Methodology article

A new way to rapidly create functional, fluorescent fusion proteins: random insertion of GFP with an *in vitro* transposition reaction

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Abstract

Background: The jellyfish green fluorescent protein (GFP) can be inserted into the middle of another protein to produce a functional, fluorescent fusion protein. Finding permissive sites for insertion, however, can be difficult. Here we describe a transposon-based approach for rapidly creating libraries of GFP fusion proteins.

Results: We tested our approach on the glutamate receptor subunit, GluR1, and the G protein subunit, α_s. All of the in-frame GFP insertions produced a fluorescent protein, consistent with the idea that GFP will fold and form a fluorophore when inserted into virtually any domain of another protein. Some of the proteins retained their signaling function, and the random nature of the transposition process revealed permissive sites for insertion that would not have been predicted on the basis of structural or functional models of how that protein works.

Conclusion: This technique should greatly speed the discovery of functional fusion proteins, genetically encodable sensors, and optimized fluorescence resonance energy transfer pairs.

Background

The discovery that the jellyfish green fluorescent protein (GFP) can form a functional fluorophore without other gene products or co-factors [1] was rapidly followed by reports that GFP can be used to create fluorescent fusion proteins [e.g. [2,3]]. For the first time, it became possible to create a wide variety of genetically encodable fluorescent fusion proteins that could be followed in living systems [reviewed in:[4]]. Most GFP fusion proteins have been built by placing GFP at either the N- or C-terminus of the host protein. This can, however, destroy the function of some host proteins. The alternative is to insert GFP into the middle of the host protein [5–8]. Unfortunately, finding a permissive location for insertion of the GFP can be problematic and time consuming.

One way of speeding the process is to randomly generate libraries of GFP fusion proteins and then screen for clones
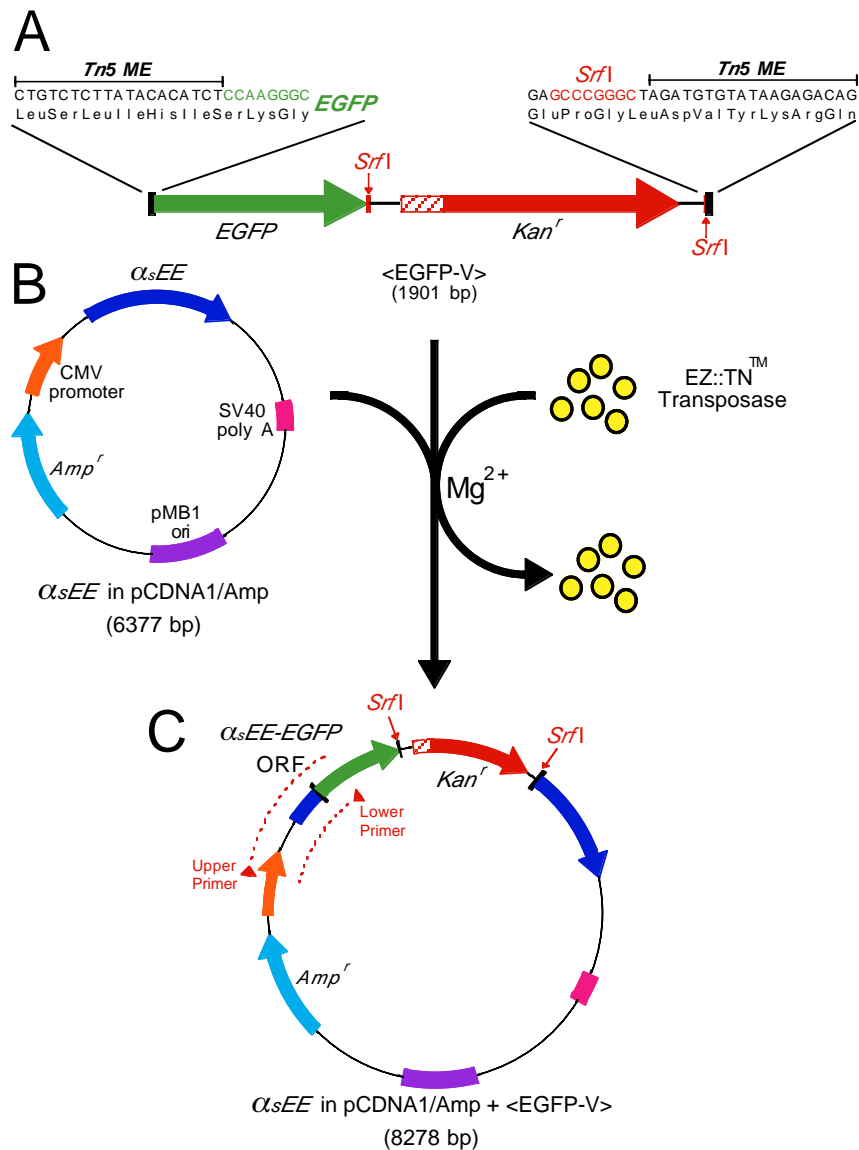
Figure 1
Transposition with <EGFP-V> (A) The transposon, <EGFP-V>, is flanked by 19 bp inverted repeats, the MEs. The EGFP coding region is positioned such that when <EGFP-V> inserts between the codons of a target gene, a fusion protein will be produced. <EGFP-V> also carries*Kan^r*. There is a stop codon in the 5' end of the *Kan^r* cassette in the same frame as the EGFP coding sequence, so if the transposon lands in an open reading frame, in the correct orientation and frame, a truncated, EGFP-tagged, protein will initially be produced. Removal of the*Kan^r* cassette by *Srf* I digestion and re-ligation produces a reading frame that extends across the entire transposon. (B) The target plasmid, $\alpha_s EE$ in pcDNA1/Amp, encodes an epitope tagged version of the G protein subunit α_s. (C) Transposed plasmids carry *Amp^r* and *Kan^r*. <EGFP-V> insertions within the target gene produce a PCR product when <EGFP-V> is inserted in the correct orientation, and the size of the PCR product reveals which <EGFP-V> insertions are in the coding sequence.

that encode functional, fluorescent proteins. One group used a combination of nick translation and nuclease S1 treatment to randomly insert GFP into a cAMP-dependent protein kinase regulatory subunit from *Dictyostelium*[6]. A surprisingly large number of the resulting fusion proteins were fluorescent and retained cAMP binding, demonstrating that this can be a powerful approach. A weakness of this strategy, however, is that it can produce deletions in the host sequence. Another approach is to use the random behavior of a transposon to insert GFP into many different places in a target protein. Two synthetic transposons have been reported that can produce GFP fusion proteins [9,10]. The design of these transposons included additional protein domains or linkers between the GFP and the target protein, however, and little is known about how many of the resulting proteins continued to function. We reasoned that a *Tn*5 transposon [11,12] could be designed that would generate GFP fusion proteins with relatively short linkers (~7 amino acids) between the GFP and the host protein analogous to GFP fusion proteins that have already been shown to function [6,8]. To test this approach, we targeted the G protein subunit α_s and the glutamate receptor subunit GluR1.

Results

Changes have been made to the *Tn*5 transposon, and its transposase, that in concert produce a hyperactive transposon capable of a 1% insertion frequency in an *in vitro* reaction [reviewed in: [12]]. This hyperactive *Tn*5 transposon is defined as any sequence flanked by the inverted 19 base pair repeats known as mosaic ends (MEs). The recombinant *Tn*5 transposase binds these ME sequences and, in the presence of Mg^{2+}, catalyzes the random insertion of the transposon into target DNA in a complex process that involves generating a 9 base pair staggered nick in the target. This staggered nick is subsequently repaired to produce a 9 base pair duplication of the target sequence that flanks the inserted transposon. Two possible reading frames extend through the MEs of the *Tn*5 transposon. Our initial GFP transposon, <EGFP-V>, was created by placing the sequence encoding enhanced green fluorescent protein (EGFP) in one of these frames such that if the transposon landed in another coding sequence, in the correct orientation and frame, it would produce a GFP fusion protein (figure 1A). The low probability of transposition in an *in vitro* reaction made it necessary to include antibiotic resistance, so *Kan^r* was added to the transposon flanked by *Srf* I restriction sites that can be used to subsequently remove it.

An epitope tagged version of the G-protein subunit α_s (α_s*EE*) was chosen as the first target (figure 1B). Previous studies have shown that the N- and C-termini of α_s are important for its interactions with receptors, G-protein β and γ subunits, and the plasma membrane [13,14], so placing

GFP within internal regions of α_s is more likely to generate a functional, fluorescent subunit [8]. Moreover, the structure of α_s has been solved [15], making it possible to interpret the results in the context of the three-dimensional structure. After transposition and transformation, colonies expressing dual antibiotic resistance were screened with PCR to identify clones in which <EGFP-V> had landed in the correct orientation within the coding region (figure 1C). Assuming that *Tn*5 behavior is random, the probability that <EGFP-V> will land in the α_s*EE* coding sequence during transposition should be the ratio of the coding sequence to the size of the total plasmid (18.5%). However, transpositions that disrupt critical elements of the plasmid (the plasmid origin or the *Amp^r* gene) should not be recovered after transformation, so the predicted probability of observed transpositions within the α_s*EE* coding sequence increases to 23.8%, with half of these (11.9%) being in the correct orientation. PCR screening of 384 *Amp^r* + *Kan^r* resistant colonies identified 44 clones with <EGFP-V> insertions within the α_s*EE* coding region in the correct orientation (11.4%).

Each clone containing an in-frame insertion should encode a truncated α_s protein with GFP at the carboxy-terminus due to a stop codon in the *Kan^r*. Thirty-five of the PCR-positive clones were transiently expressed in HEK 293 cells, and 13 were fluorescent. Sequencing confirmed that the 13 fluorescent constructs were truncated α_s-GFP fusion proteins (with 12 being unique insertions) and that the remaining 22 <EGFP-V> insertions were out of frame (figure 2A). The 12 clones encoding unique α_s-*GFP* fusion proteins were digested with *Srf* I and re-ligated to create full-length fusion proteins (figure 2B). Transient expression of each of the 12 α_s-GFP fusion proteins in HEK 293 cells produced a fluorescent signal. This is surprising because several insertions appear to be in internal and/or rigid secondary protein structures (figure 3). It appears that the folding of GFP to form a fluorophore is thermodynamically favorable at most insertion sites.

To determine what effect the GFP insertions had on α_s localization, the full-length fusion proteins were transiently co-expressed in HEK 293 cells with G protein subunits β_1 and γ_7, which have been shown to mediate signaling between the β-adrenergic receptor and G_s[16]. Amino- and carboxy-terminus GFP fusions, α_s-GFP(N) and α_s-GFP(C), respectively, were also co-expressed with β_1 and γ_7 for comparison. The end-labeled GFP fusions and two of the transposon insertions, α_s-GFP(18–20) and α_s-GFP(92–94), showed clear localization to the plasma membrane (figure 4A). The remaining 10 fusion proteins displayed a uniform fluorescence signal throughout the cytoplasm (figure 4B).
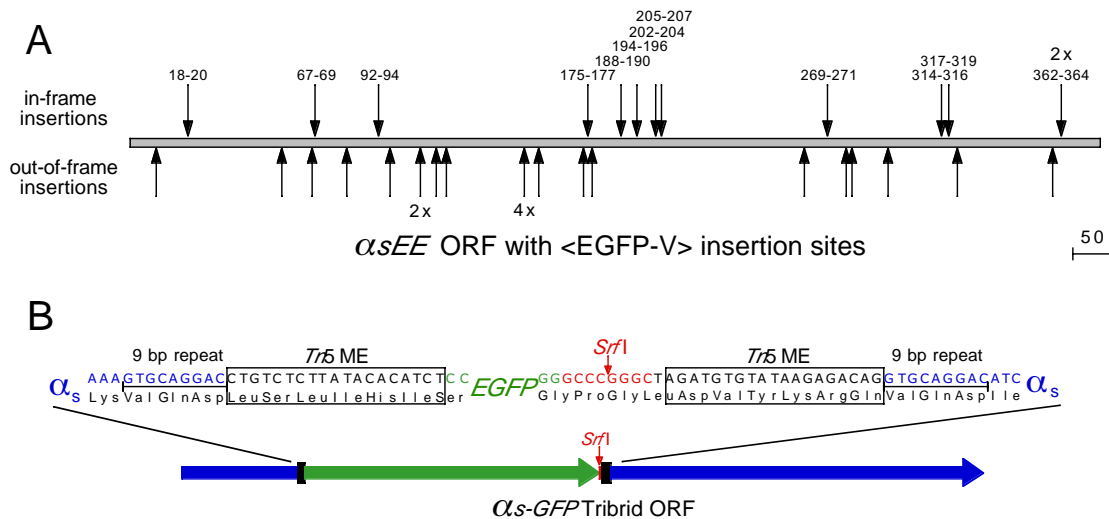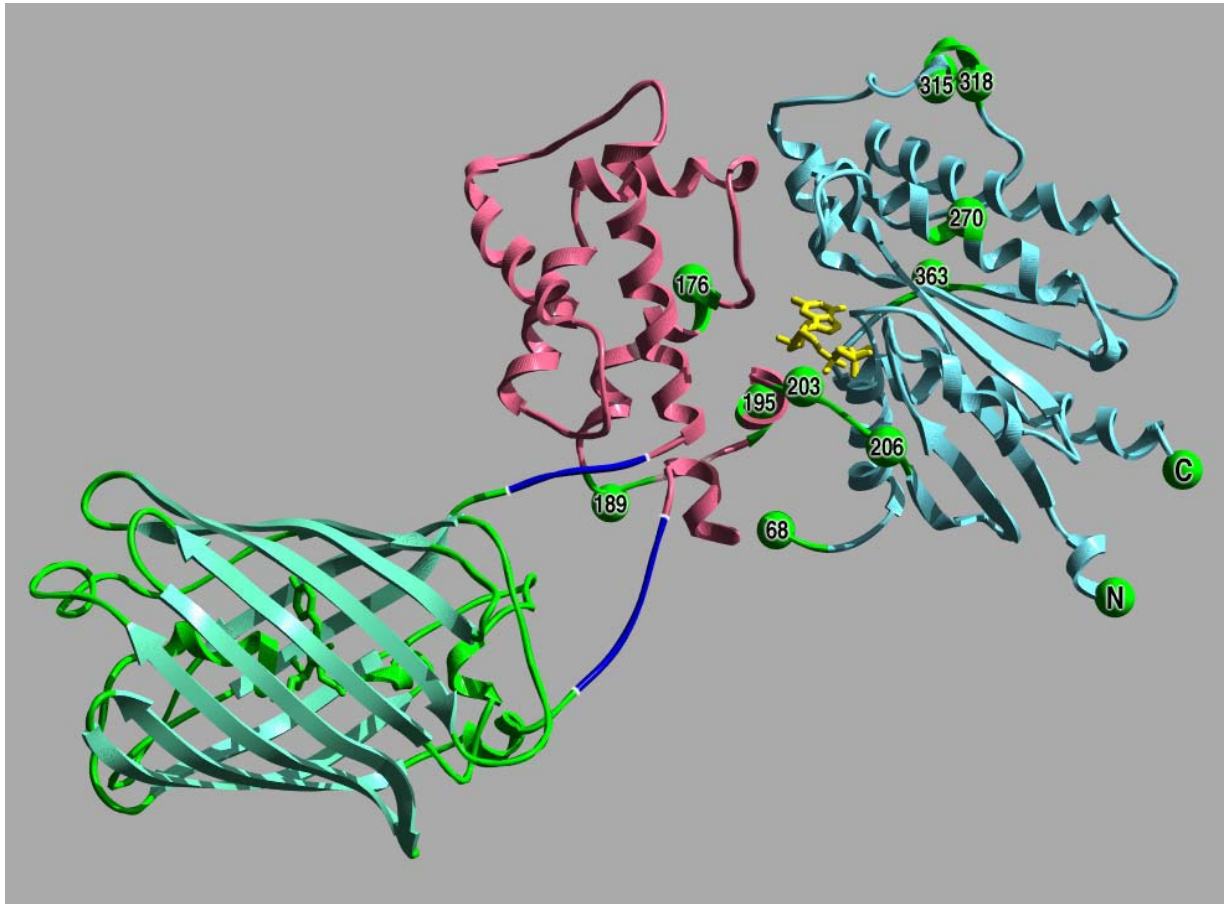
Figure 2
<EGFP-V> Insertions in α_s**EE.** (A) Location of 35 <EGFP-V> insertions in the α_sEE coding region. The 13 labeled insertions marked above the coding region are in the correct reading frame and encode fluorescent fusion proteins (12 unique insertions, 1 duplication). The insertions are named for the 3 amino acids of α_s duplicated during transposition. The 22 unlabeled insertions (18 unique) marked below the coding region are those in which <EGFP-V> landed out-of-frame with respect to α_sEE. Redundant insertions are indicated by the number of clones recovered at that site (e.g. 2X, 4X). (B) *Srf* I digestion of the transposed clone, followed by religation, removes the *Kan*r selection cassette and produces the full-length fusion protein. In the final fusion protein, the EGFP domain is bordered by amino acid linkers encoded by the *Tn*5 MEs as well as the 9 bp duplication of the target sequence that is generated during the transposition process.

The fusion proteins were tested for function by assaying their abilities to stimulate adenylyl cyclase in response to receptor stimulation. They were co-expressed with the luteinizing hormone (LH) receptor in HEK 293 cells and cAMP accumulation was measured in both the presence and absence of the LH receptor agonist, human chorionic gonadotropin (hCG). Basal and stimulated cAMP accumulation in cells expressing α_s-GFP(18–20), α_s-GFP(92–94), α_s-GFP(N), or α_s-GFP(C) were higher than in cells expressing vector alone (figure 5A). However, only in cells expressing α_s-GFP(92–94) were these differences statistically significant (p < 0.05). The basal and stimulated activities of α_s-GFP(92–94) were less than those of α_s, although these differences were not statistically significant (p < 0.05). The remaining 10 of the12 fusion proteins exhibited no detectable activity. One possible explanation for the decreased activities of the α_s-GFP fusion proteins relative to α_sEE would be a decrease in protein expression level. Cell fractionation and immunoblotting with an anti-EE monoclonal antibody showed that both α_s-

GFP(92–94) and α_s-GFP(18–20) were expressed at lower levels than α_sEE, in contrast to α_s-GFP(N) and α_s-GFP(C) (figure 5B).

Interpreting these results in the context of the structure of α_s leads to a surprising result. A rational approach to designing a fluorescent, functional α_s-GFP fusion protein would have most likely targeted the exposed loops [e.g. [8]], yet these insertions were not functional. The most functional protein was produced by the insertion of GFP into an α-helix that one would have avoided (figure 3).

The discovery that all of the in-frame insertions in α_s produced truncated fluorescent fusion proteins suggested that we could identify in frame insertions by transiently expressing all of the transposed clones and visually screening them for fluorescence. This alternative screening strategy could be particularly useful for large coding regions where a PCR-based screen might fail. To reduce the number of transient transfections required, a second

Figure 3
Model of α_s-GFP(92–94). The GFP insertions into α_s can be interpreted in the context of the structures of GFP (PDB file: 1EMA) and α_s-GTPγ S (PDB file: 1AZT). In this image, the structure of GFP [42] is green, while the helical domain of the $\alpha_s$ub-unit [15] is pink, and the GTPase domain is blue. GTPγ S is yellow. The GFP insertion α_s-GFP(92–94) that produced a functional G protein subunit is illustrated by the short linkers (encoded by the *Tn*5 MEs) between GFP and α_s (dark blue). The other sites of <EGFP-V> insertion are shown as green spheres. The numbers on the spheres indicate the second of the three duplicated residues that flank the transposon insertions (the numbers are based on the long form of α_s).

transposon was created with enhanced cyan fluorescent protein (ECFP). Two separate transpositions with the different colored transposons, followed by co-transfections in the visual screen (one potential green clone and one potential cyan clone per well), can identify twice as many in-frame insertions in a given number of transfections. This approach could be expanded to encompass many different fluorophores.

In the experiments with α_s, several clones were recovered with identical transposon insertions. This is consistent with previous reports of *Tn*5 preferentially inserting into particular locations in the target sequence [17,18]. Since these "hotspots" could become a limiting factor in the number of unique insertions recovered within a target sequence, the second reading frame through the *Tn*5 MEs was used for the ECFP transposon. This doubles the
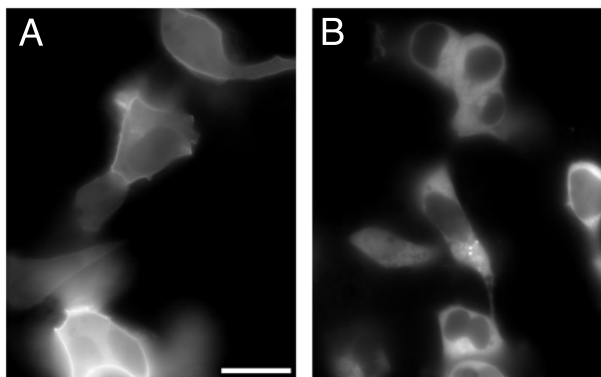
Figure 4
Localization of α_s-GFP Fusion Proteins in Living Cells. (A) Membrane localization of tribrid fusion, α_s-GFP(92–94), in HEK 293 cells ~24 hr after co-transfection with β_1 and γ_7. Similar localization patterns were observed when α_s-GFP(18–20), α_s-GFP(N), or α_s-GFP(C) fusions were co-expressed with β_1 and γ_7 (A non-linear representation of the image brightness was used to illustrate both the dimly fluorescent cells in the upper right corner and the very bright ones at the bottom, scale bar = 20 μm). (B) The remaining 10 tribrid fusion proteins were evenly distributed throughout the cytosol (with little fluorescence in the nucleus) as seen here in HEK 293 cells transiently expressing α_s-GFP(362–364), β_1 and γ_7.

number of potentially useful insertion sites within a given target sequence.

The glutamate receptor subunit GluR1 [19] was used to test the new transposons and the visual screening process. Independent transpositions of the GluR1 plasmid were performed with the EGFP and ECFP transposons (<TgPT-0> and <TcPT-1>, respectively). In 288 co-transfections, there were 20 wells with EGFP fluorescence, 21 wells with ECFP fluorescence, and 2 wells with both EGFP and ECFP fluorescence. Sequencing revealed 35 unique insertions (17 <TgPT-0> and 18 <TcPT-1>) and 10 repetitive insertions (figure 6A). The recovery of 45 fluorescent clones from 576 colonies (7.8%) agrees with the predicted frequency of transpositions resulting in GluR1-EGFP/ECFP fusions (7.7%), which is consistent with the interpretation that all in-frame insertions produce a fluorescent protein. Clones representing unique fluorescent fusion proteins were digested with *Srf* I to remove the *Kan^r* selection cassette and re-ligated to generate full-length GluR1-EGFP/ECFP fusions. These fusion proteins were screened, in transiently transfected HEK 293 cells, for glutamate-gated ion channel function. Of the 29 unique tribrid fusion constructs tested, all produce detectable fluorescence and 6 were functional (figure 6B).

Discussion

Creating functional, fluorescent fusion proteins involves finding a permissive site for the insertion of GFP, a process that in most cases still involves some guesswork. The results of both the α_s and GluR1 transpositions illustrate this point. Based on previous studies with the G protein subunit α_q [8] we anticipated that an insertion within an exterior flexible loop region of α_s would be most likely to produce a functional fusion protein. Surprisingly, the α_s fusion protein that was the most functional, α_s-GFP (92–94), resulted from an insertion into an α helix (figure 3), while the insertions in exposed loops, α_s-GFP (67–69) and α_s-GFP (188–190), were not functional. Similarly, in the case of GluR1, one of the insertions that produced a functional channel, GluR1-GFP(526–528), was within the hydrophobic region thought to be the first transmembrane domain (see Additional File: Figure 7). Additionally, within a given region of GluR1, one insertion will produce a functional channel while another nearby insertion does not (e.g. the intracellular carboxy-terminus region or the amino terminus between amino acids 210 and 330). The reasons for these discrepancies are not obvious.

The discovery that GFP will still fold and form a fluorophore when placed virtually anywhere in another coding region suggests that the limiting step in the process is whether the target protein it is inserted into folds and functions correctly. Indeed, GFP fusion constructs have been used to assay, and improve upon, the folding of a variety of proteins in a bacterial expression system [20]. The relatively random nature of the transposition events we recovered in this study suggests that it might be possible to insert GFP at nearly every position in a given protein, but there are two potential limits. First, the laws of probability predict that there will be rapidly diminishing returns in the search for unique *Tn5* insertions as one recovers each additional clone. Second, the behavior of the *Tn5* transposon is not entirely random. Goryshin and colleagues [18] have shown that there is a weak consensus site for *Tn5* insertion which is consistent with our results. It appears that the resolution limit will be an insertion each three amino acids on average in a target protein.

Inserting a reporter domain such as GFP into another protein always has the potential of perturbing the target and destroying it's ability to function. In this study 16% of the tribrid fusion proteins were still functional. One explanation for why GFP can be used for internal insertion is that the N- and C-termini of GFP exit the structure quite close to one another and are unlikely to displace the surrounding domains of the target protein a great deal. This is analogous to the use of the bovine pancreatic trypsin inhibitor for internal insertions [21]. The transposons described here could potentially be improved upon by optimizing the length and flexibility of the linkers between the target
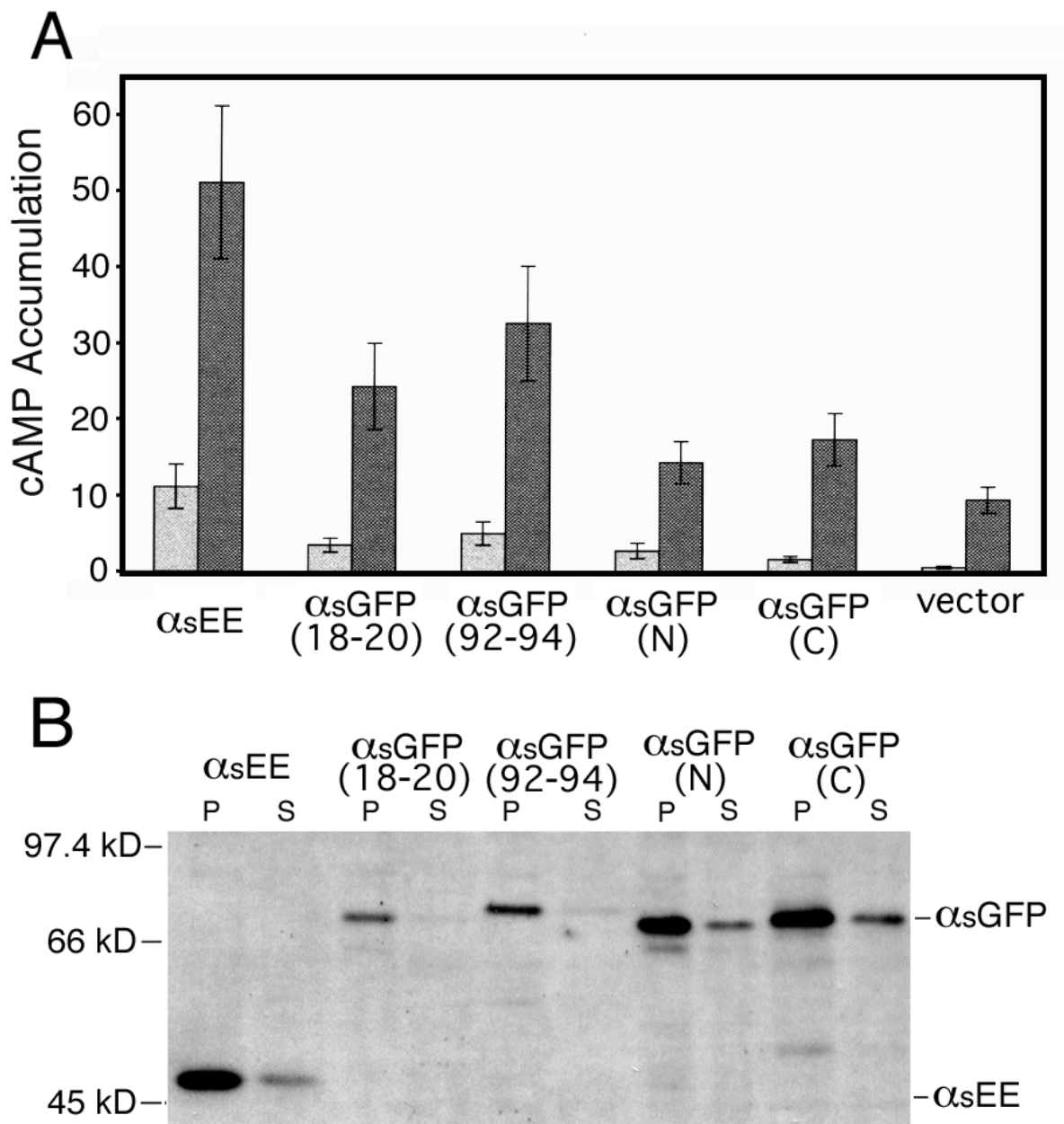
Figure 5
Activity and Expression Levels of α_s-GFP Fusion Proteins. (A) HEK 293 cells were transfected with 2 μg/10^6 cells of the indicated α_s-GFP constructs or vector alone (pcDNA1/Amp) and 0.2 μg/10^6 cells of plasmid encoding the LH receptor. cAMP accumulation was measured in the presence (dark gray bars) or absence (light gray bars) of hCG, an LH receptor agonist. Cells expressing each of the α_s-GFP fusion proteins exhibited increased basal and stimulated cAMP accumulation relative to cells expressing vector alone, but only the increases in cells expressing α_s-GFP(92–94) were significantly greater (p < .05). Values represent the mean $\pm$ S.E. of 5 independent experiments. (B) Immunoblots of the membrane pellets (P) and supernatant (S) fractions from transiently transfected HEK 293 cells. Expression levels of tribrid fusion proteins α_s-GFP(18–20) and α_s-GFP(92–94), but not of amino- and carboxy-terminus fusions, α_s-GFP(N) and α_s-GFP(C), respectively, were decreased in both fractions relative to that of unlabeled α_sEE. Similar results were obtained in an additional experiment.
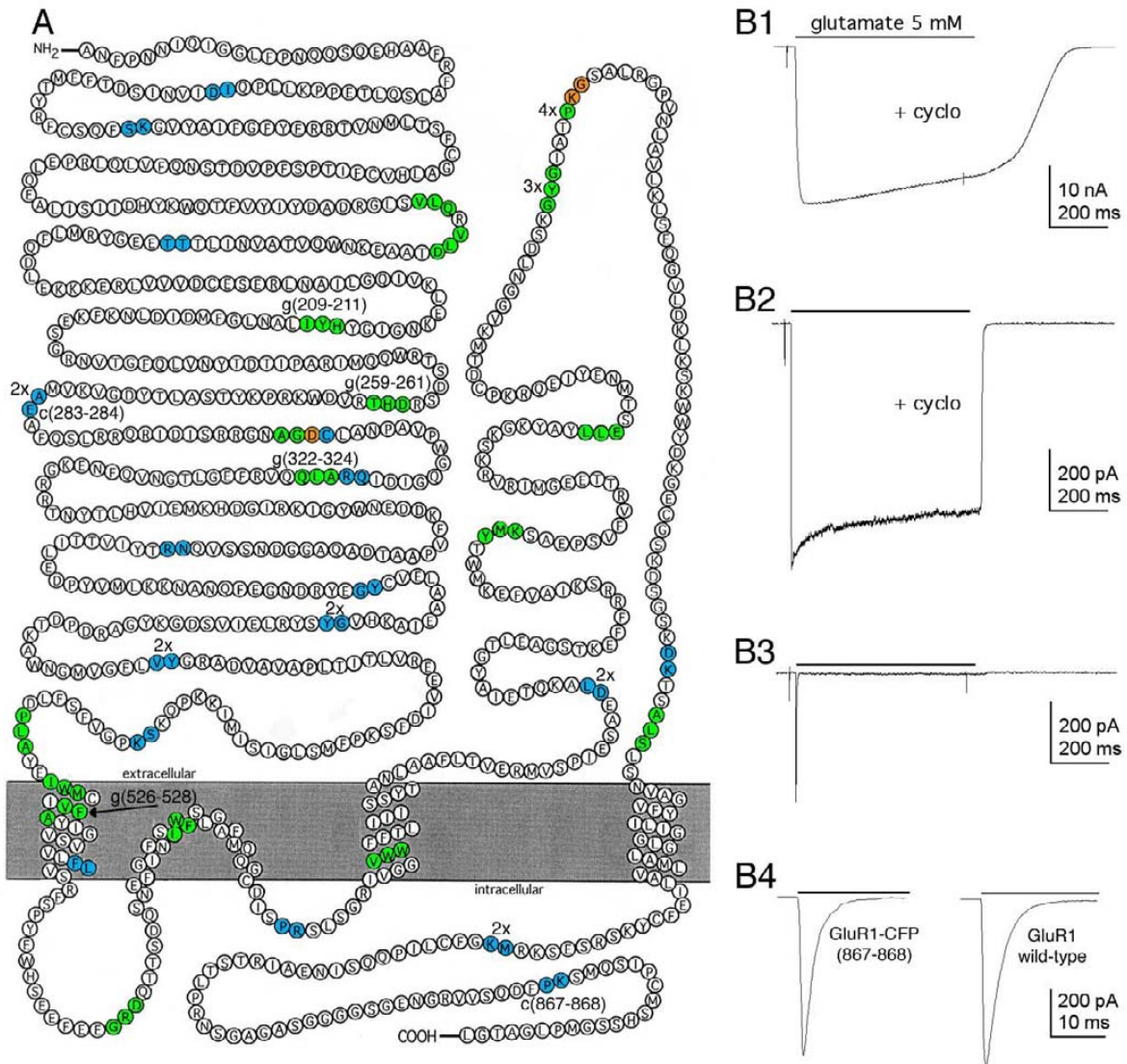
Figure 6
Insertion Sites and Functional Screening of GluR1-GFP Fusion Proteins (A) A model of GluR1 topology showing the locations of the GFP insertion in 45 fluorescent fusion proteins. In-frame insertions of <TgPT-0> result in a 3 amino acid duplication (green) flanking the insertion site. In-frame insertions of <TcPT-1> generate only a 2 amino acid duplication (cyan) in the target because of the different reading frame. The orange amino acids are overlapping insertion sites of the two transposons (See supplemental diagram for the two reading frames). Multiple clones with identical transpositions are identified as 2x, 3x, etc. The six insertions resulting in functional, fluorescent GluR1-GFP/CFP tribrid fusion proteins are identified by the duplicated target amino acids (e.g. g209–211, c867–868). This figure was adapted from [43]. (B) AMPA receptor-mediated current from GluR1-CFP(867–868). (B1) Large whole-cell current elicited by the rapid sustained application of 5 mM glutamate (bar) in a cell transiently expressing GluR1-CFP(867–868) after reducing desensitization with cyclothiazide (100 μM). (B2) Current elicited by 5 mM glutamate in an outside-out patch pulled from a cell transiently expressing GluR1-CFP(867–868). (B3) Current elicited in the same patch as B3, but in the absence of cyclothiazide. Note the rapid and nearly complete desensitization of the current. (B4) The trace on the left is an expanded time scale of B3, the trace on the right is from an outside-out patch pulled from a cell transiently expressing wild-type GluR1 in the presence of 5 mM glutamate without cyclothiazide.

and the GFP. Another potential improvement to the process would be to use bacterial expression to screen for transposon insertions that produce a fluorescent protein. This could, however, be problematic with proteins from the mammalian nervous sytem, such as ion channels, that are difficult to express in bacteria.

The approach described here should speed the discovery of genetically encodable fluorescent sensors. The pioneering work of Siegel and Isacoff showed that GFP placed within a portion of the Shaker K^+ channel C-terminus produced a fluorophore that responded to changes in membrane voltage [5], but they built a number of different constructs before finding one that worked. Similarly, Ataka and Pieribone created an EGFP-Na^+ channel fusion protein that changes fluorescence in response to membrane depolarizations on a time-scale that would be sufficient to image action potentials. This discovery, however, was the result of designing, building, and testing eight different tribrid fusion proteins [22]. Little is known about the mechanism whereby changes in channel conformation are converted to changes in the fluorophore, so it remains to be determined whether GFP can signal conformational changes in other kinds of proteins. Nevertheless, the use of the transposons described here should shift the work from building the constructs to devising high throughput assays for function.

Finally, random GFP tagging will facilitate the creation of potential fluorescence resonance energy transfer (FRET) reagents to study protein interactions in living systems. To date, a few studies have demonstrated the potential power of GFP-FRET by labeling different proteins [6,23–26] or by fusing two different fluorophores to the same protein [27–32]. Creating efficent donor and acceptor fusion proteins is difficult, however, because FRET only occurs when the two fluorophores are attached to surfaces that are very close to one another. The approach described here makes it possible to rapidly generate libraries of potential donor and acceptor tribrid fusion proteins that can be screened, in pairwise combinations, for function and FRET signals.

Conclusions
The transposons described here make it possible to rapidly generate large numbers of different GFP fusion proteins. The results show that GFP can be inserted into a wide variety of other protein domains and it will continue to fold and form a fluorophore. The rapid and random nature of the transposition process makes it possible to generate and screen many different fusion constructs to identify those that continue to function. In the case of the two proteins tested here, roughly 1 in 6 of the fusion proteins retained their signaling function, and the random nature of the transposition process revealed permissive sites for insertion that would not have been predicted on

the basis of structural or functional models of how that protein works. This simple tool should speed the search for a wide variety of new biological probes for the study of nervous system.

Materials and Methods
PCR and standard subcloning procedures were use to create the initial transposon, <EGFP-V> (full sequence at: [http://momotion.med.yale.edu]). The *Tn*5 MEs were added to the 5' and 3' ends of an EGFP coding sequence, with a *Srf* I restriction site at its 3' end, such that one continuous reading frame extended through both MEs and EGFP (figure 2). To add antibiotic selection, the Kan^r gene from pUniV5-His-TOPO™ (Invitrogen, Carlsbad, CA) was flanked with *Srf* I sites and inserted into the transposon. The improved transposons, <TgPT-0> and <TcPT-1>, were created in the same way as <EGFP-V>, but *Asc* I sites were added to facilitate changing the fluorescent protein at a later date (supplemental material). In addition, the two different reading frames present in the MEs were used to create the two different transposons, and ECFP was used in place of EGFP in <TcPT-1>. A primer complementary to the19 bp *Tn*5 ME (5'-CTGTCTCTTATACACATCT-3') was used to amplify the transposons (1 cycle at 95°C for 3:30 min., 24 cycles of 95°C for 30 sec 47°C for 30 sec 72°C for 1 min., 1 cycle at 72°C for 5 min.) with *Pfu* polymerase (Stratagene, La Jolla, CA). The PCR product was purified and concentrated with the Geneclean II kit (Bio101 Inc., Vista, CA) and eluted in 1X TE buffer. 0.2 fmoles of transposon were incubated with 5.0 μL of EZ::TN™ transposase (Epicentre Technologies) in 25% glycerol at 25°C for 30 min.

Molar equivalents of transposon and target plasmid (0.4 fmoles ea.) were incubated in reaction buffer (50 mM Tris-acetate (pH 7.5), 150 mM potassium acetate, 10 mM magnesium acetate and 4 mM spermidine) at 37°C for 2 hr in a 10 μL reaction. Transposition was stopped by adding 1 μL of 1% SDS and incubating at 70°C for 10 min. Top 10 F' *E. coli* (Stratagene) were transformed with 1 μL of the transposition reaction and plated on LB agar with either ampicillin (100 μg/mL) and kanamycin (50 μg/mL) to recover transposed clones, or ampicillin (100 μg/mL) alone to establish the transposition efficiency.

The cDNA encoding the rat α_s [33], modified to carry the EE epitope [34], was in pcDNA1/Amp (Invitrogen). GFP was added to the N- or C-terminus of α_s to create end-labeled constructs for comparison with the transposed GFP tribrid fusion proteins. The amino-labeled clone, GFP-[GGGPSGGGGS]-α_sEE, and carboxy-labeled clone, α_sEE-[SGGGGSGQH]-GFP, were generated via overlap extension [35]. Linker sequences are in brackets. The flip variant of rat GluR1 was in the CMV expression plasmid pRK5

(a generous gift from Derek Bowie, Emory University, Atlanta, GA).

PCR screening for <EGFP-V> insertions within the $\alpha_s EE$ coding region was performed using a protocol described by Cease et al. [36] using an upper primer complimentary to the 5' UTR (5'-GCTCCCGCGGCTCCTGCTCTGCTC-3'), and a lower primer complimentary to EGFP (5'-GCCGTCGCCGATGGGGGTGTTCTG-3'. The clones that produced clear PCR products within the expected size range were then miniprepped (QIAgen, Germantown, MD).

Insertion sites were identified for all PCR-positive <EGFP-V> transposed clones and all fluorescent <TgPT-0>/<TcPT-1> transposed clones by sequencing out of the transposon with a primer complimentary to the EGFP/ECFP coding region (5'-tggccgtttacgtcgccgtcca-3'). *Srf* I restriction digestion was then used to remove the *Kan^r* cassette from the clones carrying in-frame insertions, thereby creating a sequence encoding a full-length fusion protein. After digestion and re-ligation, Top 10 F' *E. coli* were transformed with 1 μL of the ligation reaction and plated on LB agar containing ampicillin. The colonies were re-plated the following day on ampicillin and kanamycin to verify loss of the *Kan^r*.

The fusion proteins were transiently expressed in HEK 293 cells [37]. Transfections were done using Lipofectamine 2000 (Gibco BRL). Images were collected from live cells 20–48 hr later on an inverted Zeiss microscope fitted with computer controlled (IPLabs, Scanalytics) filter wheels (Ludl Electronics) on the excitation and emission paths. EGFP was imaged with an FITC filter set, while ECFP was distinguished from EGFP in co-expression experiments by changing both the excitation and emission filter sets (Exciters: 440AF21 & 500AF25, Dichroic cat# XF 2063, Emitters 480AF & 545AF35; Omega, Brattleboro, VT).

α_s-GFP fusion proteins were assayed for the ability to stimulate adenylyl cyclase in response to luteinizing hormone (LH) receptor stimulation [38]. 10^6 HEK 293 cells/60 mm-dish were co-transfected with 2 μg of plasmid DNA encoding the α_s-GFP fusion protein, and 0.2 μg of plasmid DNA encoding the rat LH receptor in pCIS [39], using 10 μL of Lipofectamine 2000. [^{3}H]-adenine-labeled cells were assayed for cAMP accumulation after incubation at 37°C for 40 min. in the presence of 1 mM 3-isobutyl-1-methylxanthine (IBMX) a phosphodiesterase inhibitor, and in the presence or absence of 7.5 ng/mL human chorionic gonadotropin (hCG), as described previously. Conversion of ATP to cAMP was expressed as:

$$10^3 \times [^3H]cAMP/([^3H]ATP + [^3H]cAMP).$$

12×10^6 HEK 293 cells were transfected, using DEAE-dextran [40], with 25 μg of plasmid DNA. Forty-eight hours after transfection, cells were lysed and membrane and supernatant fractions harvested as described previously [8]. 10 μg of membrane proteins and normalized volumes of the supernatants were resolved by SDS-polyacrylamide electrophoresis (10%), transferred to nitrocellulose, and probed with a monoclonal antibody to the EE epitope [34]. The antigen-antibody complexes were visualized with ECL chemiluminescence (Amersham Biosciences, Piscataway, NJ).

Whole-cell patch clamp recording was used to test the GluR1 fusion proteins for function in transiently transfected HEK 293 cells as previously described [41]. The external solution was (in mM): 150 NaCl, 3 KCl, 2 CaCl$_2$, 1 MgCl$_2$, 5 glucose, 0.002 glycine and 10 HEPES (pH 7.4). Patch pipettes were filled with a solution containing (in mM): 120 CsF, 33 KOH, 2 MgCl$_2$, 1 CaCl$_2$, 0.1 spermine, 10 HEPES, and 11 EGTA (pH 7.4). Cyclothiazide was prepared as a 20 mM stock solution in DMSO and diluted to 100 μM in external solution. All chemicals were purchased from Sigma. Drugs were applied with a rapid superfusion system made from a pulled theta capillary. The open tip responses obtained with this system had 10–90% rise-times of 150 μs to 300 μs.

Authors' contributions

Author 1 D. L. Sheridan carried out the design and construction of the transposons, conducted the biochemical assays for G-protein signaling, imaged the living cells, and drafted the manuscript. Author 2 C. H. Berlot provided critical reagents and advice for all portions of the G-protein work. Author 3 A. Robert screened the Glutamate receptor subunits for function. Authors 4 F. M. Inglis and 5 K. B. Jakobsdottir provided help in the minipreparation of plasmid DNA for each of the constructs. Authors 6 J. R. Howe and 7 T. E. Hughes participated in the study design, coordination, and analysis.

All authors read and approved the final manuscript.

Additional material

Additional File
Figure 7
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Acknowledgments

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