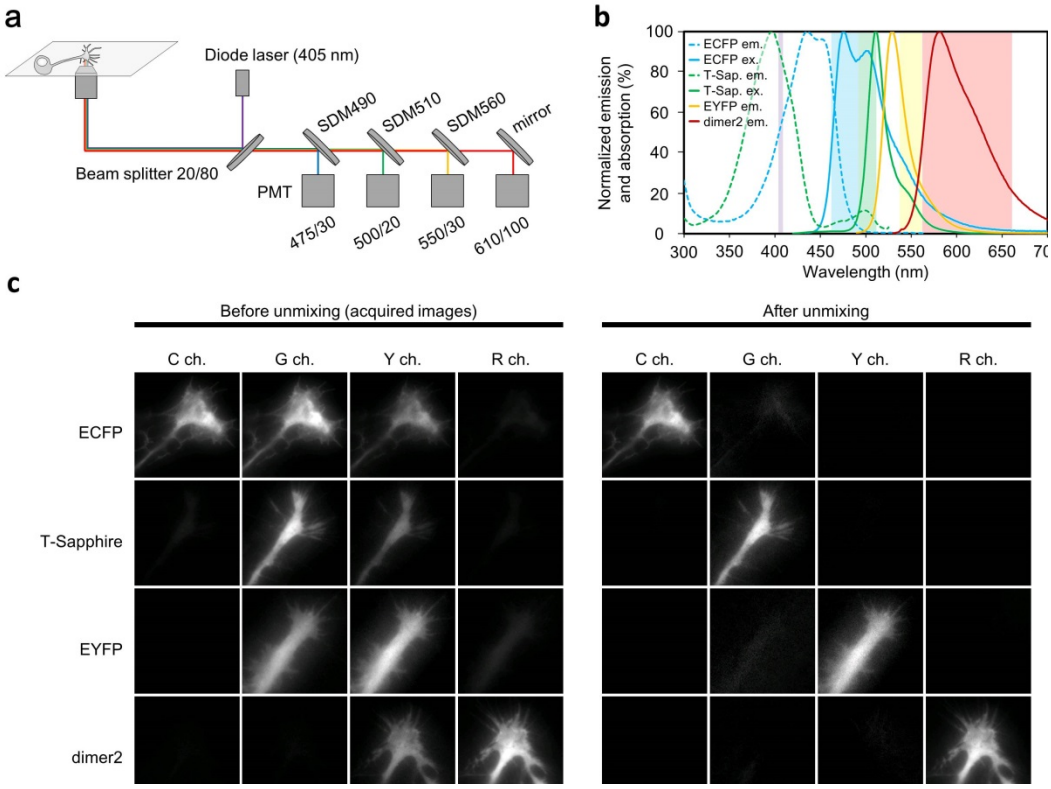
Supplementary Information

Title: Crosstalk between Second Messengers Predicts the Motility of the Growth Cone

Author names: Takahiko Kobayashi, Fumiaki Nagase, Kohji Hotta and Kotaro Oka*

Supplementary Figure 1



Supplementary Figure 1

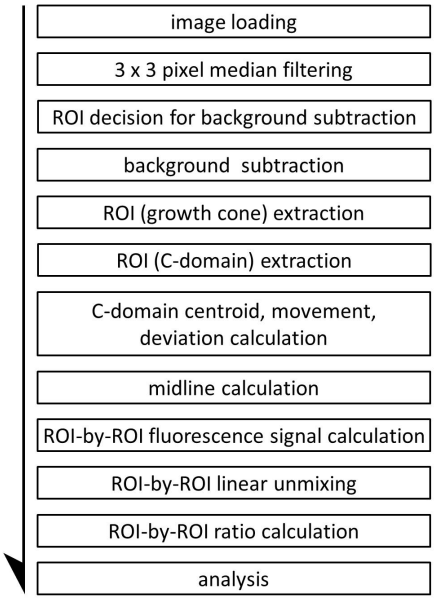
Microscope for simultaneous imaging of dual FRET pairs.

(**a**) Schematic representation of the optical setup with single excitation and four-color fluorescent channel detection. The numbers after "SDM" and below the photomultiplier tube (PMT) indicate the wavelength and wavelength/half-bandwidth (nm), respectively.

(**b**) Excitation spectra of FRET donors (broken line) and emission spectra of FRET donors and acceptors (solid line). The cyan, green, yellow, and red areas represent each detection channel.

(**c**) Acquired images in each detection channel (left) and the images after linear unmixing (right) of DRG growth cones expressing each fluorescent protein.

Supplementary Figure 2



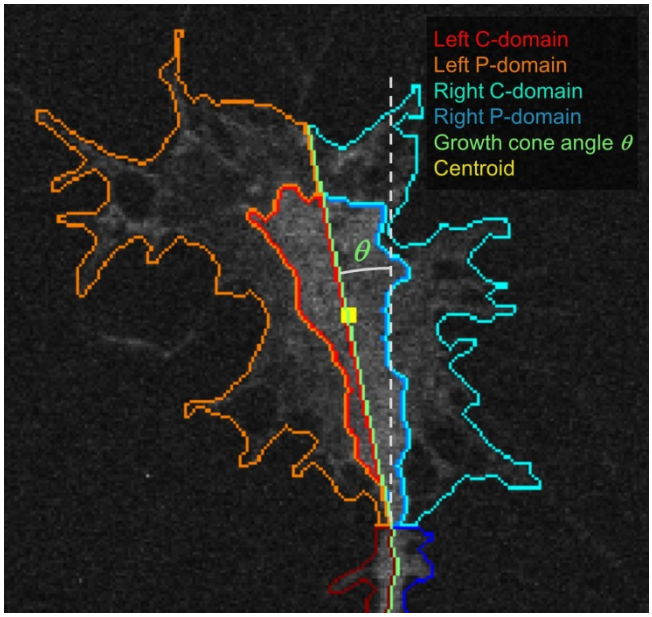
$$R = \frac{\left(F_{cell} - F_{background}\right)_{donor}}{\left(F_{cell} - F_{background}\right)_{acceptor}}$$

Supplementary Figure 2

Flow chart of image processing and analysis.

All processing and analysis software were written in MATLAB. Any regions except cell region were chosen to decide ROI for background subtraction. Background intensity was averaged within the ROI and subtracted from each pixel. ROIs of growth cone and C-domain were determined by thresholding the fluorescence images using thresholds that are selected manually. Movement, deviation and midline were calculated as described in **Supplementary Fig. 3**. ROI-by-ROI linear unmixing was applied to fluorescence signal averaged within the ROIs as previously described[11]. ROI-by-ROI ratio was calculated as represented formula in case of Epac1-camps and red cGES-DE5. Numerator and denominator were replaced when using SapRC2.12.

Supplementary Figure 3

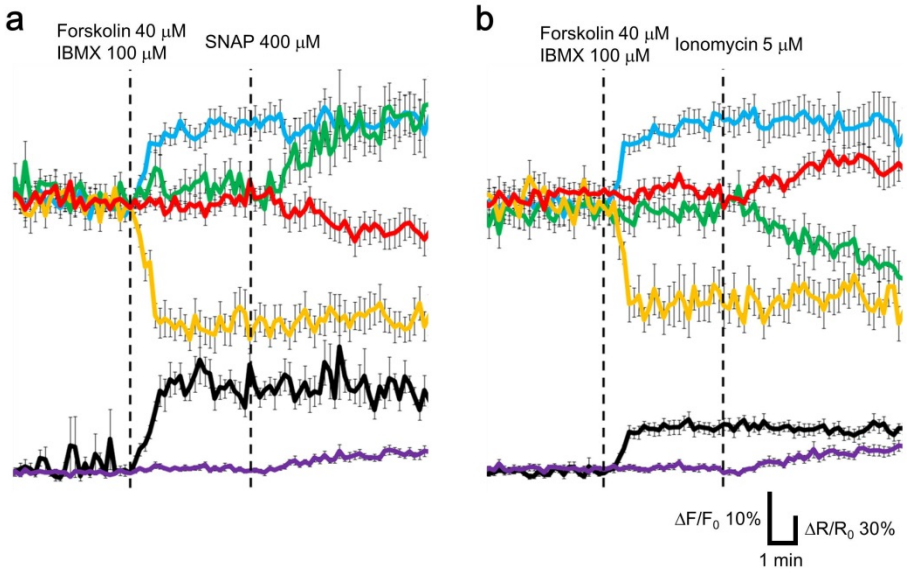


Supplementary Figure 3

Definition of regions, angles, and movement of growth cones.

The growth cone edge and central-domain (C-domain) edge were determined as described in **Supplementary Fig. 2**. The midline of the growth cone was defined using a straight line that links the center of the growth cone-side terminal of the axon to the centroid of the C-domain. The left- and right-sides of the growth cone were divided by the midline of the growth cone. The growth cone angle was defined as the angle between the vertical line and the midline of the growth cone. Growth cone movement was calculated from the position of the centroid of the C-domain.

Supplementary Figure 4



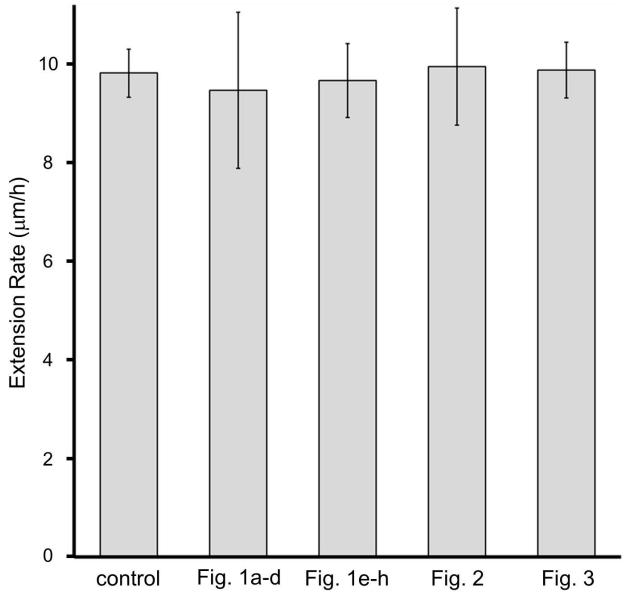
Supplementary Figure 4

Responses of the dual FRET sensors to pharmacological stimuli in DRG neurons.

(**a**) Simultaneous imaging of cAMP and cGMP in DRG neurons. The cyan, green, yellow, red, black, and purple lines indicate the fluorescence intensity of ECFP, T-Sapphire, EYFP, and dimer2, and the cAMP and cGMP levels, respectively. The cells were first stimulated with 40 μM forskolin and 100 μM isobutyl-methylxanthine (IBMX; Sigma-Aldrich), and subsequently with 400 μM S-nitroso-N-acetylpenicillamine (SNAP; Sigma-Aldrich; n = 16).

(**b**) cAMP and Ca^{2+} responses to pharmacological stimuli in DRG neurons revealed by simultaneous imaging. The black and purple lines indicate the levels of cAMP and Ca^{2+}, respectively. The other colored lines are the same as in **a**. The cells were first stimulated with 40 μM forskolin and 100 μM IBMX, and subsequently with 5 μM ionomycin (Sigma-Aldrich; n = 16).
Data are presented as means±s.e.m.

Supplementary Figure 5

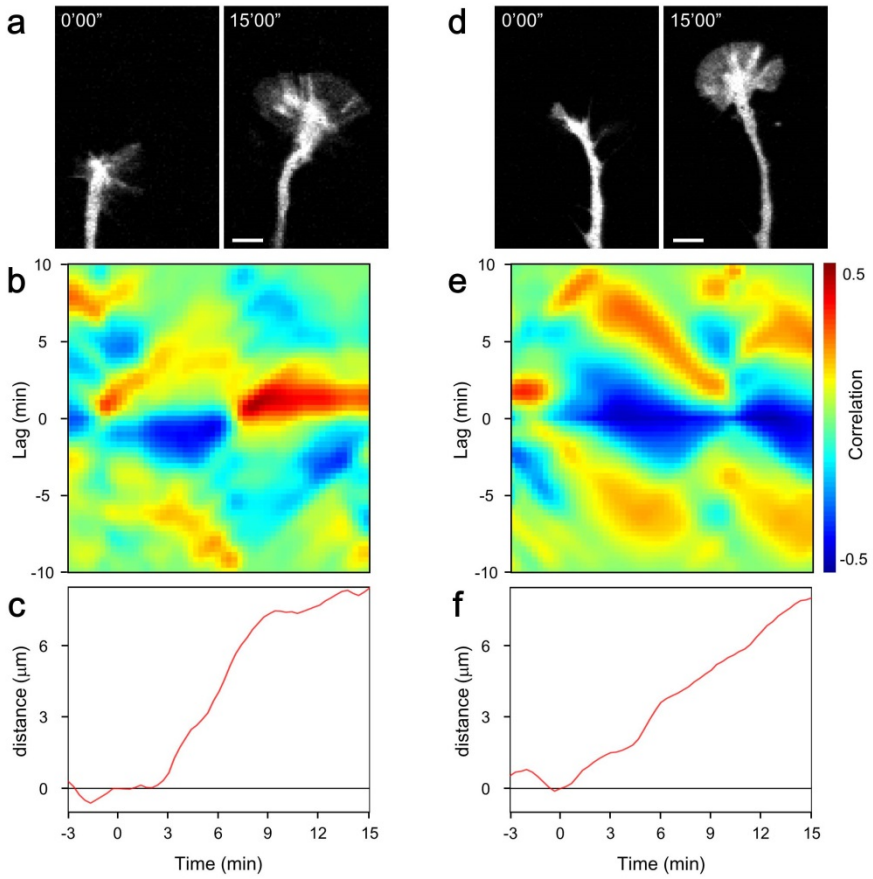


Supplementary Figure 5

Influence of imaging and expressing FRET sensors on axon extension.

The rate of axon extension was unaffected by dual FRET imaging in each experiment (15, 13, 27, 14, and 11 extensions for control, Figs. 1a–h, 2, and 3). Control data were acquired in the absence of transfection and excitation.

Supplementary Figure 6



Supplementary Figure 6

Typical correlations between cAMP, Ca^{2+}, and extension in freely extending growth cones.

(**a**) Time-lapse fluorescence images of a freely extending growth cone co-expressing Epac1-camps and SapRC2.12 at the onset of extension and at 15 min after. Scale bar represents 5 μm.

(**b**) Sequential time course of the cross correlation function between the short-term dynamics of cAMP and Ca^{2+}. The start of growth cone extension was as defined at 0 min (**Fig. 3e**).

(**c**) Cumulative distance during observation of the growth cone.

(**d–f**) Another example of **a–c**.