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Fluorescence resonance energy transfer analysis of subunit assembly of the ASIC channel

Ying Gao [a], Shuang-Shuang Liu [a], Shuang Qiu [a], Wei Cheng [b], Jie Zheng [b], Jian-Hong Luo [a,*]

[a] Department of Neurobiology, Institute for Neuroscience, Zhejiang University School of Medicine, Hangzhou 310058, China
[b] Department of Physiology and Membrane Biology, University of California, Davis, School of Medicine, Davis, CA 95616, USA

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Abstract

Acid-sensing ion channels (ASICs) are believed to be homo- or heteromeric complexes, which have been verified by classical methods such as co-immunoprecipitation or electrophysiological assays. However, the exact subunit combinations of ASICs in living cells have not been established yet. Here, we apply assays based on fluorescence resonance energy transfer (FRET) between GFP color mutants CFP and YFP to investigate ASIC assembly directly in living cells. Homomerization as well as heteromerization of different combinations of ASIC subunits were found. In addition, our results suggest the formation of heteromeric 1a/2a channels of stoichiometry consisting of at least two 1a subunits and two 2a subunits. Similar stoichiometry was observed from heteromeric 1a/2b and 2a/2b channels. Our results imply that these heteromeric ASIC channels contain at least four subunits.

Keywords: FRET; ASIC; Assembly; Homomer; Heteromer; Stoichiometry; CHO

Acid-sensing ion channels (ASICs), a class of ligand-gated cation channels activated by protons and highly expressed in both peripheral sensory neurons and central neurons, play important roles in mechanosensation, nociception, learning-memory, and brain ischemia [1,2]. ASICs belong to the ENaC/DEG superfamily, which also includes the amiloride-sensitive epithelial sodium channels (ENaCs), the mechanically gated degenerins of *Caenorhabditis elegans* (DEGs), and a neuropeptide-gated channel of *Helix aspersa* (FaNaC). The transmembrane topology of all channels in this superfamily features cytosolic amino and carboxy termini, two hydrophobic transmembrane segments and a large cysteine-rich extracellular domain [3]. So far, six ASIC subunits encoded by four genes have been cloned, ASIC1a (BNaC2α), ASIC1b (BNaC2β), ASIC2a (MDEG1), ASIC2b (MDEG2), ASIC3 (DRASIC), and ASIC4 (SPASIC). Homo- and heteromeric ASIC complexes have been verified by classical methods such as co-immunoprecipitation, or functional assays applying electrophysiology [4–9]. However, it is at present largely unknown how many subunits are required to form an ASIC channel. Stoichiometric studies with other ENaC/DEG channels yielded conflicting results that suggest four to nine subunits per channel [10–13].

To directly determine the assembly of ASIC subunits of intact channels in living cells, we have used a biophysical approach based on fluorescence resonance energy transfer (FRET). FRET is a physical phenomenon in which energy absorbed by a fluorophore (CFP, the donor) is transferred to another fluorophore (YFP, the acceptor) through a non-radiative process. Because distances closer than about 10 nm between appropriate fluorophores are required to elicit FRET, it is a sensitive measure of the close proximity of fluorescent molecules. The efficiency of energy transfer falls off with the sixth power of the distance between the donor and acceptor molecules [14]. FRET has been successfully employed as a molecular ruler in investigations

* Corresponding author. Fax: +86 571 88208045.
E-mail address: luojianhong@zju.edu.cn (J.-H. Luo).

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of the stoichiometry, assembly, and conformational arrangement of many types of receptors and channels [13,15–19]. Another advantage of FRET over biochemistry approaches such as co-IP is that FRET measurement is non-invasive, allowing determination of subunit stoichiometry from intact channels in the cell membrane.

In the present study, we found that ASIC subunits can form homomeric and heteromeric channels in living cells. We also found that the stoichiometry of heteromeric ASIC channels is most consistent with at least two of each type of subunit. Our observation that each ASIC subunit is capable of interacting with similar subunits also supports the idea that the fully complexed channels contain at least four subunits.

Materials and methods

Molecular biology. ASIC subunits with a YFP or CFP-tagged at the C terminus (ASIC-YFP/CFP) were generated as follows. cDNAs encoding ASIC 1a, 2a, 2b were amplified by polymerase chain reaction from the original plasmids ASIC 1a, 2a, 2b and the stop codons were removed. These fragments were inserted into pEYFP-N1 or pECFP-N1 (Clontech, Palo Alto, CA). The vector pECFP-YFP coding for the CFP–YFP fusion protein used as a positive control, in which CFP and YFP were linked by a 15-amino acids peptide, was generated previously [18]. As negative controls, we co-expressed CFP-tagged ASICs with unrelated membrane proteins, including mGluR1α-YFP and GIRK4-YFP. As in ASIC fusion constructs, YFP was attached to the C terminus of mGluR1α and GIRK4. The GIRK4 contained S143T to form functional homomeric channel [20]. All plasmids were sequenced to ensure correct reading frame, orientation, and sequence.

CHO cell culture and transfection. Chinese hamster ovary (CHO) cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, antibiotics, and glutamine (all from Invitrogen) in a 5% CO_2 incubator. For transient transfection, cells were seeded at 2×10^5 cells/35 mm dish in 2 ml of culture medium, and plated on glass coverslips. CHO cells were transfected with appropriate plasmids (3–4 μg/35-mm dish) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The transfection mixture was replaced 3–5 h later with fresh culture medium. For FRET imaging, transfected cells were observed within 2 days in extracellular medium composed of (in mM): 150 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 glucose at pH 7.3 with Tris base.

Electrophysiology. CHO cells were used for whole-cell recordings 24–48 h after transfection. The standard external solution was buffered to various pH levels with 10 mM either HEPES (pH 6.0–7.4) or MES (pH < 6.0). The pipette solution was composed of (in mM): 136.5 K-gluconate, 17.5 KCl, 9 NaCl, 1 $MgCl_2$, 10 HEPES, 0.2 EGTA, and 4 Na_2ATP, pH 7.2. Patch pipettes were pulled from glass capillaries with an outer diameter of 1.5 mm on a two-stage puller (PP-830, Narishige, Tokyo, Japan). The resistance of the recording electrode filled with the pipette solution was 4–6 MΩ. Membrane currents were measured using a patch-clamp amplifier (Axon 700A, Axon Instruments, Foster City, CA, USA), sampled and analyzed using a Digidata 1322A interface and a personal computer with Clampex and Clampfit software (Version 9.0, Axon Instruments). The membrane potential was held at −60 mV throughout the experiment. All the experiments were carried out at room temperature (22–25 °C).

FRET imaging. The procedure of FRET imaging was similar to that described previously [18]. Briefly, we used a Nikon (Tokyo, Japan) TE2000 inverted microscope equipped with a mercury lamp light source (100 W), Dual-View™ (Optical Insights, LLC, Santa Fe, NM) and a SNAP-HQ-cooled charge-coupled device camera (Roper Scientific, Trenton, NJ). The excitation control and image acquisition were achieved using MetaMorph version 5.0 software (Universal Imaging, West Chester, PA). We used the following filters (Chroma) for acquiring CFP, YFP, or FRET images (excitation; dichroic; emission): CFP (S430/25 nm; 455dclp; S470/30 nm), YFP (S500/20 nm; Q515lp; S535/30 nm), and FRET (S430/25 nm; 455dclp; S535/30 nm). Binning 2 × 2 modes and 200 ms of integration time were used.

Data analysis and statistics. MetaMorph software was used to analyze the cell image data. Average background signal was determined as the mean fluorescence intensity from a blank area and was subtracted from the raw images before carrying out FRET calculations. FRET ratio (*FR*) was calculated with a method described previously [18]. The following equation was used: $FR = [\text{FRET} - (b \times \text{CFP})]/(a \times \text{YFP})$, in which a and b represent the fractions of bleed-through of YFP and CFP fluorescence through the FRET filter channel, respectively. Apparent FRET efficiency, E_{app}, was calculated from *FR* using the equation $E_{app} = (FR - 1)\frac{\varepsilon_{YFP}}{\varepsilon_{CFP}}$, in which ε_{CFP} and ε_{YFP} are the molar extinction coefficient for CFP and YFP, respectively. The dependence of E_{app} to the intensity ratio between CFP and YFP, Fc/Fy, was used to estimate the FRET efficiency between two subunits. This was done using a recently reported method [21,22].

Results

Normal functions of CFP/YFP-tagged ASIC subunits

To investigate the assembly of ASIC subunits by FRET in living cells, we fused YFP and CFP onto the cytosolic C-terminus of ASIC subunits to generate ASIC (1a, 2a, 2b)-YFP and ASIC (1a, 2a, 2b)-CFP, respectively, (Fig. 1A and B). Current recordings confirmed that expression of ASIC 1a-YFP or 2a-YFP alone could result in functional channels, while ASIC 2b-YFP was nonfunctional by itself but interacted with ASIC 2a to form heteromers (Fig. 1C). Similar results from untagged subunits have been previously reported [5–7,9]. CFP-tagged ASIC subunits exhibited functional properties indistinguishable from YFP-tagged ASIC subunits (data not shown).

Detection of FRET in living cells using three-cube FRET

We used a convenient, microscope-based three-cube FRET assay. Three-cube FRET employs three filter cubes to isolate CFP and YFP signals from a cell expressing both fluorophores. As expected, exciting CFP in cells that only expressing CFP showed no fluorescence signal with the YFP filter set but detectable signal with the FRET filter set (due to bleed-through of CFP). Similarly, exciting cells expressing only YFP with the CFP filter set yielded no fluorescence signal while exciting with the FRET set did produce detectable signal (due to direct excitation of YFP) (Fig. 2A). We calculated the FRET ratio (*FR*) as a unitless index equal to the fractional increase in YFP emission due to FRET, from which the FRET efficiency was determined. Cells co-expressing CFP and YFP, which were used as a negative control, showed no FRET ($E = 1.9 \pm 0.2\%$, $n = 159$; Fig. 2B and Table 1), indicating no appreciable interaction between free CFP and YFP in CHO cells. Cells expressing a CFP–YFP concatemer, in which CFP and YFP were joined together with a 15-amino acids linker, showed significant energy transfer ($E = 33.9 \pm 0.4\%$, $n = 177$; Fig. 2C, and Table 1). Therefore, these control

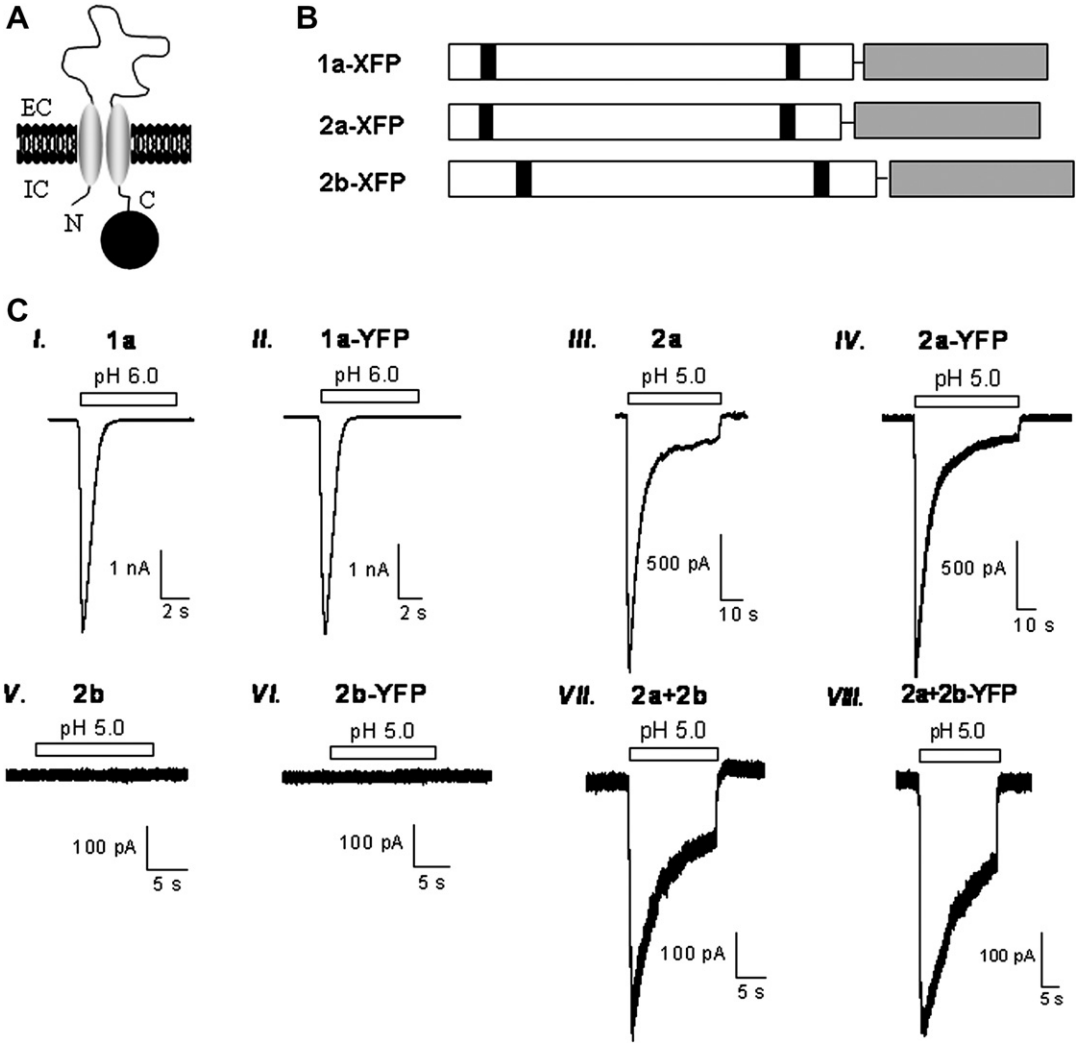

Fig. 1. ASIC subunits tagged with CFP or YFP. (A) Diagram representing the presently understood membrane topology of a ASIC subunit, with a fluorophore attached to the C-terminal tail. N, N terminus; C, C terminus; EC, extracellular; IC, intracellular. (B) Schematic representation of the ASIC fusion proteins used in this experiment. *Black boxes* show the transmembrane domains and *gray boxes* indicate CFP or YFP. *Left* and *right sides* are the N and C termini, respectively. CFP or YFP (XFP) was ligated in-frame behind the subunits at the C terminus. (C) Representative acid-evoked current waveforms from cells expressing 1a, 1a-YFP, 2a, 2a-YFP, 2b, 2b-YFP, 2a + 2b, 2a + 2b-YFP. Transfected cells were hold at −60 mV and pH 6.0 or pH 5.0 applied for the times indicated by the bars.

experiments verified that three-cube FRET provides sensitive and selective detection of FRET.

FRET measurement from homomeric ASIC channels

To measure steady-state FRET in ASIC channels, CHO cells transfected with different fusion proteins were imaged using the same system described above (Fig. 3A). We found that co-expression of 1a-CFP and 1a-YFP produced significant FRET signals ($E = 37\%$; $n = 163$) (Fig. 3B and Table 1). Co-expression of 2a-CFP and 2a-YFP yielded less but significant FRET ($E = 27\%$; $n = 136$). Surprisingly, co-expression of 2b-CFP and 2b-YFP also yielded significant FRET ($E = 35\%$; $n = 63$), even though no functional current was observed from expressions of only 2b subunits (Fig. 1C). These results suggest that fluorescence protein-tagged ASIC subunits are capable of co-assembly as untagged subunits. In addition, all the three types of ASIC subunits can form homomeric ASIC channels.

In order to confirm that FRET signals observed in these experiments represent genuine subunit assembly rather than non-specific signals due to spontaneously close proximity of different channel proteins, we co-expressed ASIC subunits with various non-related membrane protein subunits and repeated the FRET measurement. For these tests, we attached YFP to the G-protein-coupled inward rectifier potassium channel GIRK4 or the metabotropic glutamate receptor mGluR1α, and co-expressed them with each of the CFP-tagged ASIC subunits. Example data sets from these experiments are shown in Fig. 3D and E. We found that the apparent FRET efficiency values were mostly less than 10%, much lower than those measured from ASIC-expressing cells (Table 1).

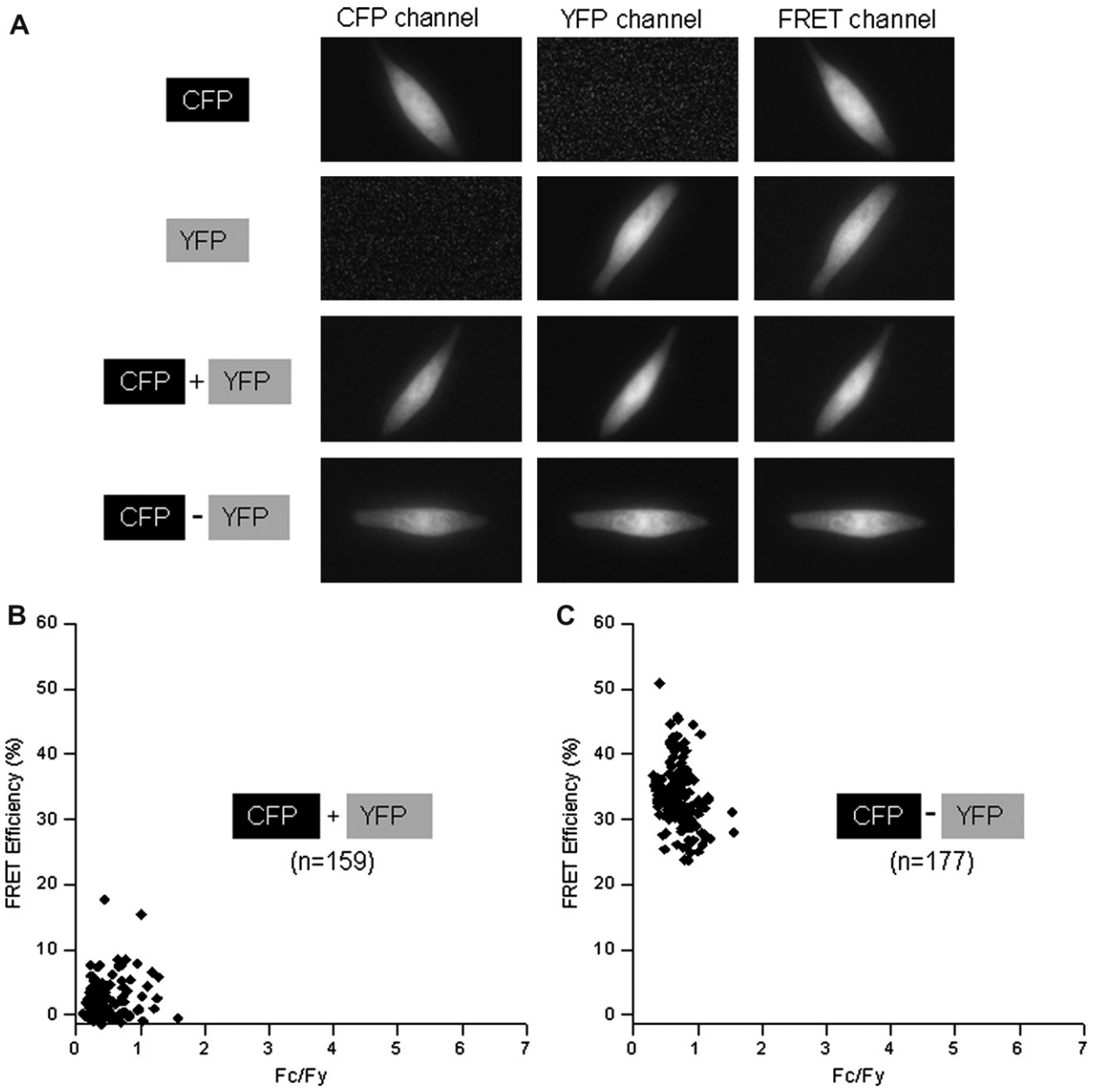

Fig. 2. Images of CHO cell expressing fluorescence protein used for FRET analysis. (A) The indicated fusion proteins were transiently expressed in CHO cells, images were taken by the CFP channel (*first column*), YFP channel (*second column*), and the FRET channel (*last column*). *Black boxes* indicate CFP, whereas *gray boxes* indicate YFP. (B,C) FRET efficiency measurements from cells co-expressing CFP and YFP (negative control) or CFP–YFP (positive control). Each symbol represents a single cell.

FRET measurement from heteromeric ASIC channels

Next we tested the assembly of heteromeric ASIC channels. Co-expression of 1a-CFP and 2a-YFP yielded a significant FRET signal in living cells ($E = 30\%$, $n = 121$) (Fig. 3C). Similarly, co-expression of 1a-CFP and 2b-YFP, or 2b-CFP and 2a-YFP also yielded significant FRET ($E = 36\%$, $n = 93$; and $E = 33\%$, $n = 113$, respectively). These results are consistent with the notion that these ASIC subunits can associate with each other, as previously suggested by co-immunoprecipitation experiments [4,7]. We conclude that different ASIC subunits can co-assembly into heteromeric ASIC channels.

Stoichiometry of ASIC channel

Our experimental design and rationale for determining subunit stoichiometry closely followed that of Zheng and colleagues [16,17], Staruschenko and colleagues [13], and Kerschensteiner and colleagues [19]. Zheng et al. [16] and Kerschensteiner et al. [19] successfully used a FRET method in the determination of a fixed stoichiometry of 3:1 for the rod cyclic-nucleotide gated channels and heteromeric Kv2.1/Kv9.3 channels, respectively. Staruschenko and colleagues [13] also used this method to establish that epithelial Na^+ channels (ENaC) have a 3α:3β:3γ stoichiometry. Briefly, the experimental design is as follows, taking

Table 1
Summary of FRET measurements

Combinations	FRET efficiency (%)	*n*
CFP + YFP	2	159
CFP–YFP	34	177
1a-CFP + 1a-YFP	37	163
2a-CFP + 2a-YFP	27	136
2b-CFP + 2b-YFP	35	63
1a-CFP + 2a-YFP	30	121
1a-CFP + 2b-YFP	36	93
2b-CFP + 2a-YFP	33	113
1a-CFP + GIRK4-YFP	6	57
2a-CFP + GIRK4-YFP	6	65
2b-CFP + GIRK4-YFP	8	28
1a-CFP + mGluR1α-YFP	5	49
2a-CFP + mGluR1α-YFP	13	61
2b-CFP + mGluR1α-YFP	12	56

heteromeric A/B channels for example, in which A or B represents different ASIC subunits. Untagged B subunits were co-expressed with A-CFP and A-YFP. Under these conditions, FRET should only occur if heteromeric channels contained two or more A subunits. In the reverse experiment, fluorophores were attached to B and co-expressed with untagged A. In this case, FRET should only occur if heteromeric channels contained two or more B subunits (Fig. 4A).

Results from these experiments are shown in Fig. 4B–D. FRET was observed from either co-expression of 2a with 1a-CFP and 1a-YFP ($E = 34\%$, $n = 72$), or co-expression of 1a with 2a-CFP and 2a-YFP ($E = 22\%$, $n = 60$), corroborating a stoichiometry of at least two 1a and two 2a. Similarly, in heteromeric 1a/2b or 2a/2b channels, FRET occurred when fluorophores were attached to either subunit (1a-CFP + 1a-YFP + 2b, $E = 44\%$, $n = 69$; 2b-CFP + 2b-YFP + 1a, $E = 39\%$, $n = 60$; 2a-CFP + 2a-YFP + 2b, $E = 21\%$, $n = 48$; 2b-CFP + 2b-YFP + 2a, $E = 31\%$, $n = 116$). Interestingly, the FRET efficiency values from these heteromeric channels are compatible to those measured from homomeric channels (Table 1). Particularly, when 2a-CFP and 2a-YFP were co-expressed with another ASIC subunit, a consistently lower FRET efficiency was obtained. It is noticed that co-expression of 2a-CFP and 2a-YFP also yielded lower FRET comparing to all other ASIC subunit pairs (Table 1). Positive FRET signals from these experiments indicate that in heteromeric ASIC channels, there are at least two copies of each of the different subunit types. While FRET can also come from co-existence of homomeric channel populations, we consider this unlikely to contribute to all the observed FRET signals given that each pair of different ASIC subunits can co-assemble to give strong FRET signals (Fig. 3C and Table 1).

Discussion

To the present, the exact subunit combinations of functional acid-sensing ion channels (ASICs) have not been established yet, but both homomeric and heteromeric channels seem to exist. Here we addressed this issue directly in living cells by the FRET technique. When 1a-CFP and 1a-YFP, or 2a-CFP and 2a-YFP were co-expressed in CHO cells, significant FRET signals were observed, indicating the formation of specifically assembled homomeric ASIC channels. Current recording data are consistent with FRET data supporting the formation of functional channels, as have been previously reported [5,6,8,9]. Surprisingly, when 2b-CFP and 2b-YFP were co-expressed in CHO cells, significant FRET signals were also observed. Combined with the absence of functional currents in these cells, these results indicate that ASIC 2b subunits can also co-assemble, though they do not form functional homomeric channels. Indeed, no functional channel was observed when ASIC 2b alone was expressed in *Xenopus laevis* oocytes or mammalian cells [5,7,8].

Previous studies have shown that immunoprecipitation of ASIC 2a causes co-precipitation of ASIC 1a in heterologous expression systems [4]. Moreover, co-expression of ASIC 1a and ASIC 2a subunits in *Xenopus* oocytes or CHO cells generates an amiloride-sensitive H^+-gated Na^+ channel with novel properties (different kinetics, ionic selectivity, and pH sensitivity) [4,8]. Heteromeric assemblies of both ASIC 2a and 2b proteins in heterologous expression systems have been previously described [5]. In addition, co-immunoprecipitation studies have also shown that ASIC 2a/ASIC 2b complexes are detected in circumvallate papillae extracts. Electrophysiological studies have shown that ASIC 2b associates with ASIC 2a to form a heteromeric complex, modifying the pH sensitivity of the ASIC 2a channel [7]. It still remains elusive whether heteromeric channels can form between ASIC 1a and 2b subunits. When ASIC 2b was co-expressed with ASIC 1a, minor changes to channel functional properties such as pH dose-response curves were observed, but nothing significant [8]. However, since the electrophysiological method can only identify the presence of heteromeric ASIC channels that displayed biophysical properties distinct from the homomeric channels, heteromeric ASIC 1a/2b channels may exist. Here, we took advantage of FRET between CFP and YFP that were genetically linked to the C-terminus of ASIC subunits to identify the presence of heteromeric ASIC channels directly. We found that significant FRET signals were yielded between the three different ASIC subunits, which further indicated that heteromeric ASIC 1a/2a, ASIC 2a/2b and ASIC 1a/2b could form.

ASIC, FaNaC, and ENaC all belong to the ENaC/DEG superfamily. Previous studies have shown that the FMRFamide-gated sodium channel (FaNaC), one closest relative to the ASIC channel, is a tetramer [12]. The subunit stoichiometry of ENaC remains uncertain, with biochemical and biophysical experiments supporting either a tetramer with a $2\alpha{:}1\beta{:}1\gamma$ stoichiometry [10] or a higher ordered channel with a $3\alpha{:}3\beta{:}3\gamma$stoichiometry [11,13]. Here we used a FRET method to test the stoichiometry of ASIC channels. We found significant FRET signals when different combinations of fluorescence protein-tagged ASIC sub-

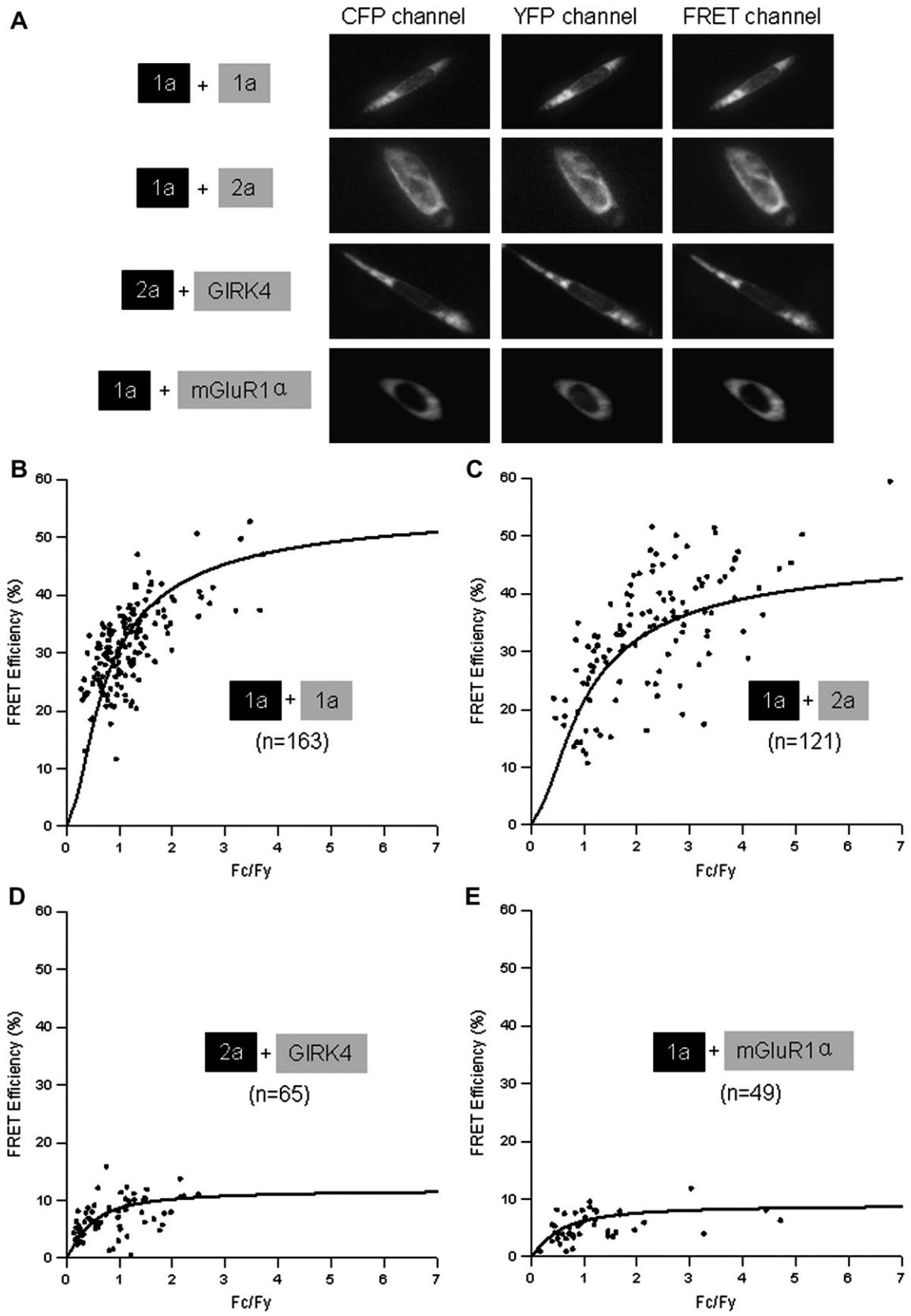

Fig. 3. Homo- and heteromerization of ASIC subunits in living cells. (A) Fluorescence microscopic images of CHO cells expressing the fusion proteins were taken through a CFP channel, a YFP channel, a FRET channel. *Black boxes* indicate CFP, whereas *gray boxes* indicate YFP. (B,C) The FRET efficiency measured from cells expressing 1a-CFP + 1a-YFP (B) or 1a-CFP + 2a-YFP (C) is plotted as a function of the fluorescence intensity ratio between CFP and YFP. Each symbol represents a single cell. The solid curve represents the FRET model that yields the best fit. (D,E) Measurements from cells expressing 2a-CFP + GIRK4-YFP (D) and 1a-CFP + mGluR1α-YFP (E), which serve as negative controls.

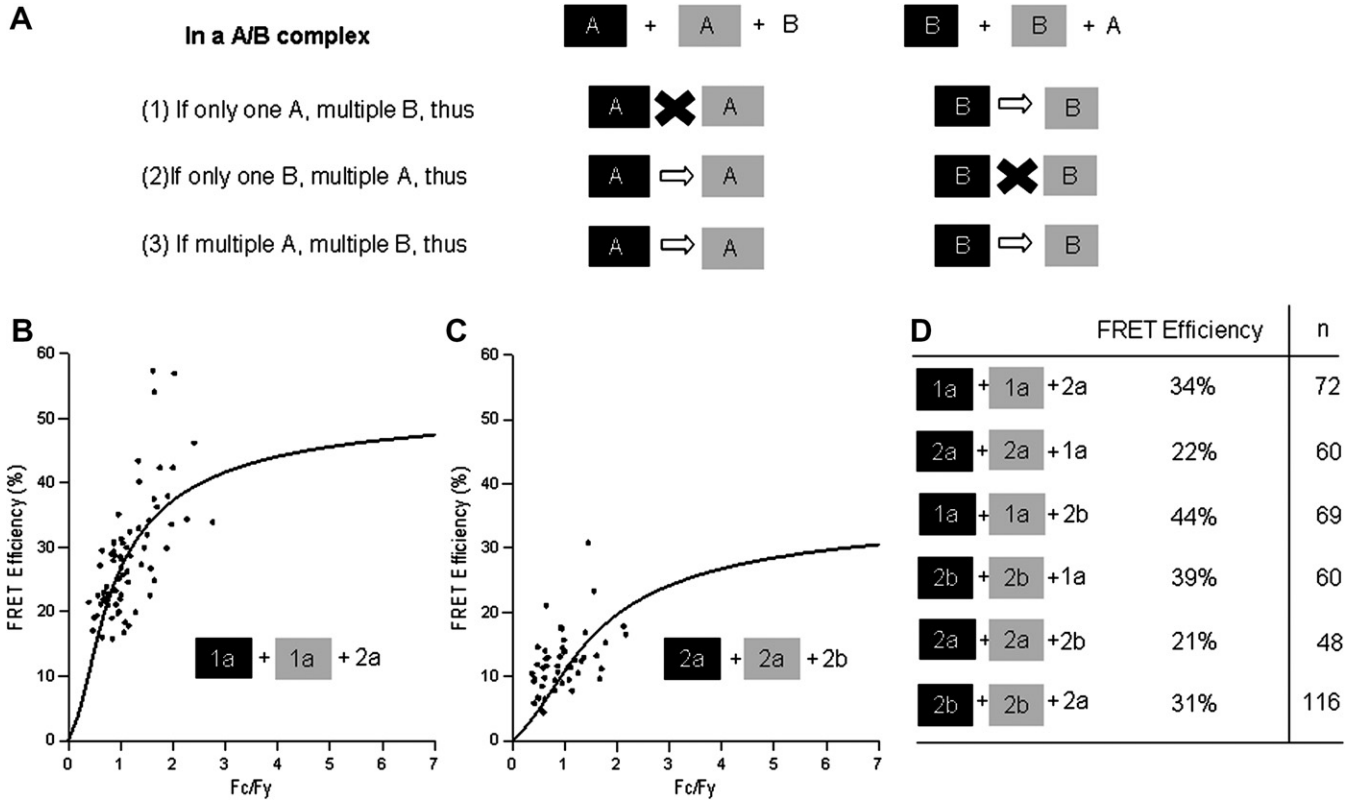

Fig. 4. Stoichiometry of heteromeric ASIC channels. (A) Schematic representation of the experimental paradigms and the resulting relevant subunit assemblies used to determine the stoichiometry of ASIC channels. Different ASIC subunits are represented by A or B, while CFP and YFP are indicated by *black boxes* or *gray boxes,*, respectively. *Arrows* denote FRET, and *X* denotes a lack of energy transfer. (B,C) FRET efficiency measurements from cells expressing 1a-CFP + 1a-YFP + 2a (B) or 2a-CFP + 2a-YFP + 2b (C). (D) Summary data of FRET efficiency from different combination of heteromeric ASIC channels. The FRET efficiency value is determined by fitting the FRET model to each co-expression experiment and is given as a percentage; *n* represents the number of cells. The ASIC subunits *without boxes* indicate the wild-type subunits without fluorescence protein.

units were co-expressed with untagged subunits, indicating that these heteromeric ASIC channels very likely contain at least two of each type of subunit. Combined with the observation that each ASIC subunit is capable of interacting with like subunits, our results also support the idea that the fully complexed channels contain at least four subunits. Nonetheless, the present data can not distinguish whether ASIC channels are tetramers or multimers with more than four subunits. Further experiments, such as blue native PAGE system, are required to address this issue.

In conclusion, the present study provides further evidence for the existence of different homomeric and heteromeric ASIC channels, which may contribute to the diversity of acid-evoked currents displayed in sensory neurons as well as in neurons of the central nervous system. In addition, our results suggest that heteromeric ASIC channels consist of at least two of each type of subunit.

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