The following procedure describes the acquisition and processing of confocal fluorescent and bright field images of live cells expressing yellow fluorescent protein (YFP), with a spinning disk confocal head on a Zeiss Axiovert 200 M microscope.

**Description of Microscope and Imaging Setup**

1. Live cells, which have been prepared on glass coverslips or coverslip chambers, are washed 2 to 5 times with a phenol-red–free buffer. The phenol-red–free buffer includes (but is not limited to) Ringer's solution, pH 7.4, 1X, for WEHI-231 cells and HEPES buffered Hanks' balanced salt solution, pH 7.35, 1X (low endotoxin), for RAW 264.7 cells.

2. The microscope is an automated Zeiss Axiovert 200 M with a 1.4 NA 100x oil objective.

3. A Yokagawa confocal spinning disk containing a dual laser transmitting dichroic is attached to the left camera port of the microscope.

4. Helium-cadmium and argon-ion lasers are focused onto two respective laser-to-fiber couplers and then onto the respective ports of a Y-shaped optical fiber that aligns the laser beams onto a 460 long-pass dichroic. The combined laser beams are brought into the confocal head through the third port of the combining fiber.

5. Excitation and emission wavelengths are controlled with filter wheels attached to the Yokagawa spinning disk head and controlled with a lambda-10-2 filter wheel controller. This modification of the Yokagawa head is available from PerkinElmer.

6. A 515/15 nm band pass filter is used for exciting YFP.

7. Laser power is measured at objective port with an optical power meter. Adjust the laser power supply to set the 514 nm light to between 190 and 210 µWatts. Since these values may deviate as laser components change with time, specific laser power values are tracked in the database.

8. A cooled charge-coupled-device (CCD) camera is attached to the Yokagawa head for image acquisition.

9. A 565/75 nm band pass emission filter is used for YFP.

10. Hardware control and image acquisition are performed with Metamorph software.

**Description of Acquisition Parameters**

11. Twelve-bit images (intensity values of 0 to 4095) are acquired with the camera binning set to 2 x 2. This, combined with the 100x objective and 6.5 x 6.5 µm CCD chip pixel dimensions, results in images that have pixel dimensions of 0.13 x 0.13 µm.

12. Camera exposure time is set to 2000 msec for YFP images. Specific exposure times may vary because of changes to laser power, and these are tracked in the database.

13. Bright field images are acquired with the halogen lamp set to 4 volts and a camera exposure time of 500 msec.
Image Processing
14. A series of background images (20) of an empty chamber are acquired on each
day of experimentation for YFP imaging. The images are averaged to create an
average background image.
15. The average background is subtracted from each plane of the experimental
image.
16. Images are cropped to generate a panel that is 300 pixels or 38.4 µm on each
side.
17. The 12-bit images are scaled between 0 and the highest intensity prior to
converting to 8-bit images (0 to 255) for display.
18. The original 12-bit intensity scale is shown on the bottom left of each image.
19. Additional information such as the construct name and the scale bar are stamped
on the images.
20. Montages of the images are generated to view relevant planes simultaneously.
21. Montages are presented horizontally as black and white images, with the YFP to
the left and the bright field to the right.

Reagents and Materials
Ringer's solution, pH 7.4, 1X: AfCS Solution Protocol ID PS00000426

HEPES buffered Hanks' balanced salt solution, pH 7.35, 1X (low endotoxin): AfCS
Solution Protocol ID PS00000578

Axiovert 200 motorized microscope stand: Carl Zeiss, Inc.; catalog no. 0000001005820

Zeiss immersion 518F objective oil: VWR International; catalog no. 41800-488

Plan-Apo NA 1.4 100x oil objective: Carl Zeiss, Inc.; catalog no. 440782

Yokagawa spinning disk head with excitation and emission filter wheels, model CSU10
UltraVIEW live cell imager confocal scanhead: PerkinElmer; catalog no. 611005

CFP/YFP dual dichroic in Yokagawa head: Chroma Technology Corp.; 442-514T PC

Helium-cadmium laser, 100 mW: Kimmon Electric Co., Ltd.; catalog no. IK4101R-F

DR1601C-F

Argon-ion laser, 300 mW: Melles Griot; catalog no. 35LAL030-220

Dichroic in fiber: Chroma Technology Corp.; catalog no. 460DCLP

Laser-to-fiber coupler (514 nm): OZ Optics Ltd; part no. HPUC-23AF-514-S-3.9AS-2
Laser-to-fiber coupler (442 nm): OZ Optics Ltd; part no. HPUC-23AD-442-S-6.2AS-11
Laser fiber combiner: OZ Optics Ltd.; part no. WDM-12P-111-442/514-3.5/125-SSS-40-3AF3AF3S-3-3-SP
YFP excitation filter: Chroma Technology Corp.; catalog no. D515/10x
Optical power meter and sensor: Advantest; catalog nos. TQ8210 and Q82017A, respectively
Cooled CCD camera, Photometrics CoolSNAP HQ: Roper Scientific; catalog no. CoolSNAP HQ
YFP emission filter: Chroma Technology Corp.; catalog no. HQ565/75m
Image acquisition and analysis software, the latest version of Metamorph: Universal Imaging Corp.; catalog no. 31290
1 GHz Pentium III computer manufactured by Omni Tech Corporation with 1 GigaByte of RAM: Universal Imaging Corp.; catalog no. OT1010

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