SI Methods

Simulation of Bimolecular Kinetics. We modeled binding kinetics of Calcium Green-5N and Ca^{2+} ions according to the method of Salmon *et al.* (1). Numerical simulations were used to model a bimolecular reaction such as

$$A + B \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} C$$

in the mixing device. The flow velocity $\vec{V}$ in the device is governed by the steady-state incompressible Navier–Stokes equation,

$$\rho(\vec{V} \cdot \nabla)\vec{V} = -\nabla P + \eta \nabla^2 \vec{V} \qquad \textbf{[3]}$$

where ρ is the fluid density, P is the pressure, and η is the viscosity. The local concentrations of the reactants, C_A, C_B, and C_C, are obtained from the diffusion and convection equation,

$$\vec{V} \cdot \nabla C_A = -k_{on} C_A C_B + k_{off} C_C + D_A \nabla^2 C_A$$

$$\vec{V} \cdot \nabla C_B = -k_{on} C_A C_B + k_{off} C_C + D_B \nabla^2 C_B$$

$$\vec{V} \cdot \nabla C_C = +k_{on} C_A C_B - k_{off} C_C + D_C \nabla^2 C_C \qquad \textbf{[4]}$$

where D_A, D_B, and D_C are the diffusion coefficients of the reactants, k_{on} is the association rate constant of A and B, and k_{off} is the dissociation rate constant. These partial differential equations with boundary conditions appropriate for the mixer are numerically solved with a finite element method, using the commercial program COMSOL Multiphysics.

Mutagenesis, Expression, and Purification of Calmodulin. The calmodulin (CaM) expression plasmid pCR2, containing the sea urchin CaM cDNA (identical in predicted amino acid sequence

to vertebrate CaM) in the bacterial expression vector pET23d (Novagen), was generously provided by Carol Rohl (University of California, Santa Cruz) and Rachel Klevit (University of Washington, Seattle). Introduction of a single Cys residue by conversion of Lys at amino acid position 75 to Cys to produce CaM(C75) was accomplished with the QuikChange site-directed mutagenesis kit (Stratagene). The presence of the desired mutation and the absence of artifacts were verified by DNA sequencing. BL21(DE3)plysS (Novagen) cells expressing CaM were grown and harvested, and recombinant CaM was purified as described previously (2) with minor modifications. Cell pellets were resuspended in lysis buffer [50 mM Tris (pH 7.5), 10 mM EDTA], freeze-thawed three times, and sonicated. After heating to 70°C in a boiling water bath, the lysate was clarified by centrifugation at $100{,}000 \times g$ for 1 h at 4°C. 2.5 M ammonium sulfate was added to the supernatant, which was then centrifuged at $12{,}000 \times g$ for 10 min at 4°C. The supernatant was retained, and an additional 2.5 M ammonium sulfate was added. After centrifugation at $12{,}000 \times g$ for 10 min at 4°C, the pellet was dissolved in 100 ml of buffer A [50 mM Tris (pH 7.5), 1 mM EDTA, 200 mM ammonium sulfate] and loaded onto a 30-ml phenyl-Sepharose column preequilibrated in buffer A. 2.5 mM $CaCl_2$ was added to the collected flow-through and applied to a second phenyl-Sepharose column (20 ml) preequilibrated in buffer A plus 2.5 mM $CaCl_2$. The second column was washed in buffer A plus 0.5 mM $CaCl_2$, followed by elution with 50 mM Tris (pH 7.5)/1 M NaCl/5 mM EGTA. CaM was detected by monitoring the absorbance at 276 nm. After purification, the pooled fractions were concentrated (Ultrafreespin-15 Centrifugal Units, Biomax-5; Millipore) and dialyzed in 50 mM Mops (pH 7.0). The yield was 100 mg of CaM per 500 ml of bacterial culture. CaM was quantified by a modified Bradford protein assay (Bio-Rad) and was homogeneous as judged by SDS-PAGE. Purified CaM preparations were stored at –20°C or –80°C. Wild-type CaM was prepared in a similar manner.

Labeling of CaM(C75) with Acrylodan. CaM labeling was performed as described previously (3) with the following modifications. CaM(C75) was reduced in equimolar DTT in 50 mM Hepes (pH 7.5) and slowly shaken for 30 min at 30°C. Immediately before addition to the labeling reaction, Acrylodan (Molecular Probes) was dissolved in *N*,*N*'-dimethylformamide at a concentration of 20 mM and was added in 6.5-fold molar excess over both protein and DTT. The reaction was allowed to proceed overnight at 4°C and was quenched using 10 mM 2-

mercaptoethanol. The labeled protein was purified using a 15-cm Bio-Gel P6-DG (Bio-Rad) column followed by exhaustive dialysis against 50 mM Mops (pH 7.0). Final protein concentrations were determined using the BCA assay (Pierce) with wtCaM as the standard. The amount of bound probe was determined by absorbance spectroscopy using an extinction coefficient at 391 nm of 20,000 $M^{-1}\bullet cm^{-1}$. Probe to protein ratios were 0.5 to 0.9. Full characterization of the fluorescently labeled protein was performed to determine its ability to activate αCaM-kinase II and its spectral properties over a physiological range of parameters, such as pH, calcium concentration, and ionic strength.

Protein Kinase Assay. The activity of recombinant αCaM-kinase II (10 ng per assay) was determined in the presence of increasing concentrations of recombinant bacterial-expressed CaM (wtCaM) or CaM_{Acr}. Serial dilutions of the CaM stocks were made in 5 mM Mops buffer with 0.1 mg/ml BSA. CaM at the final concentrations indicated in SI Fig. 7 were added to reaction mixtures containing 25 mM Hepes (pH 7.0), 70 mM KCl, 0.5 mM $CaCl_2$, 10 mM $MgCl_2$, 100 μM ATP, 2 μCi of [γ-^{32}P]ATP, 50 μM syntide (PLRRTLSVAA), and 0.4 mM DTT. αCaM-kinase II was diluted in 10 mM Mops (pH 7.0), 200 mM KCl, 0.1% Tween 20, and 1.0 mg/ml BSA. Reactions were prewarmed for 1 min at 30°C, initiated by the addition of enzyme (10 ng), and terminated after 30 s. Twenty microliters of the reaction mix was spotted onto a Whatman P-81 filter paper and the bound radioactivity was quantified by Čerenkov counting. Activity is expressed as μmol/min/mg as described previously (4). The experimental data were fit to the following form of the Hill equation

$$A = \frac{A_{\max}}{1 + 10^{N(\log CaM_{50} - \log[CaM])}} \quad \textbf{[5]}$$

where A is the activity at a given CaM concentration, A_{max} is the maximal activity, N is the Hill coefficient, and CaM_{50} is the concentration of CaM that yields 50% of maximal activity.

Characterization of Microfluidic Mixer. Mixing devices were fabricated and characterized as described (5). Briefly, the microstructures were imprinted on Zeonor 1020R (Zeon Chemicals) and sealed with a glass coverslip coated with a thin layer of RTV 615 (GE Silicones). Flow-

speed profiles inside the mixer were measured with fluorescence correlation spectroscopy (FCS). 10 nM Rhodamine Green (Invitrogen) in CaM dilution buffer was injected into the five inlet ports of the mixer. The device was mounted onto an inverted microscope (IX71; Olympus) equipped with an *x*–*y* translation microscope stage (MS2000; Applied Scientific Instrumentation) to allow FCS measurements at regularly spaced locations in the entire region of fluid mixing. The back aperture of a water immersion objective (UApo/340 ×40/1.15 N.A.; Olympus) was overfilled with 488-nm light of a solid-state diode-pumped laser (Sapphire 488-20; Coherent). The laser power at the sample was 18 μW and 70 μW for diffusion and flow-speed measurements, respectively. The fluorescence passed through a dichroic beam splitter and a long-pass filter (510DCLP and 510LP; Chroma Technology) and was imaged onto a 50-μm core, 50/50 fiber splitter (Oz Optics). The two output ends of the fiber were coupled to two identical avalanche photodiodes (PerkinElmer Optoelectronics). The signals from two outputs were cross-correlated by a Correlator card to eliminate the artifacts from detector afterpulsing at short time scales. The data acquisition time at each location within the mixer was 10 s, and five curves were averaged.

The FCS curves were taken in the vertical center plane of the device at 5-μm increments. In the absence of the driven flow, we measured the characteristic diffusion time τ_{diff} by fitting the FCS curve with

$$G_{diff}(\tau) = \frac{1}{N}\left(1+\frac{\tau}{\tau_{diff}}\right)^{-1}\left(1+\frac{\tau}{w^2\tau_{diff}}\right)^{-\frac{1}{2}} \qquad \textbf{[6]}$$

where N is the average number of molecules in the focal volume, and w is the ratio of axial to lateral dimensions of the focal volume. We determined the $1/e^2$ focal radius of the beam, r_0, using the diffusion coefficient of Rhodamine Green, $D_{RG} = (3.01 \pm 0.08) \times 10^{-6}$ cm^2/s (6), in the absence of the driven flow. For the flow-speed measurement, FCS curves were fit using a model of uniform translation with diffusion:

$$G_{diff\,\&\,flow}(\tau) = \frac{1}{N}\left(1+\frac{\tau}{\tau_{diff}}\right)^{-1}\left(1+\frac{\tau}{w^2\tau_{diff}}\right)^{-\frac{1}{2}} \exp\left\{-\left(\frac{\tau}{\tau_{flow}}\right)^2\left(1+\frac{\tau}{\tau_{diff}}\right)^{-1}\left(1+\frac{\tau}{w^2\tau_{diff}}\right)^{-\frac{1}{2}}\right\} \quad \textbf{[7]}$$

where τ_{flow} is the characteristic flow time (7, 8). τ_{diff} and w were fixed at the values obtained from the measurement in the absence of the flow. N and τ_{flow} were obtained from the best fit to each FCS curve. Finally, the flow speed V was calculated from

$$V = \frac{r_0}{\tau_{flow}}.$$

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