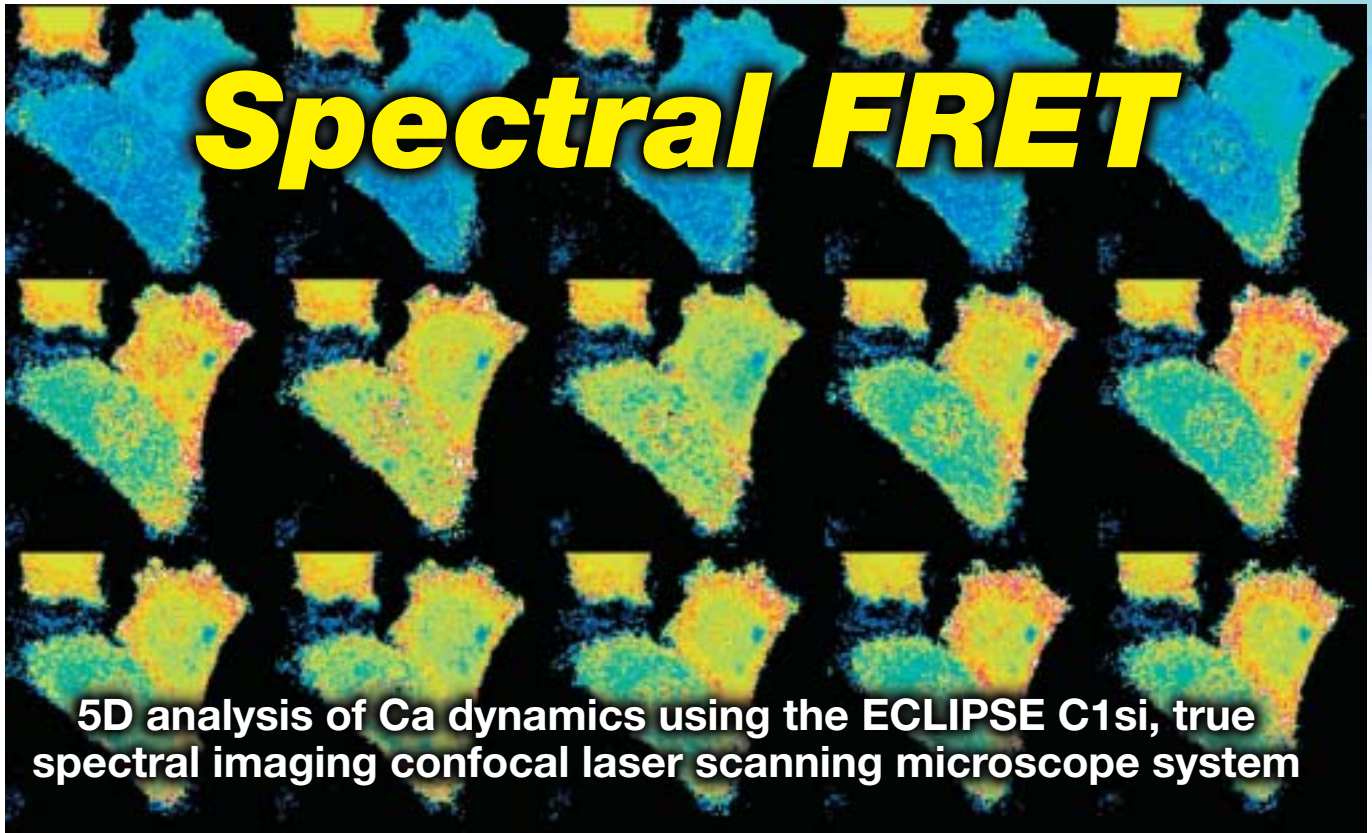




# Cell Imaging Press

Application Notes ~ June 2005

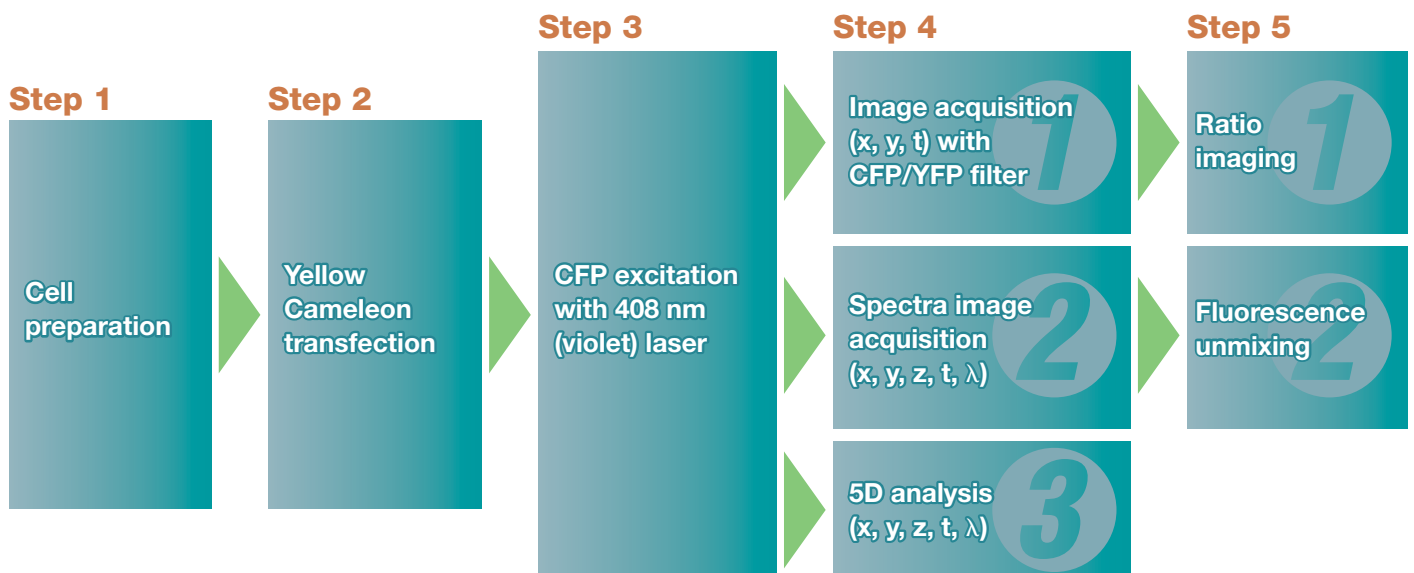


## Summary of This Issue

The C1si Confocal Laser Scanning Microscope System was used in FRET\* experiments where the FRET pair, CFP/YFP, is part of the Yellow Cameleon calcium indicator. 5-dimensional analysis ( $x, y, z, t, \lambda$ ) of changes in the fluorescence intensity of CFP/YFP and their spectra, as well as intracellular calcium dynamics was demonstrated.

※: Fluorescence Resonance Energy Transfer

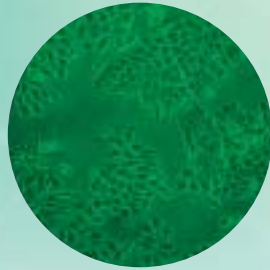
### FRET Imaging Flow with Yellow Cameleon 3.6



## Step 1: Cell preparation

Culture cells in a glass-bottom culture dish. Cells usually become subconfluent in 2-3 days.

\* Avoid plastic dishes because they obscure visualization by introducing optical aberrations and polarization artifacts.



Monitor cell cultures by phase contrast microscopy.

## Step 2: Transfection of cells with Yellow Cameleon

Mix optimal quantities of plasmids and liposomes in the solution, then add to the cultured cell. Expression of fluorescent proteins can be seen in about 24-36 hours following transfection (about 10% with lipofection) (Fig. 1).

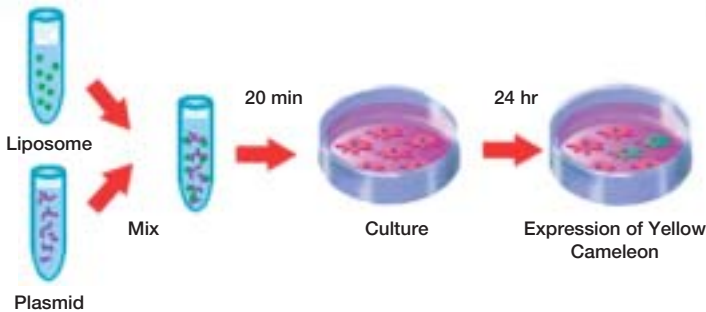


Figure 1 Genetic insertion through lipofection

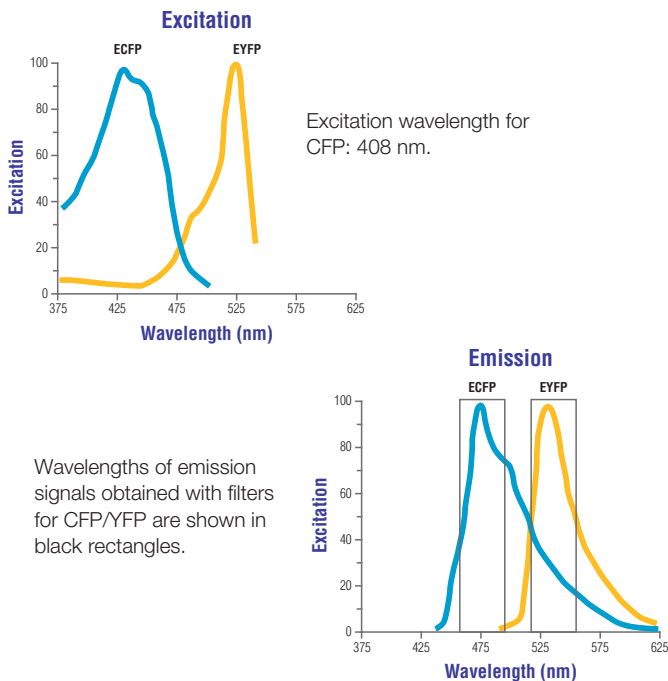
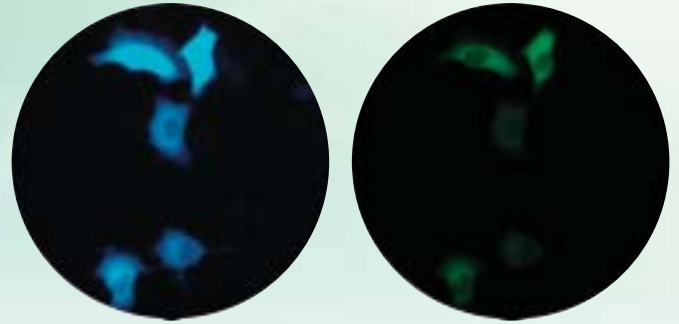


Figure 2 Yellow Cameleon 3.6\* excitation and emission spectra

\* The Cameleon calcium indicator was invented by Atsushi Miyawaki MD., Ph.D. (RIKEN). With this calcium indicator, FRET occurs as the end of the Cameleon molecule is drawn toward the end with YFP as bound calcium increases. Nagai, T et al; Proc Natl Acad Sci, USA. 101:10554 (2004)

## Step 3: CFP excitation with 408 nm (violet) laser

Confirm fluorescent protein expression with a fluorescence microscope. Use a wavelength range of 400-430 nm (violet laser) to excite Yellow Cameleon (Fig. 3).



Plan Apochromat VC water immersion 60x/NA 1.20

Fluorescence intensity distribution on an individual cellular basis can be visualized with an objective of 20x (NA: 0.5 or above) or higher. The VC Series of objectives is recommended for observations at 60x or higher because of their excellent color correction.

Excite cells expressing Yellow Cameleon with a violet laser (408 nm), then acquire fluorescence images with a standard CFP/YFP detector (sample image in Fig. 4) and a spectral detector (sample image in Fig. 6).

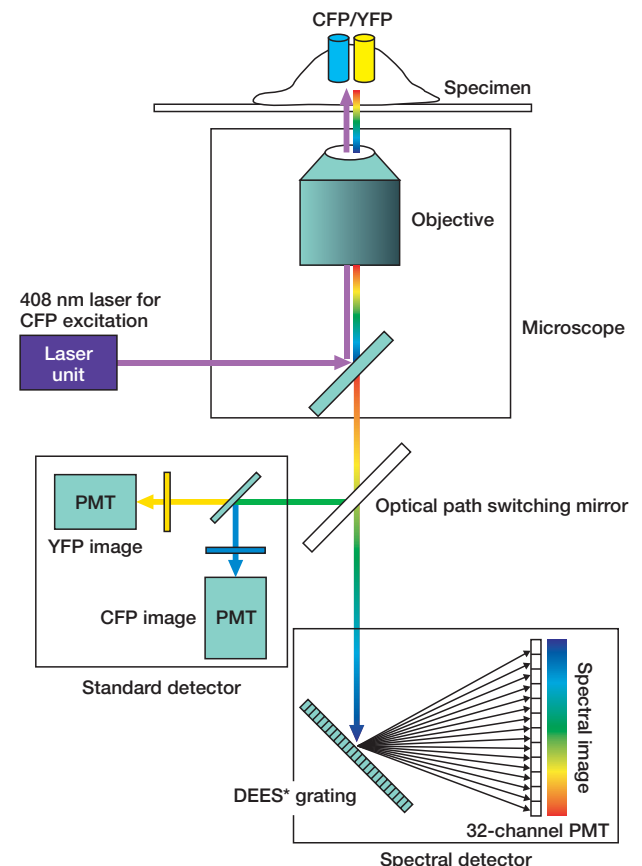


Figure 3 Optical paths for excitation and fluorescence detection

\* DEES (Diffraction Efficiency Enhancement System) is Nikon's proprietary polarization correction method.

### Step 4 (1): Vector image acquisition with CFP/YFP filters(x, y, t)

Changes in fluorescence intensity of CFP and YFP can be visualized by capturing the image with a standard detector using dedicated filters for each color.

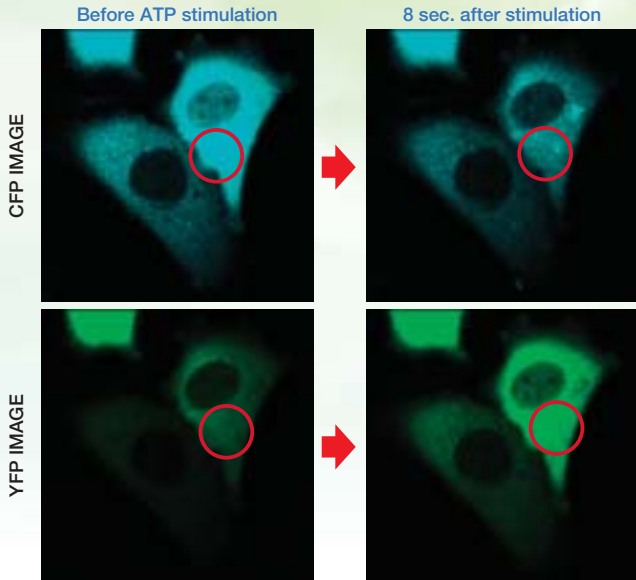


Figure 4 CFP and YFP images

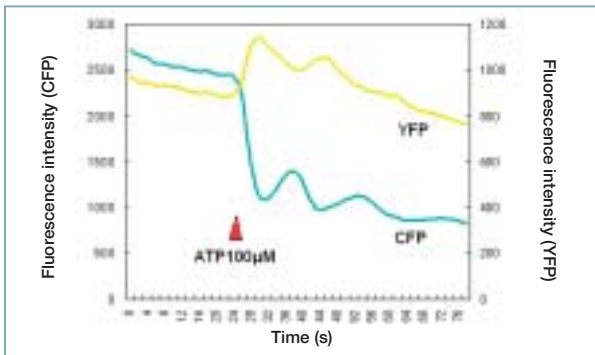


Figure 5 Changes in fluorescence intensity over time of CFP and YFP

### Step 4 (2): Spectral image acquisition (x, y, t, λ)

It is possible to investigate changes in emission spectra over time within a defined wavelength range by acquiring spectral data over a broad range with a spectral detector.

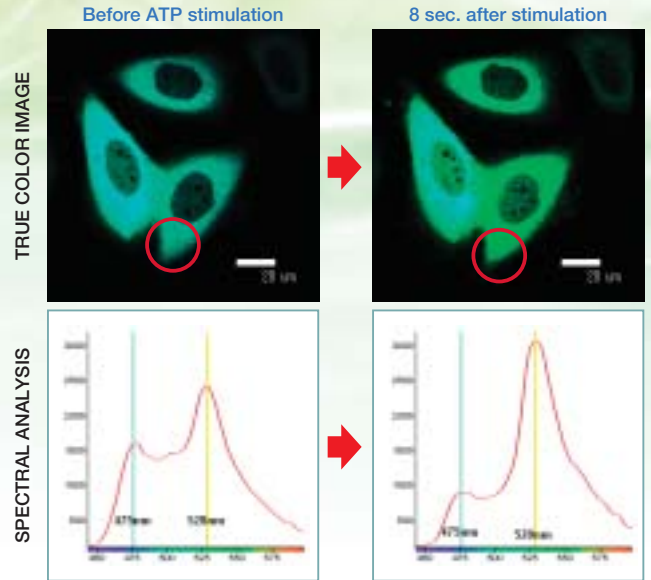


Figure 6 True color images of CFP/YFP (above) and spectral analysis (below)

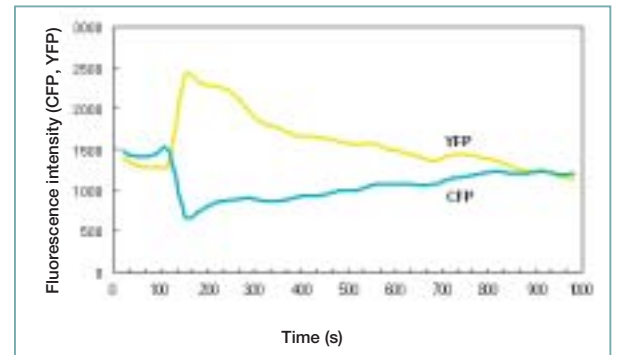


Figure 7 Changes in fluorescence intensity at CFP and YFP peak emission wavelengths over time

### Step 5 (1): Ratio imaging

It is possible to analyze intracellular  $Ca^{2+}$  concentration change over time (FRET) in separate ROIs (1, 2) without influence of fading.

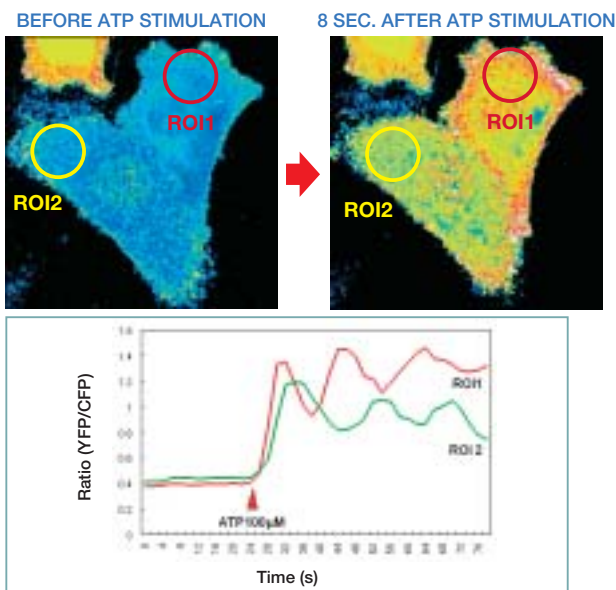


Figure 8 CFP/YFP fluorescence intensity ratio analysis

### Step 5 (2): Fluorescence unmixing

Separation (unmixing) of overlapping spectra is now possible. Unmixing using spectral data permits the assignment of emission intensities correctly to the donor and acceptor probes in a FRET pair. Analysis of the shift in emissions from the donor to the acceptor probe with increasing calcium concentration is possible without corruption of the data by acceptor photobleaching.

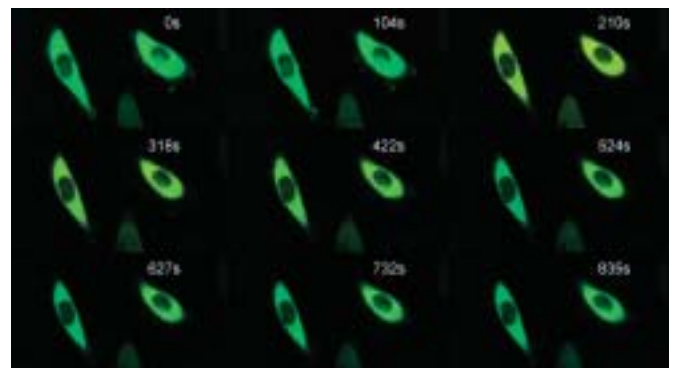


Figure 9 FRET image after spectral unmixing

## Step 4 (3) 5-Dimensional analysis (x, y, z, t, $\lambda$ )

The true spectral imaging capabilities of the C1si confocal laser scanning system make it possible to analyze spectral changes over time (t) in 3 spatial dimensions (x, y, z), in true color ( $\lambda$ ).

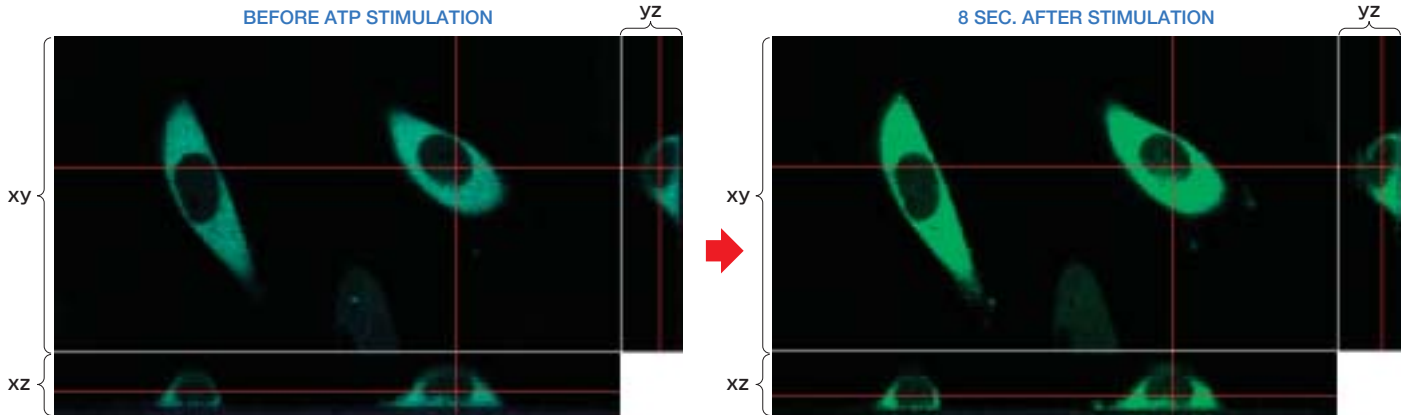


Figure 10 True color ( $\lambda$ ) analysis of spectral changes over time (t) in 3 spatial dimensions (x, y, z)

### Editor's Note

#### FRET analysis with confocal spectral imaging

There are three benefits to FRET analysis with confocal spectral imaging.

First, the confocal effect allows a **high S/N ratio** and **3D analysis** (Fig. 4, 10). Fluorescence intensity distribution can be detected not only adjacent to the coverglass, but also within the cytoplasm, nucleus, or even on the opposite side of the cell. This allows measurement of  $\text{Ca}^{2+}$  concentration, distribution, and other parameters wherever FRET occurs in full 3D.

Second, spectral imaging allows simple **individual spectral analysis of donor and acceptor probes** (Fig. 6). Even with fluorophores with overlapping spectra such as CFP/YFP, simple unmixing is possible leading to easy FRET analysis.

Third, **quantitative FRET analysis** is possible with spectral unmixing and ratio calculation (Fig. 8, 9) between CFP and YFP signals (YFP/CFP), since fluorescence intensity changes of CFP and YFP can be accurately detected.

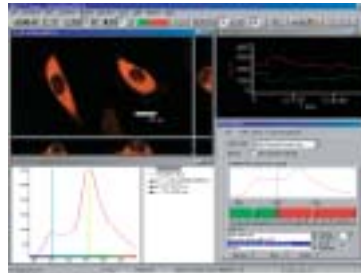
In this issue, we have described the 5-dimensional analysis of  $\text{Ca}^{2+}$  concentration as an actual research example that exemplifies the three benefits discussed above.

## True Spectral Imaging Confocal Laser Scanning Microscope System

### Nikon Digital ECLIPSE C1si



"One-shot" acquisition capability of wide wavelength range over a 320nm.



C1si analysis software: EZ-C1 spectral analysis screen

Contact Cell Imaging Press: [cell.imaging@nikon.co.jp](mailto:cell.imaging@nikon.co.jp)

#### CELL IMAGING PRESS APPLICATION NOTES

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