enable pre-1.30 software. Therefore, newer dongles work with older software but not vice versa. Contact PerkinElmer if you have older (pre-1.30) software and wish to upgrade to the most recent software.

Specifications

See the Nuance User's Manual, revision 1.1 for the most recent table of specifications for Nuance systems with the FireWire interface.

Mechanical Dimensions

See the Nuance User's Manual, revision 1.1 for the most recent mechanical drawings of the enclosure for Nuance systems with the FireWire interface.

Miscellaneous
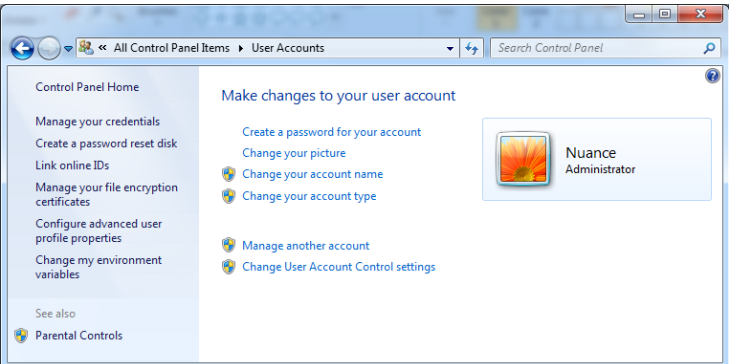
Unlike present Nuance enclosures, legacy enclosures do not allow the movement of the liquid crystal tuning element out of the light path. Legacy Nuance systems hardware require a different hardware-specific driver to enable the camera to function properly. The installer will be named accordingly.
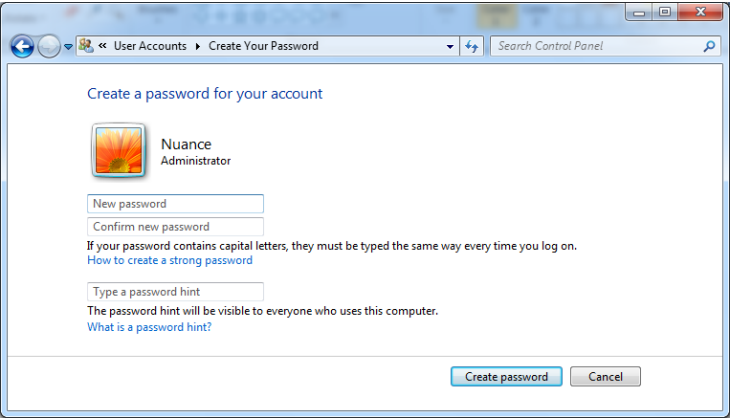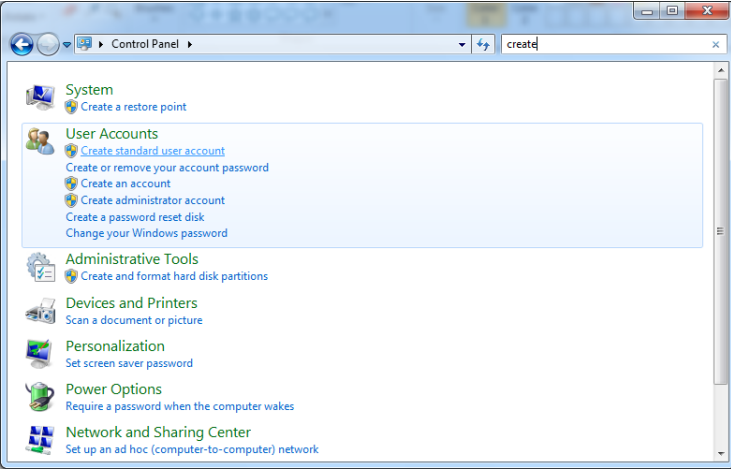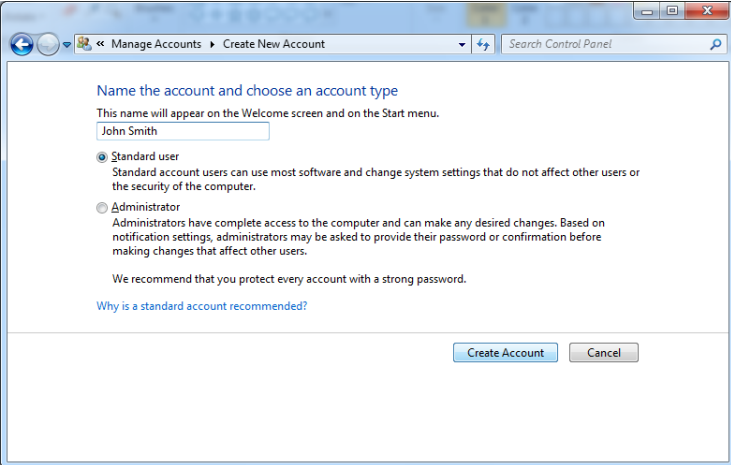
18 Windows User Management
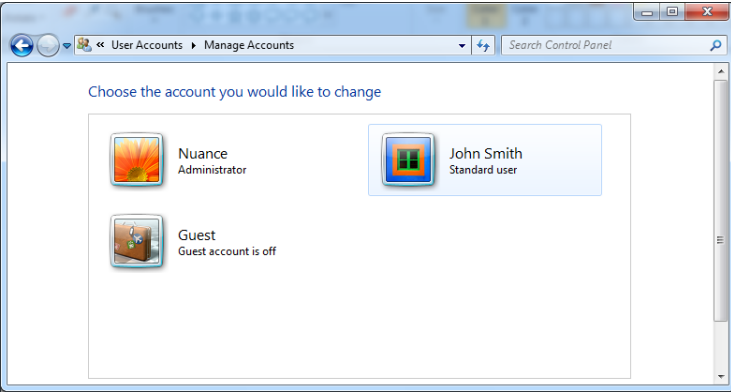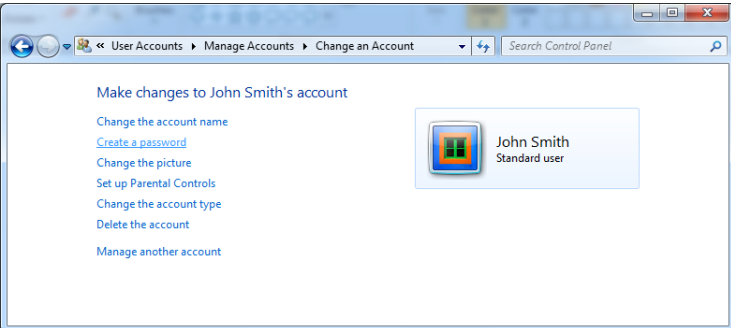
In most instances, the computer used to run the Nuance system will be shared by multiple users. Each user will have his or her own Nuance protocols, cubes, spectral libraries, and other documents and files associated with his or her work with the Nuance system.

Each system user should be assigned a personal Windows user account on the computer. There should be one *Computer administrator* account for (in most cases) the lab director or similar person. All other users should be given *Standard* accounts that should be password protected for increased security. Users should be instructed to save their work within their own My Documents directories, which are private and cannot be accessed by other *Standard* users. This prevents other users from accessing Nuance files or documents that belong to their peers (i.e., other *Standard* users).

The following table contains the required steps for password protecting the *Computer administrator* account and setting up *Standard* accounts and passwords for other users.

Action	User Accounts Dialog Box
The computer is set up at PerkinElmer with one *Computer administrator* account named "Nuance." This default account is not password protected. This lets you start up and use the computer without having to log on or enter a password. Create a password for the Nuance user account. Then, created *Standard* user accounts with passwords for each of your users.	
Password protect the Nuance administrator account: 1. Click the **Start** button, then select **Control Panel > User Accounts**. 2. Under **Make changes to your user account**, make sure the Nuance Administrator user is visible (as shown in this example). 3. Click on **Create a password for your account**.	

4. Type and retype the password as instructed. 5. You may also enter a password hint to use in case you need help later remembering the password. 6. Click **Create Password**. 7. A password is now required to log on to the computer as the Nuance administrator.	
Create *Standard* **user accounts:** 1. Return to the top level **Control Panel > User Accounts** dialog box. If you don't see the **Create a new account** option, click in the search box at the top of the window, type *create*, and press **Enter**. 2. Select **Create standard user account**.	
3. Name the new account: You may enter a full name (e.g., John Smith) or an abbreviation (e.g., jsmith). 4. Select **Standard User** and click **Create Account**.	

5. You will notice the new user added to the list of accounts. **6.** Click on the icon for the new user's account to select it.	
7. Create an initial password by clicking **Create a password**.	
8. Type and retype a very generic password for the user, such as "password." **9.** Click **Create Password**. **10.** The user's *Standard* account is now password protected. Only this user will have access to documents and files saved in his or her My Documents directory. Similarly, this user has no access to the My Documents directories of other users. **11.** Repeat this procedure for each user account you need to create. **12.** Exit the User Accounts Control Panel when finished. **13.** When you log off, or the next time the computer is	

turned on, the login screen appears. Users should be instructed to select their own name and enter their initial password to log on. After logging in, users should access the **User Accounts Control Panel** (as described above) and select a more personal and secure password.	

19   Software EULA

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