ISCI, Volume *15*

Supplemental Information

Genetically Encoded Fluorescent Indicator GRAPHIC Delineates Intercellular Connections

Nagatoki Kinoshita, Arthur J.Y. Huang, Thomas J. McHugh, Sachihiro C. Suzuki, Ichiro Masai, Il Hwan Kim, Scott H. Soderling, Atsushi Miyawaki, and Tomomi Shimogori

Transparent Methods

All recombinant DNA and animal experiments in this study were performed in accordance with guidelines of Research Ethic Section of RIKEN.

Probe cDNA constructs

For construction of reconstitution probe, full-length Superfolder GFP (sfGFP) cDNA was synthesized from published sequences(Cabantous et al., 2005; Pedelacq et al., 2006), except 11th β-sheet is same as EGFP. sfGFP split sites of 1-7/8-11, 1-10/11 are 157/158, 214/215 amino acid positions respectively. Single 11th β-sheet fragment is optimized for reconstitution (Cabantous et al., 2005) which DNA sequence is 5'-CGCGATCACATGGTCCTGCATGAGTATGTGAATGCCGCCGGGATCACT-3'. For generating GPI-anchored type probe molecules, sfGFP fragments were inserted between mouse preproacrosin signal peptide and mouse Thy-1 GPI-anchored domain cDNA, which were kindly donated by Dr. G. Kondoh (Kyoto University, Japan)(Kondoh et al., 1999). Spacer domains (its cDNA sequence is 5'-GGTGGAGGCGGTTCAGGCGGAGGTGGCTCTGGCGGTGGCGGATCG-3') and acidic leucine zipper domain (LZA) or basic leucine zipper domain (LZB)(O'Shea et al., 1993) were inserted between sfGFP fragment and GPI-anchored domain. Pre- and post-mGRASP cDNAs were synthesized by GeneArt Strings DNA Fragments (Thermo Fisher Scientific) from published sequences(Kim et al., 2012), except NheI sites were introduced to replace GFP fragment domains. For generating a transmembrane type molecule, pDisplay was used (Thermo Fisher Scientific). To label cell nucleus, human histone H2B (donated by Dr. T. Kanda (Aichi Cancer Center Research Institute, Japan))(Kanda et al., 1998) was fused to mCherry (donated by Dr. R. Tsien (UC San Diego, USA)), or Azurite(Mena et al., 2006) (synthesized from published sequences). To identify transfected cells, probe molecules were jointed to H2B-mCherry or H2B-Azurite with self-cleavable T2A peptide which DNA sequence is 5'-GAGGGCAGAGGAAGTCTTCTAACATGCGGTGACGTCGAGGAGAATCCTGGCCCA-3'. To label synaptic sites of transfected neurons, rat PSD95 (donated by Dr. S. Okabe (Tokyo University, Japan)) was fused to mCherry, and probe molecules were jointed to PSD95-mCherry with self-cleavable P2A peptide which DNA sequence is 5'-GGAAGCGGAGCTACTAACTTCAGCCTGCTGAAGCAGGCTGGAGACGTGGAGGAGAACCCTGGACCT-3'.

For zebrafish retina experiment, using Gibson Assembly (NEB), tdTomato that has zebrafish gap43 membrane targeting signal at the 5' end, P2A and n-GRAPHIC were connected in frame (MemtdTomato-P2A-n-GRAPHIC). TagBFP2(Subach et al., 2011) and c-GRAPHIC are connected via P2A in frame (TagBFP2-P2A-c-GRAPHIC).

Color variants of GFP fragments were synthesized by GeneArt Strings DNA Fragments or site directed mutagenesis. BFP (similar to BFP2(Park and Rhee, 2012)) type NT-fragment was

generated by 8 amino acid substitutions (I39N, T65S, Y66H, S72A, K105T, T128V, V150I, D115V) into sfGFP 1-7. YFP type CT-fragment was generated by T203Y into sfGFP 8-11.

Lentivirus expression system

Lentivirus expression system is as described(Miyoshi et al., 1998; Miyoshi et al., 1997). cDNA encoding probe molecule or nucleus color label was cloned into a pCSII-CMV-MCS vector. To produce lentivirus solution, the plasmid was co-transfected with the packaging plasmid (pCAG-HIVgp) and the VSV-G- and Rev-expressing plasmid (pCMV-VSV-G-RSV-Rev) into HEK293T cells by X-tremeGENE HP DNA Transfection Reagent (Roche).

Cell cultures

LLCPK1 cells (donated by Dr. S. Yonemura (RIKEN CDB, Japan)) were cultured in DMEM (low glucose, Wako) containing 10% fetal bovine serum (Serum Source International). LLCPK1 clonal cell lines which stably express probe molecules were generated by lentivirus infection and cell sorting. HEK293T cells (RIKEN Cell Bank) were cultured in DMEM (high glucose, Wako) medium containing 10% fetal bovine serum. All cell lines were cultured at 37 ºC under 5% CO_2.

Flow Cytometry

For analytical flow cytometry, culture medium of LLCPK1 cells were replaced to 5 mM EDTA/ phosphate-buffered saline (PBS) and cells were dissociated with pipetting. Dissociated cells were filtrated with 40 μm cell strainer (Thermo Fisher Scientific). After centrifugation (200 X *g*, 10 min) cells were resuspended into PBS, and the cell suspensions were subjected to flow cytometry. Flow cytometry and cell sorting were carried out on a FACSAria II (BD Biosciences). Flow cytometry data were analyzed with FlowJo.

Immunostaining of LLCPK1 cells

NT-probe expressing LLCPK1 cells were fixed with 4% PFA in PBS. After the fixation solution was replaced with PBS, the specimens were permeabilized with PBS containing 0.1% Triton X-100 for 5 min and blocked with PBS containing 3% bovine serum albumin (Sigma) for 30 min. The specimens were incubated with rabbit anti-GFP antibody (1:200) (MBL) for 1 h, washed with PBS, and incubated with donkey anti-Rabbit IgG antibody Alexa Fluor® 488 conjugate (1:500) (Thermo Fisher Scientific) for 30 min. All antibodies were diluted with PBS containing 3% bovine serum albumin.

Imaging of LLCPK1 cells

For time lapse imaging, cells were grown on glass-bottomed dish in DMEMgfp (Evrogen) containing 10% fetal bovine serum and GlutaMAX I (Thermo Fisher Scientific). For snap shot, LLCPK1 cells were fixed with 4% PFA in PBS, and the fixation solution was replaced with PBS. Almost all LLCPK1 images were collected with inverted microscopes Olympus IX-83 and Hamamatsu ORCA Flash 4.0 cameras. For color variation analysis (GFP and YFP), the spectrum analysis was carried out by lambda scan mode (step size: 5 nm, band width: 10 nm) of FV1000

laser confocal microscope (Olympus). For investigation of subcellular distribution of probe molecules, immunostained cells were subjected to confocal imaging with FV1000.

Quantification of GFP reconstitution activities: average of GFP intensity at cell-cell contact sites or plasma membrane were normalized with averages of nuclear fluorescent intensities of co-expressed H2B-mCherry (RFP) and H2B-Azurite (BFP).

Quantification of dissociated membrane retraction: since the most active membrane retraction was observed about 10 min after ion chelation, its velocity was calculated with time lapse images between 10-12 min after ion chelation. Nuclear centroids (N1, N2) of two cells of interest were decided by ellipse approximation of binarized H2B-mCherry or H2B-Azurite images. Membrane distance between cell pairs was decided with line scan of brightness in DIC images along N1N2 line segment. Membrane retraction velocity after disruption of adherence junctions depends on intercellular distance, therefore velocities were normalized with N1N2 distances. Cell pairs with 40-100 μm inter-nuclear distances were chosen for analysis.

Images were processed in ImageJ 2.0.0.

Label of mouse thalamocortical connection with GRAPHIC

In utero electroporation (IUE) of ICR mice were carried out as described previously(Matsui et al., 2013; Shimogori and Ogawa, 2008). For visualization of adult mouse neuronal connection, genome integrate system with Tol2 transposase(Sato et al., 2007) was used. cDNA encoding probe molecule and color label was cloned into a pT2K-CAGGS vector, and the plasmid was co-electroporated with pCAGGS-T2TP. All IUEs were done in the right side of embryonic mouse brains.

For AAV production, the AAV Helper Free System (Agilent Technologies) was used. The cDNA encoding the probe molecule and nucleic label (c-GRAPHIC-T2A-H2B-mCherry) was cloned into the pAAV-MCS vector (Agilent Technologies). To produce the recombinant AAV, pAAV- c-GRAPHIC-T2A-H2B-mCherry was co-transfected with pAAV-DJ/8 (Cell Biolabs), which supplies AAV2 replication proteins and AAV-DJ/8 capsid proteins, and pHelper (Agilent Technologies) which supplies the necessary adenovirus gene products required for the AAV production into the 293FT cell line (Thermo Fisher Scientific) utilizing the 293fectin transfection reagent (Thermo Fisher Scientific). After 72 h, the supernatant was collected and centrifuged at 2,000 × *g* for 30 minutes and then filtered through a 0.45 μm filtration unit (Millipore). Purification of the AAV was carried out by ultracentrifugation (87,000 × *g*, 2 h) with 20% sucrose cushion. The AAV was injected into the right side thalamus of electroporated adult mouse brains by stereotaxic injection (A/P -1.65 mm, M/L +1.80 mm from bregma, D/V -3.5 mm from the pial surface). Injection volume of AAV solutions was 500 nl/site, and the rate was 200 nl/min. After the injection, the needle was kept at the position for an additional 2.5 min before removal. These injection experiments were carried out with KDS Legato 130 (KD Scientific) and stereotaxic frame (Muromachi Kikai).

All electroporated and AAV injected ICR mice were anesthetized with a lethal dose of pentobarbitone, and the animals were transcardially perfused with 4% PFA in PBS. Their brains were collected and post fixed overnight at 4°C in 4% PFA in PBS. Fixed brains were sectioned in the coronal plane with vibratome Leica VT1000S at 100-120 µm. The sections were mounted with 10% glycerol in PBS. Their confocal images were collected with FV1200.

Quantification of colocalization of GRAPHIC signal (GFP) and PSD95-mCherry (RFP): 12 confocal images of electroporated S1 layer IV were taken from 2 mouse brains. Identification of overlapping regions of GFP and RFP were automatically calculated using the AND function in ImageJ (Version 2.0.0.). The number of GFP puncta overlapped with or without mCherry puncta were manually counted.

GRAPHIC signal in zebrafish retina

All the fish we used in this study were in the *roy* mutant background that lacks silvery iridophores(White et al., 2008). After 10 hours post fertilization, we maintained fish in 1x E3 medium containing 0.003% of 1-phenyl-2-thiourea (Nacalai) to prevent melanin formation.

To express n-GRAPHIC in zebrafish cone cells, a 3.2 kb vsx1 promoter(Randlett et al., 2013) and MemtdTomato-P2A-n-GRAPHIC cDNA were assembled in a Tol2 plasmid(Kawakami, 2007). To express c-GRAPHIC in off type bipolar cells, a 3.2 kb promoter fragment of the *guanine nucleotide-binding protein G(t) subunit alpha-2* (*gnat2*) gene(Kennedy et al., 2007) and TagBFP2-P2A-c-GRAPHIC cDNA were subcloned into a Tol2 plasmid.

The n- and c-GRAPHIC plasmids diluted in 1x Danieu's buffer at the concentration of 25 ng/µl and 50 ng/µl Tol2 transposase mRNA were co-injected into one-cell stage embryos.

At 5 dpf, we fixed larvae in PBS containing 4% PFA and 5% sucrose. After fixation, retinas were dissected and then mounted in 0.7% molten low melting point agarose in PBS on a coverslip with optic nerve head side down. After solidifying the agarose, the coverslip was flipped and placed on a drop of VECTOR SHIELD (Vector) on a slide glass with a spacer to prevent squeezing. Confocal images were acquired with Olympus FV1000 (Olympus). Obtained z-stack confocal images were processed using ImageJ 2.0.0 and AMIRA (FEI).

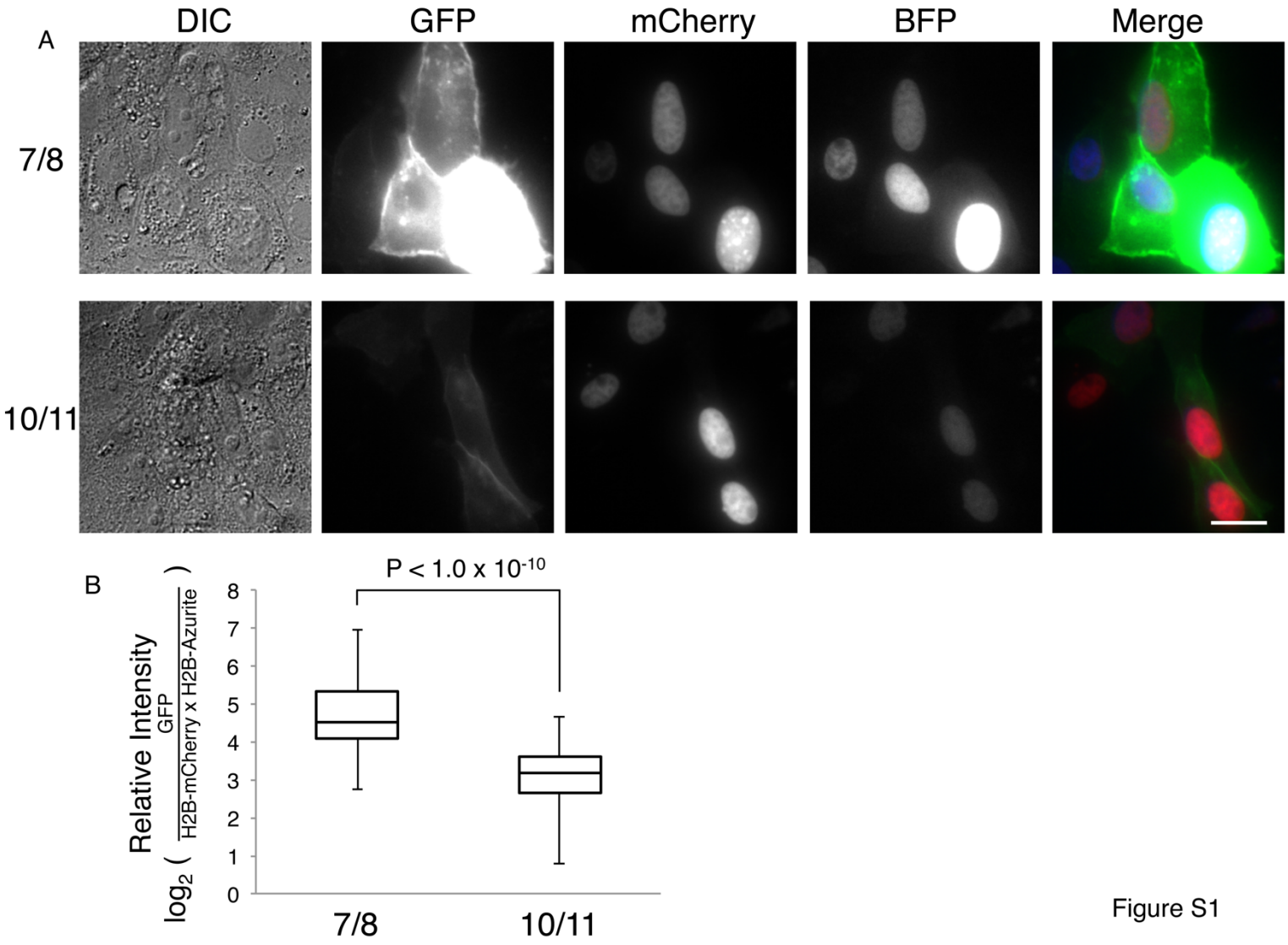
A
DIC
GFP
mCherry
BFP
Merge
7/8
10/11
B
P < 1.0 x 10-10
Relative Intensity
log2 (GFP / H2B-mCherry x H2B-Azurite)
8
7
6
5
4
3
2
1
0
7/8
10/11

Figure S1

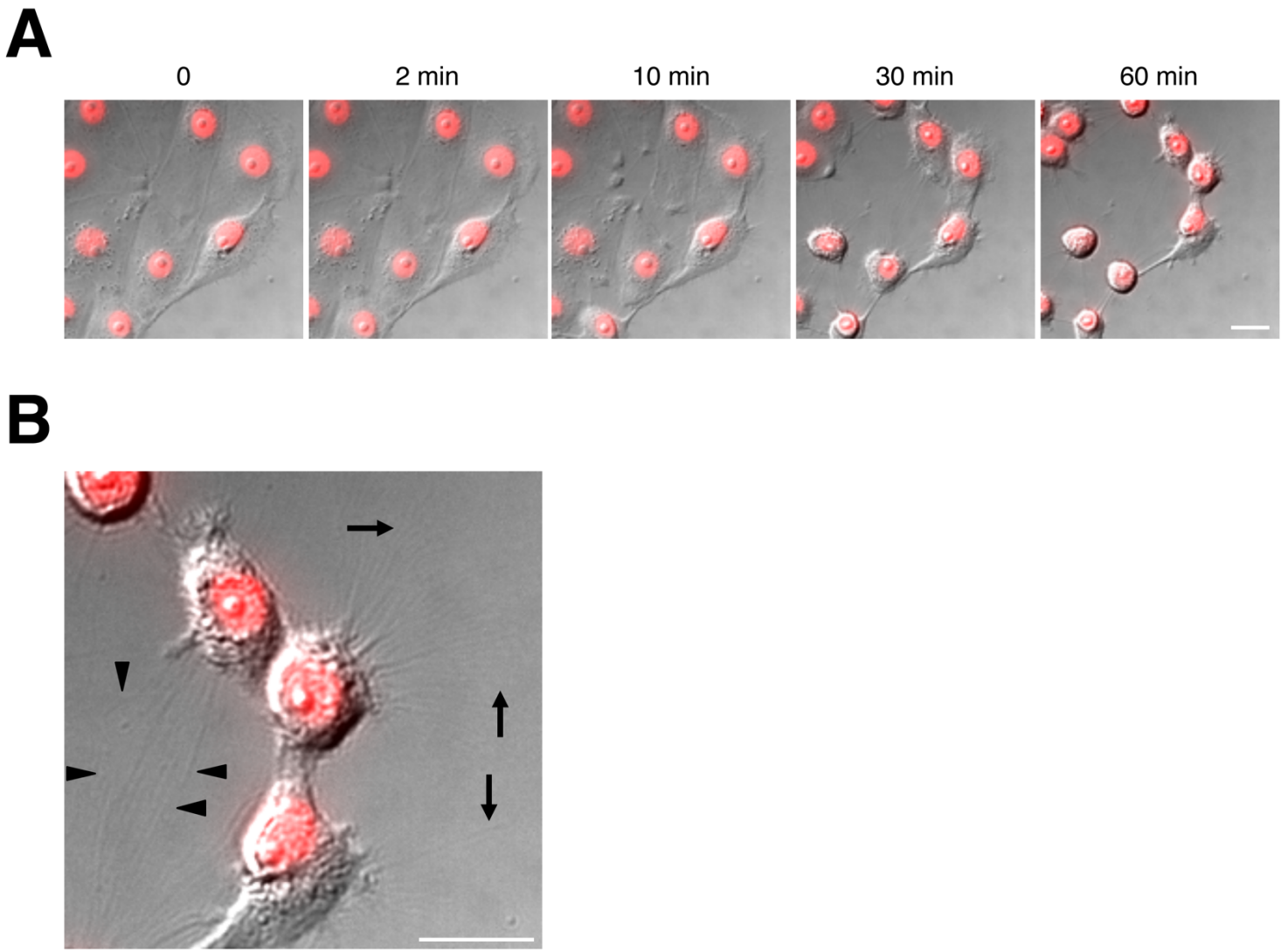
A
0
2 min
10 min
30 min
60 min
B

Figure S1. Comparison of reconstitution activity by different split site (related to Figure 1)

A. 7/8 split site of sfGFP showed more effective reconstitution activity than 10/11: To effectively label intercellular connection, we compared reconstitution activity of both 7/8 and 10/11 split sfGFP fragments. 7/8 split pair (GPI-sfGFP 1-7-2A-H2B-mCherry and GPI-sfGFP 8-11-2A-H2B-Azurite) or 10/11 split pair (GPI-sfGFP 1-10-2A-H2B-mCherry and GPI-sfGFP 11-2A-H2B-Azurite) were co-transfected into LLCPK1 cells. Co-expression of 7/8 split pair molecules showed strong reconstituted GFP signals, whereas co-expression of 10/11 split pair molecules showed relatively weak signals. **B.** Quantification and comparison of signal intensity of both spilt pairs indicates that 7/8 split pair (n =50) has more reconstitution activity than 10/11 split pair (n =60). GFP signals were normalized with co-expressed nuclei label intensities. $P = 1.23 \times 10^{-16}$; Student's unpaired t-test. Scale bar, 20 μm.

Figure S2 Ion chelation disrupt intercellular contact (related to Figure 3).

Time lapse images of control LLCPK1 cells in ion chelation experiment: To investigate the effect of GRAPHIC system on cell-cell adhesion, as a control experiment, we observed morphological changes of H2B-mCherry expressing (without GRAPHIC) LLCKP1 cells after disruption of adhesion junctions by ion chelation. (**a**) Time lapse images of control LLCPK1 cell morphology at each time points. EDTA was administrated at time 0 (final concentration; 5mM). Compared with GRAPHIC expressing cells (Fig. 2b), control cells did not show significant differences in cell morphology after ion chelation. (**b**) Higher magnification image of control cells at 60min after ion chelation. Fibers of plasma membrane were also observed at regions not previously associated with cell-cell contacts prior to ion chelation (arrows). Even at previous cell-cell contacting sites, some fibers did not connect to other cells (arrowheads). These time lapse images suggest that these fiber structures may not be largely caused by remains of cell-cell contacts but cell-substrate contacts (focal contacts). Scale bars, 20 μm.

Supplementary Movie 1. Dynamics of GRAPHIC signals during the establishment of intercellular contact of epithelial cells. (related to Figure 3A)

This movie shows the signal distribution of GRAPHIC and change in its intensity during establishment of the intercellular contact between n-GRAPHIC expressing LLCPK1 cells (red nuclei) and c-GRAPHIC expressing LLCPK1 cells (blue Nuclei). Length of video, 12 h.

Supplementary Movie 2. Dynamics of GRAPHIC signals in disruption of intercellular contact of epithelial cells. (related to Figure3B)

This movie shows dynamics in distribution and intensity of GRAPHIC signal when intercellular contact between n-GRAPHIC expressing LLCPK1 cells (red nuclei) and c-GRAPHIC expressing

LLCPK1 cells (blue Nuclei) is disrupted by ion chelation. Length of video, 1 h.