Supporting Online Material for

Orai1 determines calcium selectivity of an endogenous TRPC heterotetramer channel

Donna L. Cioffi[1,5], Songwei Wu[2,5], Hairu Chen[2,5], Mikhail Alexeyev[3,5], Claudette M. St. Croix[6], Bruce R. Pitt[6], Stefan Uhlig[7], and Troy Stevens[2,4,5]

Departments of Biochemistry and Molecular Biology[1], Pharmacology[2], Cell Biology and Neuroscience[3], and Medicine[4], Center for Lung Biology[5], University of South Alabama, Mobile AL 36688; Department of Environmental and Occupational Health[6], University of Pittsburgh, Pittsburgh PA 15219; Institute of Pharmacology and Toxicology[7], University Hospital Aachen, RWTH Aachen, Wendlingweg 2, 52074 Aachen

SUPPORTING ONLINE MATERIAL

Materials and Methods

Reagents. Reagents were purchased from Sigma-Aldrich Co. (St. Louis, MO) unless otherwise noted. TRPC antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), Sigma-Aldrich Co. (St. Louis, MO), Millipore Corp. (Billerca, MA) or received as gifts. Additionally, TRPC4 antibody was received from Dr. M. Zhu (The Ohio State University), TRPC1 antibody was received from Dr. I. Ambudkar (National Institutes of Health, Secretory Division) and protein 4.1 antibody was received from Dr. S.R. Goodman (Syracuse University). ImmunoPure® Immobilized Protein A was purchased from Thermo Scientific, Inc. (Rockford, IL).

Isolation and Culture of Pulmonary Artery and Microvascular Endothelial Cells. Extra-lobar pulmonary arteries are isolated, inverted, collagenase treated, scraped into primary cultures, and grown in standard cell culture media supported with 10-20% fetal or calf bovine serum. Cultures are intensely selected in the first weeks after isolation to eliminate contaminating cells, and enrich for and expand pure populations of pulmonary artery endothelial cells. Once expanded, these cells exhibit a cobblestone morphology that is typical of endothelium. At confluence, pulmonary artery endothelial cells reach a baseline resistance near 1200-1400 Ω, measured using the electrical cell-substrate impedance sensing approach. Upon trypsinization and reseeding, these cells grow to confluence with a doubling time of approximately 57 hours. They express typical endothelial cell markers, including endothelial cell nitric oxide synthase, vascular endothelial cell adhesion molecule (CD144), and platelet-endothelial cell adhesion molecule (CD31), and they display typical endothelial cell behaviors, such as the uptake of low density lipoprotein and formation of vascular networks on Matrigel *in vitro* and *in vivo*. Pulmonary artery endothelial cells interact with *Helix pomatia*, but not with *Griffonia simplicifolia*.

Pulmonary microvascular endothelial cells are isolated by a modification of the pleural cut technique, in which lungs are dissected approximately 0.5 mm deep along the pleural surface, minced into small pieces, exposed to collagenase and strained. Cells collected following straining are seeded onto culture plates, and grown into primary cultures in standard cell culture media supported with 10-20% fetal or calf bovine serum. Cultures are intensely selected in the first weeks after isolation to eliminate contaminating cells, and enrich for and expand pure populations of pulmonary microvascular endothelial cells. Once expanded, these cells exhibit a cobblestone morphology that is typical of endothelium. At confluence, pulmonary microvascular endothelial cells reach a baseline resistance near 1600-2000 Ω, using the electrical cell-substrate impedance sensing system. Upon trypsinization and reseeding, they grow to confluence with a doubling time of approximately 39 hours. They express typical endothelial cell markers, including endothelial cell nitric oxide synthase, vascular endothelial cell adhesion molecule (CD144), and platelet-endothelial cell adhesion molecule (CD31), and they display typical endothelial cell behaviors, such as the uptake of low density lipoprotein and formation of vascular networks on Matrigel *in vitro* and *in vivo*. Pulmonary microvascular endothelial cells interact with *Griffonia simplicifolia*, but not with *Helix pomatia*.

Isolation of Caveolin-Rich Fractions from the Intact Pulmonary Circulation. Rat lungs were perfused through the pulmonary artery at a constant hydrostatic pressure (12 cm H_2O) with Krebs-Henseleit-buffer (containing 2% albumin, 0.1% glucose, 0.3% HEPES) and ventilated with 2 mL tidal volume at 60 breaths/min.

Membrane fractions from endothelial cells were isolated by use of colloidal silica beads essentially as described. Briefly, after 40 min of lung perfusion, the flow rate was reduced to 2-3 mL/min and perfusion with 1% cationic colloidal silica beads was started. Lung tissue was homogenized and the homogenate was mixed with an equal volume of 1.02 g/ml Nycodenz (containing 20 mM KCl) and layered over 0.5-0.7g/ml Nycodenz containing 60 mM sucrose in a centrifuge tube. After centrifugation (Beckman SW 28 rotor, 20000 rpm) for 30 min at 4°C the floating tissue debris was removed and the pellet containing the silica-coated endothelial membrane fragments was resuspended with 1ml MBS buffer (25 mM MES, pH 6.5, 150 mM NaCl).

Triton-X-100 (final concentration 1%, 4°C) was added to the membranes for 60 min. After incubation the suspension was homogenized and the homogenate mixed with 80% sucrose to achieve a 40% membrane-sucrose-solution. A 30-5% sucrose gradient was layered on top. Samples were centrifuged in a Beckman-centrifuge (SW55Ti rotor, 30000 rpm) at 4°C overnight for 16-18h. Fractions were sampled from the top to the bottom. The fraction used for this study represents caveolae; it was rich in caveolin-1 and flotillin (caveolae marker proteins), but was lacking angiotensin converting enzyme (a non-caveolar plasma membrane protein).

Membrane/Cytoskeleton Fractions and Immunoprecipitation. Endothelial cell monolayers were lysed and scraped in a lysis buffer. The lysate was then centrifuged at 3000 x g, the pellet was resuspended in a sucrose buffer, homogenized and centrifuged at 3000 x g. The supernatant was then divided into aliquots and centrifuged at 50,000 x g. The resulting pellet was incubated in extraction buffer in the presence of KI for 40 minutes at 4° C, and then was centrifuged at 145,000 x g to resolve a pellet fraction and a salt-dissociated supernatant fraction.

Preparation of Isolated Immunocomplex. Protein 4.1 antibody was incubated with ImmunoPure® Immobilized Protein A beads for 4-5 hours. The antibody affixed beads were washed once in extraction buffer plus 100 mM KI. Salt-dissociated supernatant (40 μL or 8 μL) and protease inhibitor cocktail was added to the washed beads. The mixture was incubated at 4° C overnight. Beads were washed five times with extraction buffer/100 mM KI and stored under extraction buffer/100 mM KI at -80 °C.

SDS-PAGE and Western Blot. Samples were run on 4-12% Bis-Tris Gels (Invitrogen Corp.) and transferred to nitrocellulose overnight at 30V and 4° C. Membranes were rocked in blocking buffer [phosphate-buffered saline with 5% non-fat dry milk/0.1% Tween 20 or Protein-free (TBS) blocking buffer (Thermo Scientific, Inc.) with 0.05% tween] for 1 hour at room temperature, primary antibody for either 2 hours at room temperature or overnight at 4 °C and then secondary antibody (horseradish peroxidase conjugated) for one hour at room temperature. Proteins were visualized using SuperSignal West Pico or West Femto Chemiluminescent Substrates (Thermo Scientific, Inc.).

Fluorescently Tagged Antibodies. Antibodies were tagged with the cyanine reagents cy3 and cy5 following the manufacturer's directions (Cy3 mAb Labeling Kit #PA33001 and Cy5 mAb Labeling Kit #PA35001; GE Healthcare Bio-Sciences Corp.; Piscataway, NJ).

Förster resonance energy transfer (FRET) Experiments. To construct an antibody dose response curve, isolated immunocomplex (prepared above) was divided into eight aliquots. IgG (0.5 to 1.0 mg) from rabbit serum in 0.05% Triton X-100/PBS was added to bind free protein A sites on the beads. Samples were rotated overnight at 4 °C, then washed once with 0.05% Triton X-100/PBS. Fluorescently tagged antibody was added to each sample over a range of

concentrations. Samples were rotated at 4 °C for 5 – 6 hours, then washed three times with 0.05% Triton X-100/PBS. Samples were re-suspended in 0.05% Triton X-100/PBS, transferred to a 96 well plate, and fluorescence counts read on a Spectra Max M5 plate reader (Molecular Devices; Sunnyvale, CA). Fluorescence counts were plotted as a function of antibody concentration.

For FRET experiments, isolated immunocomplex was divided into eight aliquots, rotated overnight in the presence of 0.5 to 1.0 mg IgG from rabbit serum in 0.05% Triton X-100/PBS and washed once with 0.05% Triton X-100/PBS. Cy3- and Cy5-tagged antibodies to the TRPC subunits were added to the samples at their EC_{50} concentrations. Samples were rotated at 4 °C for 5 – 6 hours, washed three times with 0.05% Triton X-100/PBS and resuspended in 0.05% Triton X-100/PBS. Samples were transferred to glass slides containing mounting medium and a cover slip placed on top. FRET experiments were performed on a Leica TCS SP2 Confocal Microscope (63x oil objective) using the sensitized emission approach. Spectral bleed-through was corrected for by measuring donor only and acceptor only samples. Regions of interest (roi's) were selected and FRET efficiency calculated by the Leica software using the formula $E_A(i) = [B-Axb-Cx(c-axb)]/C$. E_A is the apparent FRET efficiency; A,B,C are the donor, FRET and acceptor channel intensities, respectively; a,b,c are correction factors. FRET signals, as determined via the sensitized emission approach, were confirmed using acceptor photobleaching.

Engineering Conditional Expression of Orai1 shRNA. Doxycycline-regulated knockdown in the rat pulmonary artery endothelial cells (PAECs) was performed using a hybrid retro-lentiviral system. Low passage PAECs first infected with retrovirus 2641, which encodes the Long Terminal Repeat (LTR)-promoter driven reverse tetracycline transactivator (rtTA) gene component of the Tet-On Advanced system (Clontech, Mountain View, CA). Infection was achieved by incubating target cells in 35 mm dishes at 20% confluence with retrovirus-containing supernatants overnight 8 µg/ml polybrene (Sigma–Aldrich Corp., St. Louis, MO). The next day supernatant was removed and cells were allowed to recover for 24 hours in DMEM, after which time cells were trypsinized, transferred into 140 mm dishes and blasticidin selection (30 µg/ml) was applied for 5 days. shRNA-encoding oligonucleotides were designed against coding sequence of Orai1 using BLOCK-iT RNAi Designer program (Invitrogen, Carlsbad, CA), and cloned into *Bfu*AI-digested lentiviral vector pMA2867. The identity of cloned oligonucleotides was verified by sequencing, and lentivirus-containing supernatants were produced from the resulting plasmids by $CaPO_4$-mediated transfection of the HEK293FT cell line using established protocols. Gag, Pol and Env functions for lentiviral constructs were provided in trans by cotransfection of the vector plasmid with two helper plasmids, psPAX2 and pMD2.G (Addgene, Cambridge, MA). Lentivirus infection of rtTA expressing cells was conducted similarly to retroviral infection described above, except puromycin selection (1 µg/ml) was applied for at least 3 days.

Patch Clamp Electrophysiology. Transmembrane current recordings were performed using the conventional whole-cell voltage-clamp configuration in single pulmonary artery endothelial cells by the standard giga seal patch-clamp technique. Confluent cells were trypsin dispersed, seeded onto 35-mm plastic culture dishes, and allowed to reattach at least 4 hours before patch-clamp experiments were performed. Patch-clamp recordings were obtained from single electrically isolated pulmonary artery endothelial cells, exhibiting a flat, polyhedral morphology consistent with the cells from a confluent monolayer. Recording pipettes were heat polished to produce a tip resistance in the range of 3 to 5 megohms in the pipette (internal) solution. Currents were recorded with a computer-controlled EPC9 patch-clamp amplifier (HEKA; Lambrecht, Germany). The step-wise voltage pulses were applied from -100 to +60 mV in 20-mV increments after the whole-cell configuration was achieved, with a 200-ms duration during each voltage step and a 2-s interval between steps. The holding potential between each step was 0 mV. For the time-dependent current decay examination, this protocol was applied

approximately every 30 s. Data acquisition and analysis were performed with Pulse/PulseFit software (HEKA), current was measured as the mean value of the current amplitude during the last 20 ms of each step. Current decay was evaluated at a time corresponding to the peak of current (fraction of peak) at the -80 mV voltage pulse. All experiments were performed at room temperature (20-22 °C).

The standard pipette solution contained (in mmol/liter) 130 N-methyl-D-glucamine, 10 Hepes, 2 Mg^{2+}-ATP, 1 N-phenylanthranilic acid, 0.1 5-nitro-2-(3-phenylpropylamino)benzoic acid, 2 EGTA, 1 $Ca(OH)_2$ (pH 7.2, adjusted with methane sulfonic acid; the free $[Ca^{2+}]$ was estimated as 100 nmol/liter, as calculated by the CaBuf program <G. Droogmans, ftp://ftp.cc.kuleuven.ac.be/pub/ droogmans/cabuf.zip>). To examine calcium currents, the bath (external) solution contained (in mmol/liter) 120 aspartic acid, 5 $Ca(OH)_2$, 5 $CaCl_2$, 10 Hepes, 0.5 3,4-diaminopyridine (pH 7.4 adjusted with tetraethylammonium hydroxide). To examine the anomalous mole fraction effect, the initial Ca^{2+}-free external solution contained (in mmol/liter) 130 sodium methanesulfonate, 10 Hepes, 0.5 3,4-diaminopyridine, 10 BAPTA (pH 7.4 adjusted with tetraethylammonium hydroxide; the free $[Ca^{2+}]$ was estimated as below 1 nmol/liter). To obtain various free $[Ca^{2+}]$ in the bath solution, the appropriate amount of Ca^{2+}, in the form of $CaCl_2$ or $CaCl_2/Ca(OH)_2$, was added in as calculated with the CaBuf program. For instance, in the presence of 10 mmol/liter BAPTA, 6.928, 9.966, 11.000, or 20.000 mmol/liter Ca^{2+} was added to obtain 100 nmol/liter, 10 µmol/liter, 1 mmol/liter, or 10 mmol/liter free Ca^{2+}, respectively. All the solutions were adjusted to 290–300 mosmol/liter with sucrose.

Text

Enriching for the TRPC Channel. By incubating the salt dissociated supernatant with antibody bound to protein 4.1 conjugated to Protein A/agarose beads, a population of channels uniquely tethered to protein 4.1 was selected. Prior studies have established that this channel possesses TRPC4, as TRPC4 binds directly to protein 4.1. Functional studies reveal the protein 4.1-bound TRPC4 channel is calcium selective and, further, that other calcium non-selective channels are not inhibited by disrupting the protein 4.1-actin or protein 4.1-spectrin interaction. Hence, utilizing protein 4.1 as bait to enrich for the TRPC4-containing channel purifies a selective population of calcium channels (**Online Figure I**).

Establishing Antibody Half-Maximal Saturation. Prior to all FRET experiments full binding saturation curves were generated to establish the EC_{50} antibody concentration, which optimizes cy3-cy5 energy transfer. The TRPC1-cy5 binding curve is shown in **Online Figure II**. From these data a 70 μg/mL TRPC1-cy5 antibody concentration was calculated to represent the EC_{50}.

Determining Specificity of Intra-Molecular Interactions using FRET. Several control approaches were developed to ensure FRET signals are due to intra-channel subunit interactions. Dilution experiments were performed where one-fifth the amount of salt-dissociated supernatant was immunoprecipitated for protein 4.1. If the FRET measurements are non-specific, then the distance between adjacent TRPC1/4 channel complexes should increase as the sample becomes more dilute. In this instance, the FRET signal would be abolished. If the FRET measurements are specific, then the distance between individual proteins in the TRPC1/4 complex should not increase as the sample becomes more dilute. In this case, the FRET signal would be retained. To do this, FRET between cy3-labeled TRPC1 and cy5-labeled TRPC4 was tested. In undiluted samples, FRET was readily apparent between TRPC1 and TRPC4. The overall fluorescence intensity was decreased in all of the diluted samples compared to the undiluted samples. However, strong TRPC1-TRPC4 FRET signals were retained in the diluted samples (**Online Figure III**), demonstrating FRET detects intra-channel subunit interactions.

To confirm efficient energy transfer between cy3 and cy5 was occurring as predicted, photobleaching studies were undertaken. In this case, the TRPC4-cy5 channel was irradiated to photobleach fluorescence. Such inactivation of the cy5 channel prevented energy transfer from cy3-labeled TRPC1 to cy5-labeled TRPC4. Consequently, increased fluorescence was measured in cy3-labeled TRPC1, indicating the signal measured in the FRET channel is specific (**Online Figure IV**). Collectively, these data confirm that our FRET approach detects intra-channel subunit interactions.

Generating shRNA to Conditionally Knockdown Orai1. A series of shRNA targeting rat Orai1 mRNA were initially transiently transfected and screened for their ability to downregulate Orai1 protein (**Online Figure V**), allowing for development of a system in which the Orai1 shRNA could be introduced into cells, and then withdrawn, to test the role of Orai1 on TRPC1/4 channel function in wild type cells. To do this, pulmonary artery endothelial cells were engineered to express the Tet-On reverse transactivator protein (rtTA). The construct was stably introduced using a retro-lentiviral system (**Online Figure VI**). rtTA was followed by an internal ribosome entry site and a fusion between enhanced green fluorescent protein and blasticidin resistance gene. Once these cells were selected to homogeneity, they were re-infected with a second virus containing the shRNA sequence driven by doxycycline stabilized Tet (**Online Figure VI**). Cells expressing shRNA were selected using puromycin, and confirmed by measuring mCherry fluorescence.

Time-lapse movies. PAECs were grown to confluence on 35 mm plates in standard growth media, washed with physiological salt solution and placed on the microscope stage. Thapsigargin (1 μmol/L) was added to the media, and pictures were taken at 15-second intervals for a period of 1-hour. Pictures were then compressed to generate the movie. Doxycycline treatment was initiated daily prior to the application of thapsigargin. **Online Figure VII** shows the typical monolayer response to thapsigargin in cells with and without Orai1 (representative of 3 experiments/treatment). Orai1 silencing prevents gap formation. Studies using wild type endothelial cells revealed no effect of doxycycline treatment; doxycycline treatment did not prevent thapsigargin from inducing gaps (data not shown).

Figure Legends

Online Figure I. Schematic representing the protein 4.1-bound TRPC channel complex interacting with agarose beads. Protein 4.1 antibodies were bound onto agarose beads and used to isolate the endogenous TRPC1/4 channel. Channel proteins were then labeled with cy3 or cy5 to enable FRET measurements using the sensitized emission approach.

Online Figure II. Binding saturation curves reveal the half maximal antibody concentration needed to optimize cy3-cy5 energy transfer. TRPC1 antibody cy3 fluorescence was tested over a range of concentrations. Using this approach, the half maximal antibody concentration is determined for subsequent experiments.

Online Figure III. Dilution experiments reveal high FRET efficiency is retained even with lower total fluorescence. Labeled agarose beads were diluted 1:5, and FRET measurements repeated. Despite a decrease in total fluorescence (data not shown) under these experimental conditions, the FRET efficiency was retained, supporting the idea that FRET detected interactions between proteins within the channel complex.

Online Figure IV. Energy transfer from TRPC1-cy3 to TRPC4-cy5 is illustrated by photobleach of the cy5 channel. A region of interest (shown in the hatched black box) was identified and irradiated in the cy5 channel. Photobleach of the cy5 florescence prevented energy transfer from cy3 to cy5 channels within this region, resulting in increased cy3 fluorescence.

Online Figure V. shRNA sequences utilized to decrease Orai1 expression. The coding region for the rat Orai1 gene is shown, with shRNA target regions highlighted in red and green. The most effective shRNA sequence is shown red. The targets were selected and prioritized (based on the probability of successful knockdown) using Invitrogen's BLOCK-iT™ RNAi Designer program. Note that targets for shRNAs #1 and #2 overlap.

Online Figure VI. Map for the conditional expression of Orai1 shRNA in endothelium. Pulmonary artery endothelial cells were first infected with pMA2641 and selected to homogeneity using blasticidin. These purified cells were then infected with pMA2879 and selected to homogeneity using purimycin. Abbreviations: 5' and 3' mir, 5' and 3' flanking sequences derived from the murine mir-155 micro-RNA gene; Bsr, blasticidin resistance gene; EGFP, enhanced green fluorescent protein; HIV RRE, human immunodeficiency virus rev response element; IRES EMV, encephalomyelocarditis virus internal ribosome entry site; LTR, retro/lentiviral long terminal repeat; PAC, puromycin resistance gene; PSV40, SV40 promoter; P_{TET}, doxycycline-regulated promoter; tTA, tetracycline controlled transactivator protein; wPRE, woodchuck hepatitis virus post-transcriptional regulatory element.

Online Figure VII. Orai1 silencing prevents thapsigargin-induced inter-endothelial cell gap formation. Pulmonary artery endothelial cells engineered for conditional expression of Orai1 shRNA were grown in the presence (+) or absence (-) of doxycycline to confluence on 25 mm glass coverslips and imaged. Images were captured every 15-seconds for 1-hour, and the images compressed into a time-lapse movie. Thapsigargin (1 µM) was applied to the monolayer to activate store operated calcium entry. Whereas thapsigargin induced transient gap formation in the presence of Orai1, gap formation was not observed in cells lacking Orai1.
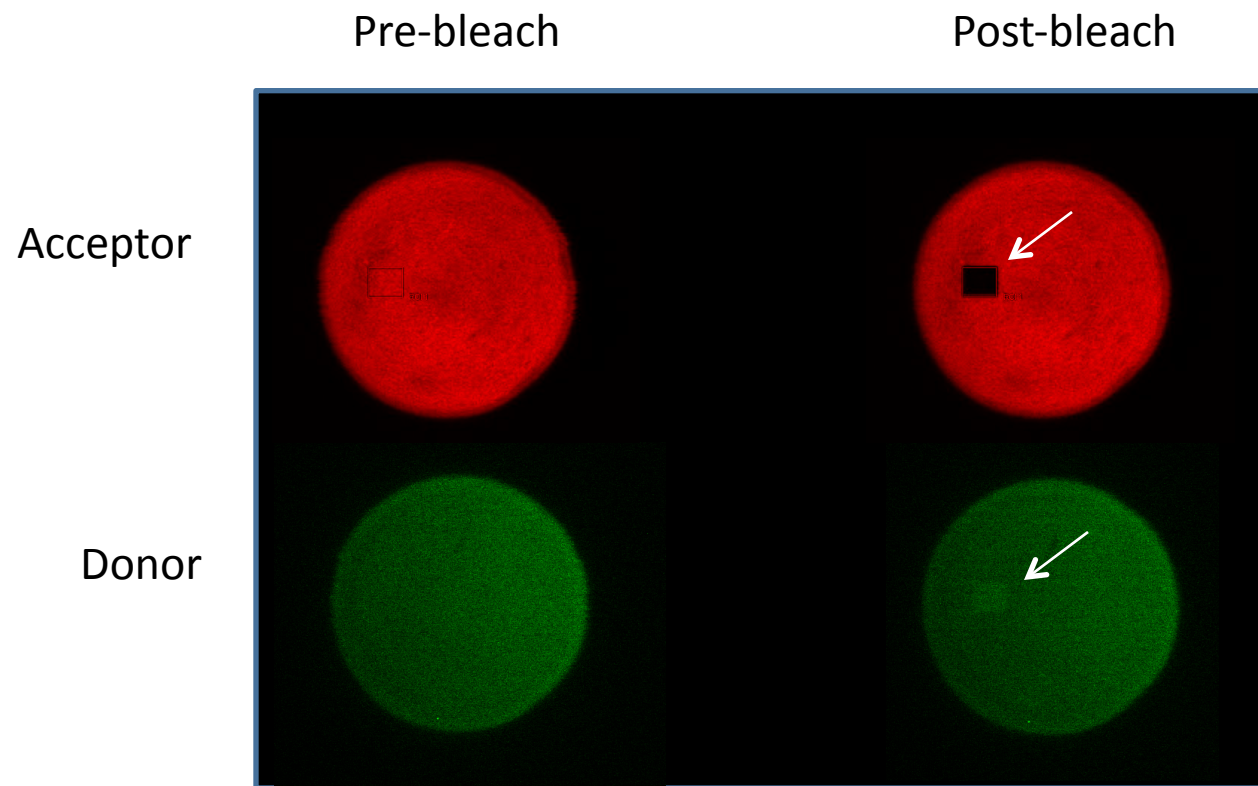
Excite Cy3

FRET

cy3

cy5

Emit Cy5

Protein A/Agarose Bead
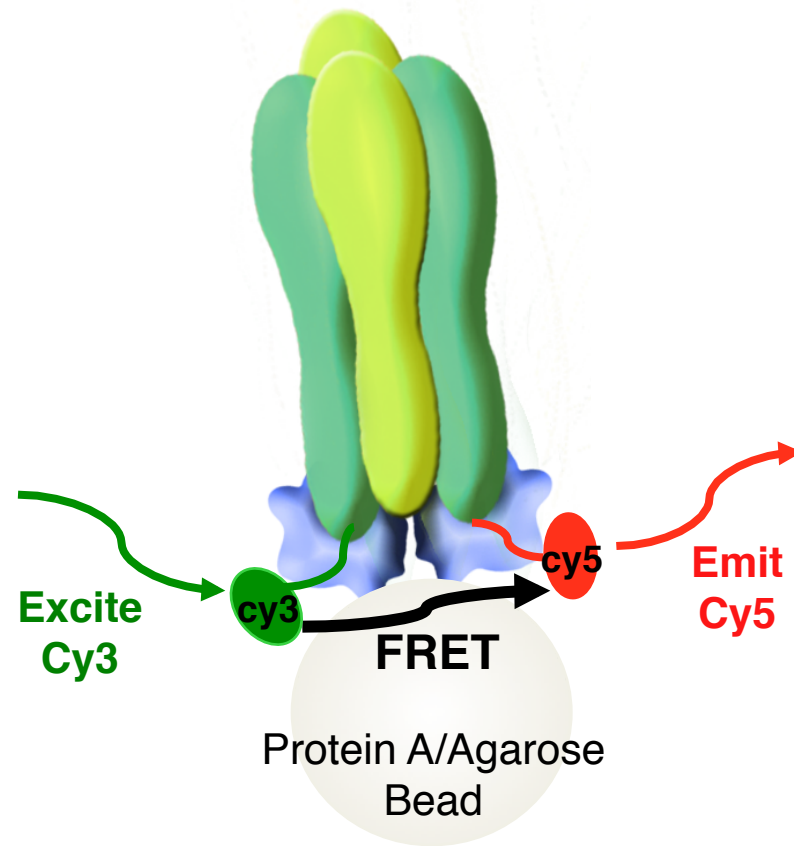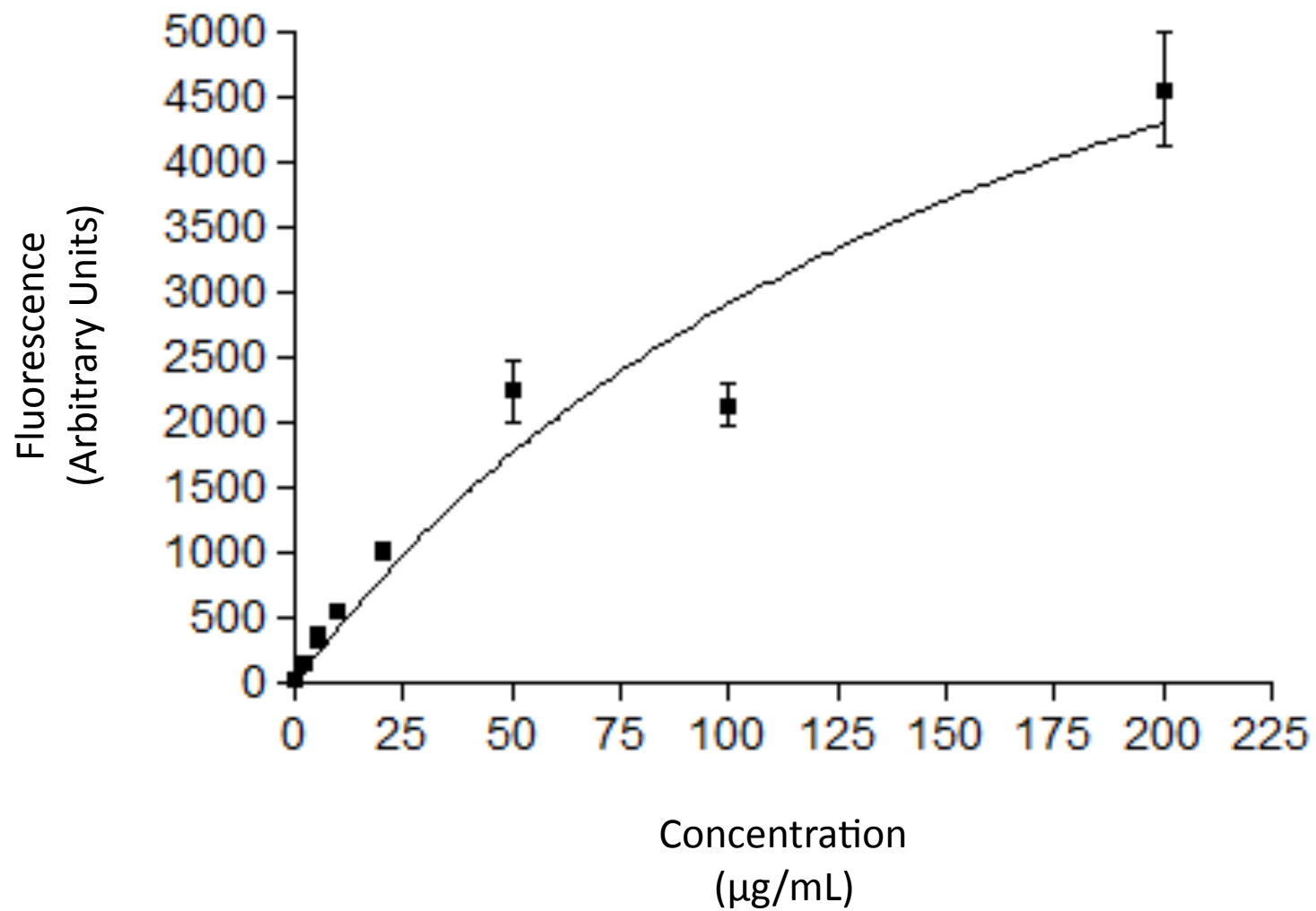
Online Figure I

Online Figure II

FRET Pair	FRET Efficiency	
	PAEC ½ Dilution	PAEC $^1/_{10}$ dilution
TRPC 1/4	0.27 ± 0.09	0.20 ± 0.04
TRPC 1/1	ND	ND
TRPC 4/4	0.30 ± 0.13	0.22 ± 0.06

Online Figure III

Pre-bleach   Post-bleach

Acceptor

Donor

Online Figure IV

5'

atgcatccggagcctgccccgcccccgaacaacagcaatccggagcttcccttgagcggcggcagcagcactagcggcagccgccggagcc
gccgccgcagcggggacggggagcccacgggggccccaccgctgccgccgccgcc

shRNA#3

cgccgtcagctacccggactggatcggccagagttactccgaggtgatgagcctcaacgagcactcgatgcaggcgctgtcctggcgcaagct
ctacttgagccgcgccaagctcaaagcctccagccgaacctccgctctgctctccg

shRNA#1

shRNA#2

gcttcgccatggtagcgatggtggaagtccagctggacacggatcatgactacccgccagggttactcatcgtcttca

shRNA#4

gcgcctgcaccacagtgctggtggccgtgcacctgttcgccctcatgatcagtacctgcatcctgcccaacatcgaggctgtgagcaacgtcca
caacctcaactccgtcaaggagtcgccgcacgagcgcatgcaccgccacatcgagctggcttgggccttctccaccgtcatcgggacgctgctt
ttcctggccgaagtcgtgctgctctgctgggtgaagttcttaccgctcaagaggcaggcgggacagccaagccccaccaagccacccaccga
accggccgtcgtcgtcgccaacagcagcaacaacggtggcatcaccccgggtgaggcggctgccatcgcctccactgccatcatggtcccctg
tggcctggtgtttatcgtctttgccgttcacttctaccgctcactggtcagccataagacggaccgacagttccaggagctcaacgagctggccg
agtttgcccgcttgcaggaccagctggaccacagaggggaccattctctaacaccgggcacccactatgcctaa

3'

Online Figure V
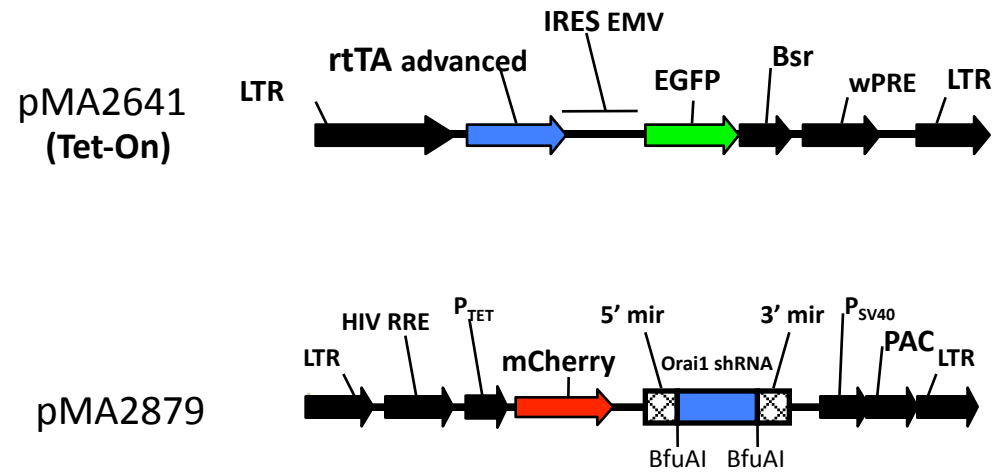
Online Figure VI

See accompanying time-lapse movie