Fluorescence signals are brighter and clearer when background noise is reduced by eliminating autofluorescence—just as city lights appear brighter as the sky darkens (in contrast to their dullness in the bright light of evening).

Fluorescence from areas where there are no fluorescent probes or conjugates present is called autofluorescence. As autofluorescence can be a significant source of background noise when acquiring images of a fluorescent specimen, it reduces signal to noise and causes low contrast. This issue of Cell Imaging Press shows how the C1si true spectral imaging confocal laser scanning microscope removes autofluorescence and improves the S/N ratio of fluorescence images acquired from live cells and fixed specimens.

**Summary of This Issue**

Fluorescence from areas where there are no fluorescent probes or conjugates present is called autofluorescence. As autofluorescence can be a significant source of background noise when acquiring images of a fluorescent specimen, it reduces signal to noise and causes low contrast. This issue of Cell Imaging Press shows how the C1si true spectral imaging confocal laser scanning microscope removes autofluorescence and improves the S/N ratio of fluorescence images acquired from live cells and fixed specimens.

**Figure 1. Comparison of autofluorescence removal by the conventional offset method and the spectral method**

Removal by the offset method

S/N ratio deteriorates as all signals less than the threshold are cut.

Removal by the spectral method

S/N ratio improves as only autofluorescence is removed while the fluorescence intensity is maintained.
**1) Removal of plastic dish autofluorescence**
Autofluorescence from the plastic container can be removed.

**Before autofluorescence removal**

**After autofluorescence removal**

Sample: HEK293 cell cultured on a plastic dish with actin labeled by Alexa 488-Phalloidin (green).
- Objective lens: Plan Apo 20x NA 0.75
- Images courtesy of Tohru Murakami, Neuromuscular and Developmental Anatomy, Gunma University Graduate School of Medicine

**2) Unmixing of multi-stained samples**
As removal of autofluorescence enables the unmixing of multiple fluorescence signals, it is effective in multi-stained samples.

**Before autofluorescence removal**

**After autofluorescence removal**

Sample: Zebrafish embryo with protocadherin labeled by GFP (green) and nuclei labeled by DAPI (blue).
- Objective lens: Plan Apo 20x NA 0.75
- Sample courtesy of Director and Professor Masatoshi Yamamoto, Drosophila Genetic Resource Center, Kyoto Institute of Technology

**3) High-sensitivity fluorescence detection with optical stimulation**
Even in case of optical stimulation such as photoconversion or photoactivation, removal of autofluorescence allows the detection of slight changes in fluorescence intensity with high sensitivity.

**Before autofluorescence removal**

**After autofluorescence removal**

Sample: Kaede expression localized to the mitochondria within Arabidopsis leaf epidermal cells. True color image before (green) and after (red) optical stimulation.
- Objective lens: Plan Apo 20x NA 0.75
- Sample courtesy of Assistant Professor Shinichi Arimura, Graduate School of Agricultural and Life Sciences, The University of Tokyo

**4) Spectral analysis of autofluorescence reveals underlying morphology in tissue sections**
Spectral unmixing, and subsequent contrast enhancement using autofluorescence clearly reveals the morphology and localization of the target.

**Before autofluorescence unmixing**

**After autofluorescence unmixing**

Sample: EGFP (green) expressed in the whole tail of drosophila sperm. Anterior pole of the egg is indicated in red (pseudo color) after unmixing the autofluorescence spectrum of the egg and spectrum of sperm.
- Objective lens: Plan Apo 20x NA 0.75
- Images courtesy of Professor Shigeo Okabe and Tatsuya Umeda, Department of Cell Biology, School of Medicine, Tokyo Medical and Dental University

**5) Observation of the deep portion of a brain hippocampus slice**
By removing autofluorescence of surrounding tissue, the three-dimensional structure of a neural network can be observed clearly.

**Before autofluorescence removal**

**After autofluorescence removal**

Sample: Fixed brain slice with neuronal cell labeled by GFP (green). Autofluorescence (yellow) observed in nuclei and cell cytoplasm is removed.
- Objective lens: Plan Apo VC water dipping 60x NA 1.20
- Sample courtesy of Professor Shigeyoshi Okabe and Tatsuya Umeda, Department of Cell Biology, School of Medicine, Tokyo Medical and Dental University

**6) Observation of a sample for an electron microscope**
By removing autofluorescence, the location of the target molecule in the sample can be easily checked before observation with an electron microscope.

**Before autofluorescence removal**

**After autofluorescence removal**

Sample: Tubulin labeled by Cy2 (green) and mitochondria labeled by Cy3 (red) after glutaraldehyde fixation. Autofluorescence derived from the fixation embedding agent is observed in nuclei and cell cytoplasm (brown). Removing autofluorescence improves the contrast.
- Objective lens: Plan Apo VC water dipping 60x NA 1.40
- Images courtesy of Jiro Usukura, D. Med. Sci., Professor of Cell Biophysics, Center for Co-operative Research in Advanced Science and Technology, Nagoya University
The spectrum obtained by actual measurement is a mixture of spectral elements with a certain proportion. An imaging algorithm is used to compare the spectra of each pixel with reference curves for each spectral element. Each fluorescent probe in the specimen is displayed in a unique color in the final unmixed image.

\[ fn = S_n \times P_n \]

- \( fn \) = Wave pattern of spectrum obtained by actual measurement
- \( S_n \) = Wave pattern of individual reference spectrum
- \( P_n \) = Ratio of elements for each wave pattern

Reference wave pattern \( (S) \) is selected from the following three depending on the experiment:

1. Spectrum obtained by actual measurement of the zone with less crosstalk in the captured image
2. Data obtained by another actual measurement using only one probe
3. Data provided by probe maker

**Editor's Note**

**Acquisition of high S/N ratio images by spectral unmixing**

Major causes of autofluorescence are proteins, including enzymes in the live cell. Other causes include oxides produced by cell fixation. The unmixing method (Fig. 2) prevents image deterioration due to background noise derived from autofluorescence. It allows the acquisition of clear images with much less deterioration in the scaling of fluorescence signals than does the conventional method, in which offset value is increased (Fig. 1).

The sample images before and after autofluorescence removal show that this method is effective to improve the images of both live cell samples (cases 1 to 3) and fixed cell samples (cases 4 to 6).

In this issue, we have introduced the spectral measurement of autofluorescence, unmixing of fluorescence signals and improvement of S/N ratio by the C1si true spectral imaging confocal laser scanning microscope.

**Footnote/Reference**

1) Cover: View of Ginza from Tokyo Tower observatory (left: before sunset, right: after dark)
2) Autofluorescence: Causes and Cures. Wright, Cell Imaging Facility, Toronto Western Research Institute, University Health Network
   http://www.uhnresearch.ca/facilities/wcif/PDF/Autofluorescence.pdf

**True spectral imaging confocal laser scanning microscope**

**C1si**