The Nikon C1si Combines High Spectral Resolution, High Sensitivity, and High Acquisition Speed

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Spectral imaging is a natural extension of the capabilities of confocal microscopes. The first confocal spectral imaging (CSI) instruments were able to acquire spectral data that allowed the emissions of overlapping fluorescent probes to be assigned to data channels representing a spectrum rather than a range of emission wavelengths. This marked a significant improvement over what could be done by channel series with standard confocal microscopes. However the performance of these earlier designs can fall short in one or more of the following areas; sensitivity, spectral resolution and reproducibility, acquisition speed, and unmixing accuracy. Nikon has recently introduced a new CSI instrument, C1si, that overcomes some of the more serious performance deficiencies of earlier designs through unique optical, electronic, and data handling advances. C1si uses a multianode photomultiplier tube (PMT) as the detector and typically acquires spectral data in a single scan. Sensitivity is enhanced over designs diffracting randomly polarized fluorescence by rotating the polarization of all emission photons to the S-plane, the plane for which the diffraction grating is most efficient. Three diffraction gratings are provided supporting wavelength sampling increments of 2.5, 5, and 10 nm. Improvements have been made in the digitization process to increase detection efficiency as well. C1si is calibrated to a high enough standard that it is possible to share and reproduce data between instruments. The algorithm implemented in the EZ-C1 software is able to accurately and repeatedly unmix fluorescent probes with emission peaks separated by as little as 5 nm. It is possible to unmix probes with emission peaks separated by 20 nm with a 10–1 brightness difference. Three probes can be unmixed with emission peaks contained within a 20 nm range. Acquisition is fast enough and the sensitivity is sufficient for C1si to acquire more than 100 frames of spectral time series data without serious photobleaching.

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ping probes can be separated, even if one probe is significantly brighter than the other(s). The spectra must be reproducible, ideally between instruments, so spectral reference libraries and data can be shared. The acquisition time must be fast enough to allow the collection of meaningful spectral time series data and stacks without measurable fading from the beginning to the end of the acquisition. The spectral bandwidth must be sufficient to allow the collection of data from fluorescent probes emitting over a wide range. While all CSI systems are limited to some degree, Nikon has gone to great lengths to produce a design that meets these spectral acquisition challenges.

LIMITATIONS OF PREVIOUS CSI SYSTEMS

Unmixing of overlapping fluorescent probes was pioneered by the commercial manufacturers of the first CSI systems. These instruments could usually unmix two overlapping fluorescent probes with emission peaks separated by 20 nm or more with little difficulty (5,6). It is impossible to separate probes with that degree of spectral overlap with a standard confocal microscope, even with a channel series. While unmixing probes with emission spectra separated by less than 20 nm can be challenging for most first generation instruments, reports abound in the literature of the successful unmixing of overlapping fluorescent proteins (7). This technology has also been successfully employed for FRET (8). The usefulness of these instruments for separating the fluorescence of probes of interest from background autofluorescence has also been demonstrated (9).

Still, there were performance limitations inherent in the designs of these earlier instruments. These limitations stem from a combination of factors, including limited sensitivity of the spectral detector, limited spectral bandwidth, and the limited spectral accuracy achievable with some systems. The sensitivity of all CSI systems is limited by channel width. The typical channel width of nonspectral confocal microscopes is 30–50 nm. The spectral channel width available on first commercial CSI systems ranges from 2 to 10.8 nm, depending upon the instrument. The reduced channel width alone limits sensitivity by restricting the number of photons that can reach the detector in a given sampling period. Earlier CSI systems relying on a diffraction grating suffer from an additional sensitivity loss at the grating because all polarizations are not diffracted with equal efficiency. The quantum efficiency of the typical photomultiplier tube (PMT) typically falls off into the red as well, compounding the problem.

Spectral accuracy is determined by two competing factors. The wavelength sampling increment (WSI) must be sufficiently small to reconstruct the analog emission spectrum. The amplitude in each detection channel must also be known with great accuracy. As a practical matter, the validity of the amplitude or intensity measurement decreases as the WSI decreases, with fewer and fewer photons contributing to the data. Therefore, signal to noise also decreases with decreasing WSI. The large fixed WSI of at least one earlier design leads to spectral aliasing, the condition where the number of data points is insufficient to accurately reconstruct the emission spectrum (10). Aliasing does not present insurmountable problems for unmixing if the emission spectra of the fluorescent probes to be unmixed are not highly overlapping. It takes three data points within the full width half maximum (FWHM) of the emission spectrum to characterize that value. It takes many more points to characterize overlapping spectra or to differentiate subtle variations in the shape of spectra. A useful guide is to use a minimum of three WSI points to define the distance between emission peaks (10). By this reasoning, a WSI of 6 nm is required to define the combined spectral profiles of GFP and YFP, with emission peaks of 509 and 527 nm, respectively. An even finer WSI is required if more than two probes fall within this range or if the brightness of the probes varies greatly.

Spectral accuracy of earlier CSI systems may also be limited by the acquisition strategy employed. One instrument acquires eight spectral channels per scan. Another older CSI system is limited to a single spectral channel per scan. When spectral data is acquired over a series of passes, the danger exists that the parts of the spectrum acquired last are subject to significantly greater photobleaching than the channels acquired in the earlier passes. This not only limits spectral accuracy, it also limits the acquisition rate that can be used in a spectral time series. Furthermore, any movement of the specimen during acquisition makes it impossible to accurately measure the spectrum at the pixel level. The size of a stack or time series that can be acquired before photodamage becomes evident is also limited.

The confocal pinhole functions as the entrance aperture of the spectrometer in some earlier CSI systems. Opening the pinhole beyond one Airy unit to increase signal to the detector results in photons from outside the selected WSI reaching the detector. Data is compromised by a broadening of the FWHM of fine spectral features that leads to a loss in spectral resolution. The peak valley ratio decreases as the pinhole is opened resulting in a reduction in contrast. While this reduction in data quality, referred to as the pinhole artifact, can be troublesome at a WSI of 10 nm or more, it becomes an especially significant contributor to spectral inaccuracies as the WSI decreases (10,11).

Previous CSI designs all suffer to some degree from limited acquisition speed. One system can acquire 512 lines in eight channels in ~1 s. A full spectrum of 32 channel acquisition takes at least four times that. CSI systems requiring more than four scans to acquire useful spectral data take longer still. Taken together, limited acquisition speed, spectral resolution, and sensitivity combine to make acquisition of meaningful spectral time series data challenging.

THE NIKON C1SI SPECTRAL CONFOCAL MICROSCOPE

The Nikon C1si spectral confocal microscope expands upon the spectral capabilities of earlier designs, overcoming problems evident in them to the greatest degree possi-
ble. A number of design improvements were introduced with C1si that enhance sensitivity, and spectral accuracy to create an instrument capable highly accurate unmixing of closely overlapping fluorescent probes and of acquiring meaningful spectral time series data. These innovations and the unmixing performance that they make possible are described in the following sections.

**Design Concept, Components, and Specifications**

The highly modular Nikon C1si spectral confocal microscope is derived from the C1 confocal microscope introduced in 2001. The spectral emission path begins with an optical coupling of the fluorescence emissions collected through the confocal pinhole into a multimode fiber with a 50 μm core (Fig. 1). This emission fiber terminates inside the spectral detector module. The detector is fiber coupled so the compact scan head design can be retained. Efficiency losses through the fiber are negligible. Reflective losses at the fiber ends are minimized by firmly butting the fiber entrance to the coupling lens and by antireflection coatings. A long focal length lens focuses the core of the fiber on the detector, a Hamamatsu™ 32 element multianode PMT. From the focusing lens, the randomly polarized emission photons pass through a polarizing beam splitter that splits them into two spatially separated and orthogonally polarized beams. The S-plane polarized beam travels directly from the beam splitter to the diffraction grating. A prism rotates the P-plane polarized beam to the S-plane. Both beams, now polarized to the S-plane, are diffracted at the same spot on one of three gratings in the detector. These gratings, supporting 2.5, 5, and 10 nm WSI’s, are mounted on a rotating drum. The full 350 nm wide bandwidth stretching from 400 to 750 nm can be utilized at any channel width by rotating the drum so that the desired band of wavelengths falls across the detector. The starting point for spectral acquisition can be adjusted in increments as small as 0.2 nm with an accuracy of 1 nm. Positioning of the grating is confirmed in a closed loop by a rotary encoder. Each diffracted beam is focused by an independently adjustable cylindrical mirror onto the multianode PMT so that the focused spots from each beam perfectly overlap. The specimen may be simultaneously illuminated by any two laser lines from the laser set. Back reflections from the longer wavelength laser are prevented from reaching detector by a movable laser mask. The normal confocal emission path is preserved in the C1si scan head so that the instrument can be operated in

![Fig. 1. C1si system drawing showing major components and the light path through the scan head, spectral detector, and standard fluorescence detector.](image-url)
three fluorescence channel confocal mode using the standard fiber coupled 3-PMT (Hamamatsu™ R-928) detector module. A fourth channel for transmitted light is available in both normal confocal and spectral acquisition modes. As in C1-Plus, the scan head dichroic mirrors and emission dichroic mirror and filter sets are easily exchangeable by the user. A 512 × 512 pixel, 32 channel spectral image is acquired in a single scan of less than 2 s. The system is controlled by a PC operating in the WindowsXP® environment.

A choice of two laser illumination units is offered with C1si. One, a three laser table featuring AOM control of laser power and direct modulation of diode lasers is the same one used with the Nikon C1-Plus confocal microscope. A four laser table featuring AOTF modulation of Argon ion and diode pumped solid state (DPSS) lasers is highly recommended. Both are coupled to the scan head through a single mode polarization maintaining optical fiber. Laser choices include 408, 440, and 638 nm modulated diode lasers, 561 and 488 nm solid state lasers, and single and multiline Argon ion lasers.

**Increasing Sensitivity**

Both optical and electronic refinements enhance C1si detection sensitivity. Optical enhancements include a Diffraction Efficiency Enhancement System (DEES) that is employed to overcome the efficiency losses associated with diffraction gratings. Because the diffraction efficiency falls as low as 40% of optimum at long wavelengths for \( P\)-plane polarized light, the polarization of all emitted photons is rotated to the \( S\)-plane before the diffraction grating. This is accomplished with little loss by focusing the beam after the confocal pinhole into a 50 \( \mu \)m core multimode optical fiber. The randomly polarized light emerging from the fiber passes through a polarizing beam splitter which separates the emission photons into two orthogonally polarized beams. The beam polarized to the \( P\)-plane is rotated through a prism to the \( S\)-plane. Rotated this way, the highest possible diffraction efficiency is maintained for the critical range from 520 nm through 660 nm. The diffraction efficiency never falls below 80% of optimum from 450 nm through 750 nm (Fig. 2). The overall improvement over the same system using randomly polarized light with the prisms removed is 10–15% depending upon the grating and wavelength.

Another advantage of coupling the spectral detector through a multimode fiber is the elimination of the pinhole artifact. Because an image of the emission fiber core rather than an image of the pinhole itself is focused on the detector, spillover of photons onto adjacent detector elements is eliminated. As when acquiring confocal data in normal wide channel mode, opening the pinhole sacrifices axial resolution. Confocal users are used to trading one acquisition parameter to improve another, in this case trading axial resolution for increased signal to noise.

While laser selection does not directly influence detector sensitivity, it can have a dramatic effect on brightness across the spectral bandwidth. Helium–Neon lasers, especially at 543 nm, are less than desirable in a CSI system because of their low power, typically 1.5 mW or less from the laser head. Nikon Instruments recommends a Melles Griot 10 mW DPSS laser emitting at 561 nm for the green excitation source. This laser is powerful enough for spectral imaging even at the smallest WSI. Many interesting and useful fluorescent probes emitting well into the red can be excited with adequate to excellent efficiency at 561 nm. The additional power and longer wavelength of this laser produce more fluorescence emission photons at longer wavelengths allowing a more chromatically balanced acquisition in a single scan. Additionally, because its wavelength is well separated from the 488 nm Argon ion laser line, wider simultaneous emission channels are available in normal confocal mode.
Increasing the sensitivity of C1si has also been addressed through advanced circuitry. The digitizing boards commonly used to convert the analog output of PMT’s sample for only part of the pixel period. The sampled and held data, voltage in the case of a PMT, is then measured and the result transferred to storage before the digitizer resets. As much as one half of the pixel period is spent with the digitizer in the hold-measure-transfer mode. C1si samples and integrates for the entire pixel period by using tandem digitization circuits. For this to happen, one digitizer circuit samples throughout the first half of the pixel period. That digitizer then enters its hold-measure-transfer phase. The second digitizer in the tandem pair samples through the second half of the pixel period. As the next pixel period begins, the first digitizer switches into its sampling phase and the second digitizer enters its hold-measure-transfer phase where it adds its measurement into storage for the first pixel and resets to sample the second half of the second pixel. Sensitivity is doubled because there is no time during the pixel period when sampling is not occurring.

**Improving Spectral Accuracy**

As mentioned previously, the spectral accuracy of a CSI system is determined by two competing factors, WSI and signal to noise. Increasing the throughput of a CSI system usually leads to increased spectral accuracy. Rotating all emission photons to the S-plane enhances spectral accuracy in two ways. The diffraction efficiency of S-plane polarized light is not only more efficient, increasing signal to noise, and the efficiency curve is nearly flat from 520 to 670 nm, the wavelengths most commonly encountered in spectral unmixing. In C1si, the WSI is fixed by the diffraction grating selected. The user can also select the number of channels to be sampled. Typically, all 32 channels are used at a WSI’s of 2.5 or 5 nm. The reduced signal to noise sometimes associated with narrow sampling channels can be compensated for by increasing the pixel dwell, or by averaging when necessary. The option also exists to average to a quality value of 12 dB. Little additional data quality accrues beyond this point. If the emission peaks of the probes are separated by 5 nm or more, or if significant features of the emission spectra to be unmixed extend over a range greater than 80 nm, 32 channels can be acquired at a WSI of 5 nm. When considering spectral accuracy, it is always important to consider the system accuracy required to perform the task at hand. High spectral accuracy is not usually required to unmix the signals from probes with emission peaks separated by 20–30 nm. Higher accuracy is required if a third probe with an emission spectrum falling between the first two is added into the mix. Still higher accuracy is required if any of the probes to be unmixed vary dramatically from each other in brightness. C1si was designed to allow the user to develop acquisition and unmixing strategies appropriate to a wide variety of specimens. The user can choose from a WSI of 2.5, 5, or 10 nm. Sensitivity can also be increased by opening the pinhole beyond the size for maximum spatial resolution.

The spectral resolution of C1si throughout the 400–750 nm specified range of the system has been evaluated using a widely recognized standard, the MIDL reference lamp (LightForm). The spectrum of a fully warmed MIDL reference lamp was recorded in a single scan at WSI’s of 2.5, 5, and 10 nm from a lamp taped to the stage of a Nikon Eclipse 90i upright microscope. The light was collected through a 10× Plan Apo objective lens focused for maximum brightness. The spectrum acquired from 400 nm through 638 nm at a WSI of 2.5 nm compares favorably with the spectrum acquired and digitized by a PARISS spectrometer (LightForm) with peaks located to within ±2 nm of their true position (Fig. 3a). The far red part of the spectrum from 670 nm through 750 nm was acquired under the same conditions at a 2.5 nm WSI within 5 s of starting a MIDL lamp cooled to room temperature (Fig. 3b). It was necessary to acquire this part of the spectrum separately because features of the spectrum at wavelengths longer than 650 nm disappear rapidly as the lamp warms. The spectra were not combined because the lamp is significantly dimmer until it fully warms and it was impossible to determine an accurate scaling factor. MIDL lamp spectra were also measured at 5 and 10 nm WSI. Aliasing comparable to published data from other CSI systems predictably occurred at these channel widths. Emission peaks could be shifted to more closely coincide with results obtained using a 2.5 nm WSI by shifting the start-
ing wavelength for the spectral acquisition 2–4 nm. While this had no measurable effect on unmixing when a single laser was used for acquisition, shifting the starting wavelength so that the second laser line falls at the center of a channel results in a slight improvement.

**Calibration of C1si**

The calibration procedures developed for C1si are intended to ensure that the performance of complete CSI system meets very stringent criteria for sensitivity and spectral accuracy. The reliability of unmixing results depends on this. An instrument that can be calibrated to stringent standards makes sharing of library spectra for unmixing possible among users of different instruments. It is also possible, albeit with somewhat greater potential for error, to use spectra provided by the manufacturers of fluorescent probes including Molecular Probes and Clontech, now Invitrogen. In fact a selection of library spectra provided by these manufacturers is included with the software.

All C1si CSI systems are assembled and calibrated as complete systems before shipment to the end user. Calibration of the positioning of each of the diffraction gratings is confirmed and adjusted if necessary against fine features of an Hg arc lamp spectrum. The tolerance for locating peaks is to within 1 nm. The position of the laser mask is checked against the smooth spectrum of a microscope quartz halogen illuminator. Positioning of the blocking plate is accurate to ±0.5 nm.

The sensitivity of the individual detector elements in a multianode PMT characteristically varies over a wide range. Nikon hand selects only those arrays where the sensitivity of each detector element falls within an acceptable range. The PMT is calibrated by the following method. The fiber is illuminated with a standard illumination source. The diffraction grating is stepped in one WSI increments so that the same spectral peak falls on each element. A calibration is made from the output voltage of each anode so that the digitized intensity from each falls within a 1% range. Nikon calls this calibration procedure the channel shift method. The offset of the multianode PMT is set as a calibration value so that the maximum gray level with zero photons falling on the detector does not exceed 40 on a 12 bit scale at a PMT gain of 142. No spurious electrons are drawn from the photocathode at this gain. The uniformity of the multianode PMT is checked and confirmed to be within 5% from 500 to 660 nm. It should be remembered that the performance of all analytical instruments can degrade over time. Annual service including recalibration is strongly recommended. Users are urged to purchase their own calibration standard so they are able to assess on the spot if an unexpected result might be due to changes in instrument performance or the experiment itself.

**Unmixing**

The proprietary unmixing algorithm employed in the EZ-C1 software supporting C1si is based on the least squares method with linear regression. Corrections are built in for noise and to eliminate negative values from the solution. The expected error in assigning intensities to overlapping probes where one is 10 times the brightness of the other and the emission peaks separated by 20 nm is <5% at a WSI of 2.5 nm. The preferred acquisition strategy is to acquire using the WSI that provides the most advantageous combination of signal to noise and spectral resolution for the probes to be unmix. This is typically 5 nm for fluorescent probes with emission peaks separated by 7 nm or more, or 10 nm for probes separated by at least 20 nm. The channels used for unmixing should cover the FWHM of both probes at the minimum, but ideally will cover as much of their emission spectra as possible. If necessary, signal to noise can be improved by extending the pixel period, averaging, and/or opening the pinhole. It is possible to unmix using spectra with peak separations less than 10 nm with a WSI of 5 nm using spectra derived from field data or library spectra matched by starting wavelength.

The power of the algorithm lies in its ability to unmix overlapping probes where the signal from one is significantly brighter than the other. This is especially important where probes are colocalized. Figure 4 shows a zebrafish tail featuring a GFP protein expressed in motor neurons. The fish was AM loaded with Calcium Green. While it is possible to unmix the signals from overlapping probes using spectra derived from regions of interest (ROI) within the image, this method has potential for error. It is impossible to get the pure spectra of each probe where one of them can only be found colocalized with the other. Figure 4a shows the true color image and the location of the ROI's from which unmixing spectra were derived. In the true color image (Fig. 4a) the brightest of the GFP expressing cell bodies is as much as 10 times the brightness of the surrounding calcium green loaded tissue. Unmixing under such conditions can cause intensities from the dimmer probe to be incorrectly assigned to the brighter one. Unmixing using spectra derived from singly labeled specimens improves the ability of the algorithm to assign intensities to both GFP and Calcium Green correctly (Figs. 4b–4d). The GFP spectrum that yielded the best unmixing result was acquired from the GFP expressing fish before Calcium Green loading. The spectra used to unmix the data presented in Figure 4 are displayed in Figure 4e. Compare the GFP spectrum derived from ROI 2 in the true color image with the spectrum derived from the Zebrafish before AM loading of Calcium Green. The GFP spectrum derived from ROI 2 has been broadened by the overlapping intensities of the Calcium Green colocalized with it. This broadening can lead to an unmixing error where Calcium Green intensities in areas of the specimen where they are colocalized with GFP are incorrectly assigned to GFP (Fig. 4c). This is noticeable as voids in the unmixed Calcium Green image in areas where bright GFP expressing cell bodies are located. The images shown were acquired using a WSI of 5 nm. Use of a WSI that is too large can also cause intensities associates with the dimmer probe to be incorrectly assigned to the brighter one.
Fig. 4. Zebrafish spinal cord with AM loaded Calcium Green expressing a GFP tagged motor neuron marker protein. (a) True color projection of the original data showing the regions that spectra selected for unmixing were derived from. (b) Unmixed image of the same field shown in (a). GFP is shown in green and Calcium Green in red. (c) Calcium Green display channel unmixed using the GFP spectrum from ROI 2 in (a). Note the uneven distribution of Calcium Green with voids appearing where the brightest GFP expressing cell bodies are located. (d) Calcium Green display channel unmixed using the GFP spectrum derived from the same specimen before Calcium Green loading. Note the even distribution of Calcium Green and lack of voids. (e) Spectra used in unmixing. The Calcium Green spectrum is shown in red, the GFP spectrum derived from ROI 2 is shown in green, and the GFP spectrum acquired before AM loading of Calcium Green in black. Note that the trailing edge of the GFP spectrum derived from ROI 2 red appears red shifted. This is the result of the contribution of photons from the colocalized Calcium Green.
Fig. 5. Eosin stained retina. (a) True color projection of eosin stained retina. (b) Unmixed image with intensities matching the spectrum with the 551 nm peak displayed in green and intensities matching the spectrum with the 561 nm peak displayed in red. From the top, the ganglionic layer in green, the inner plexiform layer showing the synaptic area unmixed to red, the inner nuclear layer unmixed primarily to green, the outer plexiform layer showing the synaptic areas in red, rods and cones in dim red, and the substantia propria of the sclera in green. (c) Spectra used for unmixing showing two distinct eosin emission spectra with a peak separation of 5 nm. The spectra are shown in the colors to which the structures spectrally matching them have been mapped.
A good measure of the success of unmixing is the brightness of the remainder of unmixed intensities. Gray levels >200 in the brightest parts of the remainder image indicate that the signal to noise of the data may not have been sufficient for the most accurate unmixing. The EZ-C1 software for C1si lets you recover to some extent by allowing the selection of a low pass filter before unmixing. This filter is applied postacquisition. A low pass filter improves unmixing accuracy at the expense of spatial resolution because the signal to noise of a group of pixels is greater than that of a single pixel. This can be especially helpful in time series where frame rate and unmixing accuracy may be the overriding concern and spatial resolution can be sacrificed. A significant remainder may also indicate that the acquisition strategy should be modified.

Sometimes the peak of the emission spectrum of a fluorescent molecule shifts under local conditions. These conditions can include variations in charge distribution of proteins and changes in pH. This change in the Stokes shift of the probe can be seen in specimens stained with eosin (Fig. 5) where cells and structures that appear to be stained identically can be separated by the small local shifts in the emission spectrum of the dye that stained them. The true color image of retina (Fig. 5a) looks almost

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**Fig. 6.** Muntjack skin fibroblasts. (a) True color image of fibroblasts stained with Bodipy-TRX™, Sytox Orange™, and Mitotracker Red™. (b) The same data unmixed with Bodipy-TRX™ displayed in red, Sytox Orange™ in green, and Mitotracker Red™ in blue. Brightness was independently adjusted for each display channel. (c) The spectra used for unmixing (b), displayed in the colors to which they were unmixed.

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identical to what would be seen looking through the eye-pieces of a fluorescence microscope with blue excitation and a long pass emission filter. All acidophilic proteins appear to be stained alike. This image can be unmixed using an ROI drawn within the inner plexiform layer and one in the substantia propria. The spectra are illustrated in Figure 5c. Pixel intensities where the eosin spectrum matches the spectrum with its peak at 551 nm are displayed in green. Intensities matching the spectrum with its peak at 556 nm are displayed in red. Overlapping intensities are not displayed in yellow or orange, but rather are displayed in their assigned colors at the appropriate brightness (Fig. 5b).

It is also possible to unmix three fluorescent probes with a high degree of overlap between their emission spectra. ROI’s are drawn the same way as they are when two probes are to be unmixed. A third color is selected to display the additional probe. A true color image of Muntzak’s skin fibroblasts stained with three closely overlapping orange/red emitting fluorescent probes, the nuclear stain Sytox Orange™, Bodipy-TRX™, and Mitotracker Red™, can be easily unmixed by this method (Fig. 6), even with the brightness of the probes varying over a fivefold range.

**Acquiring Time Series Data**

Spectral time series experiments require data acquisition at a frame rate fast enough to produce a meaningful, temporally resolved, result. The frame time for C1si acquisition of a 512 × 512 pixel 32 channel image is under 2 s. Faster acquisition rates are possible at 256 × 256 pixels or in a band scan (reduced pixel count in y). The sensitivity of the spectral detector is only partly responsible for frame rate. It is also a challenge to move the massive amount of data acquired in 32 twelve-bit data channels from the digitizer buffer into RAM before the next frame is acquired. Nikon has designed fast serial transfer circuitry to speed the process. The data from four banks of eight channel tandem digitizers is multiplexed and the information is moved into the PC by means of a newly developed low voltage differential signal transfer circuit.

The ability to move large amounts of spectral data quickly is irrelevant if the specimen does not survive the experiment. The key to survivability of the cell is using the lowest laser power possible while achieving an acceptable signal to noise. This is especially important in a time series. The sensitivity of C1si is sufficient that a 107 frame time series of a zebrafish spinal cord loaded with AM Calcium Green and expressing GFP marker for motor neurons can be acquired at 1 frame/s with a WSI of 5 nm with a photobleaching loss of 17% for the GFP and 16% for Calcium Green from the first frame to the last (on line supplementary material). The brightness of the GFP signal in the original data exceeds that of Calcium Green by nearly an order of magnitude.

**CONCLUSIONS**

C1si breaks new ground combining variable spectral resolution, high acquisition speed, and high spectral accuracy in a CSI instrument that is calibrated to a standard that allows sharing of data between instruments. Improvements in detector sensitivity, signal processing, acquisition speed, and selectable WSI make possible the acquisition of highly temporally resolved confocal spectral data. While each new technology might be very interesting in and of itself, it is their combination with an effective unmixing algorithm that allows the rapid and accurate unmixing of signal from highly colocalized fluorescent probes, even at high brightness differences. These same improvements lead to the ability to unmix the signals of three spectrally overlapping probes. Performance in the normal confocal mode has been preserved, with C1si retaining the standard 3-PMT detector module as well as a transmitted light detector. What can be easily lost in the focus on the new technology present in C1si is its versatility. A single instrument can be used for normal confocal microscopy, unmixing overlapping fluorescent probes, fluorescence spectroscopy, and imaging of many fluorescent probes over a wide spectral range at an acquisition speed typical of a simple three channel confocal microscope. C1si has addressed the most serious performance deficits of earlier CSI systems and serves as a very solid foundation for the further development of spectral confocal imaging technology.

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