Chapter IV

DEVELOPMENT OF CADHERIN FRET REPORTERS

In order to better understand the homophilic interactions of cadherins in living cells, I undertook the construction of a series of pairs of cadherin fluorescent fusion proteins to use in FRET experiments. The goal of the experiments was to detect changes in cadherin conformational and binding states by monitoring energy transfer at the cell membrane junction between adjacent cells expressing cadherins labeled with FRET donor and acceptor fluorophores, respectively.

Epitope tagging approaches

My initial efforts involved creating small-epitope-tagged cadherins with the plan of using fluorescent primary antibodies to label the epitope tags with FRET donor and acceptor fluorophores. I inserted HA and FLAG epitopes at several points in the E-cadherin extracellular region. These insertion points were chosen with the following considerations in mind: to optimize the relative distance of each molecule in an intact cellular junction for energy transfer, to use sites accessible to an exogenously introduced antibody, and to avoid regions involved in Ca^{2+} binding, cadherin dimerization, or cadherin structure (based on structural data obtained from Shapiro, Fannon et al. 1995 and Overduin, Harvey et al. 1995) (figure 4).

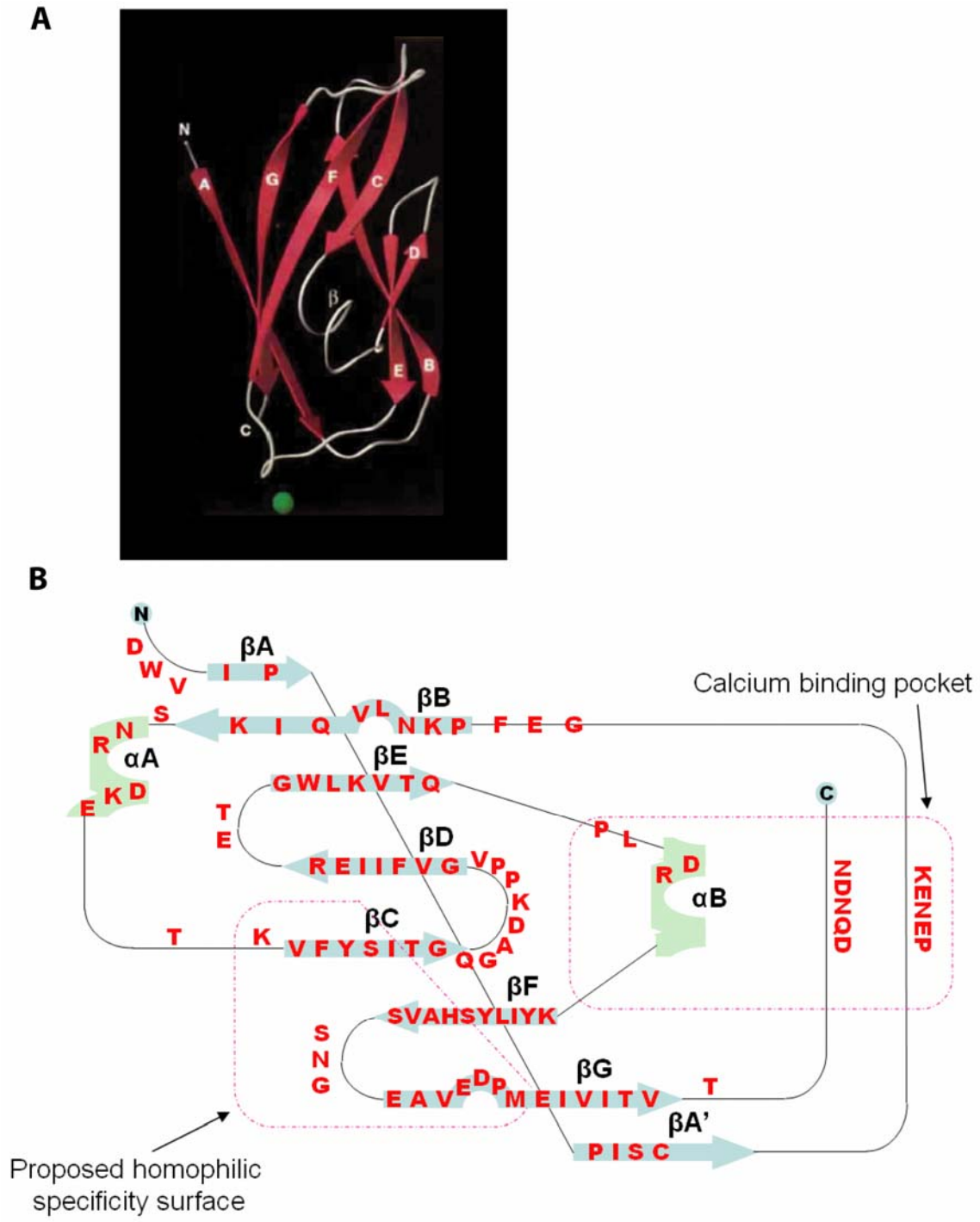


Figure 4. Structure of the N-terminal EC domain (EC1) of E-cadherin.
A. Ribbon diagram of EC1. Each β-strand is labeled and the Ca^{2+} binding site is indicated by the green sphere. B. Schematic drawing of the domain topology with the 7 β-strands (βA through βG) displayed in light blue and the two α-helices in light green. Residues involved in the proposed homophilic specificity surface and the Ca^{2+} binding

site are indicated by the purple dashed lines (adapted from Overduin, Harvey et al. 1995 and Shapiro, Fannon et al. 1995).

This approach served mostly to demonstrate the cadherin sensitivity to any molecular "mucking around," particularly when molecules were inserted near the N-terminus. Some previous work in the laboratory had generated E-cadherin N-terminally tagged with EGFP; the fusion protein could be expressed in cultured cells, and the GFP moiety was fluorescent, but the molecule was not trafficked correctly, and got stuck in the Golgi and endoplasmic reticulum (ER). Similar to this, my first generation of small-epitope tagged cadherins (with the tags inserted between EC1 and EC2) were also sequestered in the Golgi and ER and were only internally expressed, rendering them useless as FRET reporters.

For the next round of fusions I decided to insert the tags more C-terminally in the extracellular domain; on a loop in EC4 and between EC4 and EC5. These constructs were properly trafficked and inserted into the membrane, but I was not able to successfully immunolabel live cells expressing them, possibly due to the relative inaccessibility of the epitope tags in the cell junction.

Fluorescent protein approaches

Based on the lack of success described above, I decided to create fluorescent fusion protein (ECFP and EYFP) insertions instead. Unfortunately, this proved equally difficult: inserting ECFP or EYFP into the same point between EC1 and EC2 used for the small-epitope tags caused the same problems with membrane expression as seen with the epitope fusions. Inserting CFP/YFP into a loop in EC3 finally yielded a cadherin-EGFP-variant fusion that was properly expressed on the membrane, and could be

immunolabelled in live cells with either anti-cadherin antibodies or anti-GFP antibodies. However, the GFP-variant was not fluorescent, suggesting that its position in the cadherin structure resulted in misfolding.

Transposon-mediated functional insertion of fluorescent proteins in N-cadherin

Dr. Chin-Yin Tai, a postdoctoral scholar in our laboratory, used a transposon-mediated random insertion strategy to successfully create a GFP-N-cadherin intramolecular fusion that was both membrane expressed and properly folded and fluorescent. The bacterial transposon, Tn5, known for its well-known properties of high efficiency ($>10^6$) and randomness, was used. This enzyme recognizes a 19-base-pair inverted repeat of the transposon-containing DNA (linear or circular) and makes a 9-base-pair staggered nick in the target DNA. After transposition, this nick is repaired and subsequently generates a 9 base pair duplication of the target sequence immediately flanking the transposon (figure 5A). To identify clones that possess the correctly inserted GFP, colony polymerase chain reaction (PCR) was chosen since this method allowed the screening of hundreds of colonies without DNA amplification and purification (figure 5B). Three 5'-end primers covering the whole N-cadherin coding region and one 3'-end primer within GFP were designed (see materials and methods for details). To reduce the number of reactions that were required, several (~5) colonies were pooled into 1 reaction. This allowed a rapid elimination of those colonies that fail to produce a positive result. Colonies that express the in-frame fusion protein were selected by transiently transfecting all clones into HEK293 cells, to directly visualize GFP fluorescence (figure 5C). Lastly, the full-length N-cadherin with intramolecular GFP insertions was restored. During the transposition, a stop codon was inserted between GFP and the kanamycin-resistant gene,

which created a truncated N-cadherin GFP fusion. A restriction enzyme (SmaI) digestion followed by self-ligation and bacterial transformation restored the full length N-cadherin GFP fusion (figure 5D). Upon completion of the screen, all remaining clones were ready for functional assays.

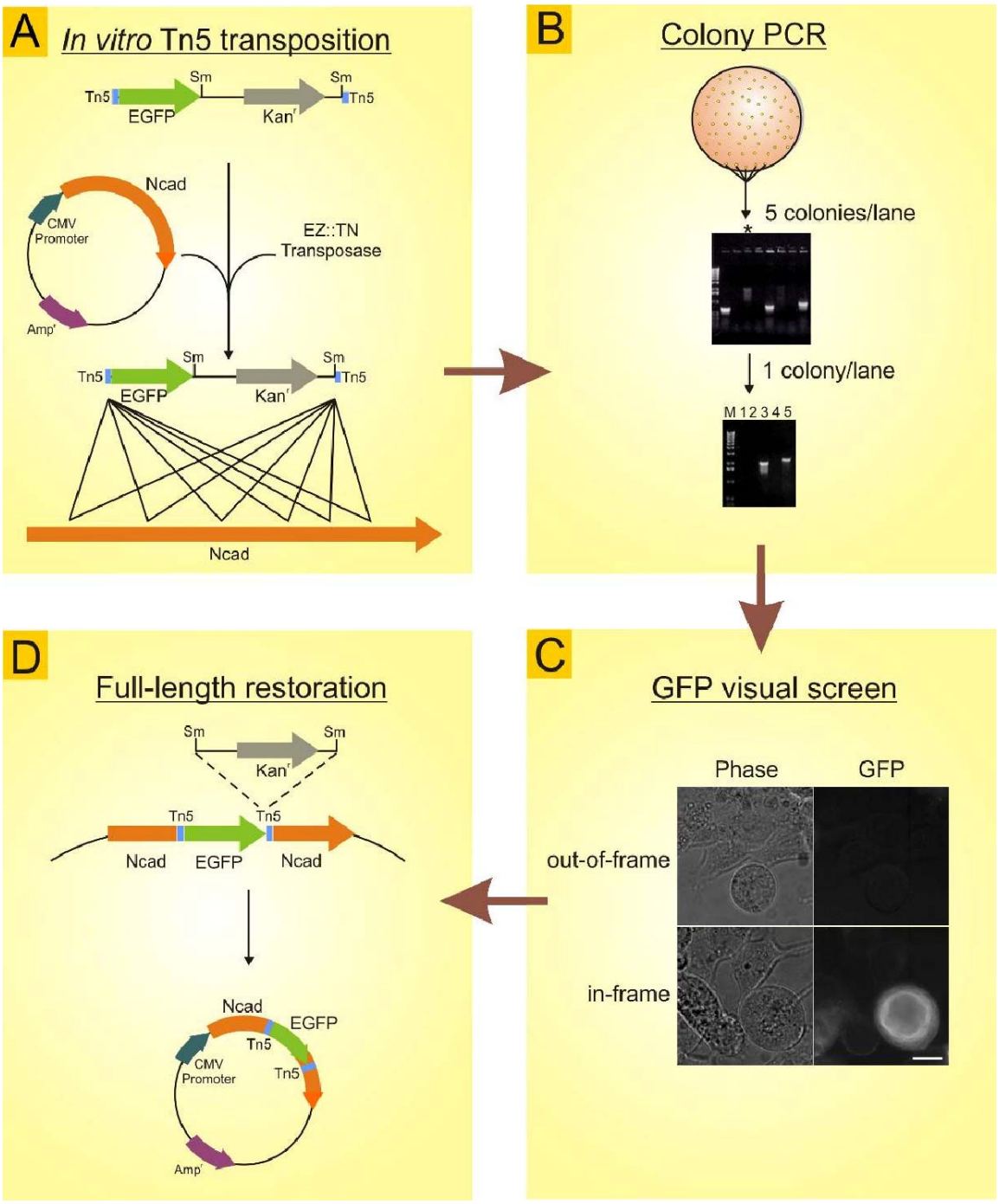


Figure 5. Overview of the bacterial transposon-mediated random insertion.
A. The target plasmid DNA (pCXN2-NCad) was incubated with the linearized transposon-containing plasmid DNA (pBNJ24.6), and EZ:TN transposase (Epicentre) at 37°C for 2 hours. The transposed products were selected by the double antibiotic resistance of ampicillin (selection marker for the target plasmid) and kanamycin (Kan^r, selection marker located downstream of EGFP). B. Correctly oriented GFP insertional clones were identified by two rounds of colony PCR reactions. C. HEK293 cells were

transfected with DNA of the selected clones and GFP signals were visualized under the fluorescent microscope. D. All fluorescent clones were digested with restriction enzyme, SmaI, and re-ligated *in vitro*. Recovered full-length clones were isolated after transforming into bacterial host strain. Scale bar: 5μm (From Dr. Chin-Yin Tai).

In order to function in FRET assays, the clones need to be expressed at cell junctions. A preliminary screen was performed as described above for 509 colonies and 23 candidate clones were obtained. All 23 clones were transfected into HEK293 cells after the full-length restoration procedure. When the fluorescence was visualized in intact transfected cells, 10 clones exhibited localization at the adherens junctions, the expected location for a functional cadherin protein. These clones were then subjected to DNA sequencing to identify the exact site of insertion - 6 out of 10 clones had GFP inserted in the extracellular domain of N-cadherin (figure 6A). In this first screen, 2 preferential insertion sites were noted: one is located between the fifth EC and the transmembrane domain, and the other is in the middle of the cytoplasmic domain. In addition, no insertion sites within the third to the fifth EC domain were found, suggesting that the Tn5-mediated transposition is not absolutely random. However, 4 clones that had GFP inserted in the distal extracellular region were found (figure 6B). Among them, clone TS25 had the brightest adherens junction signal in HEK293 cells. As such, this clone was chosen for the initial FRET experiments. After clones that express well at junctions were identified, I cloned ECFP and EYFP in the place of GFP, and the resulting constructs were transfected into L cells for an aggregation assay. Note that L cells lack endogenous cadherins but contains all catenins and have been used extensively for cadherin-dependent adhesion functional assays (Nagafuchi, Shirayoshi et al. 1987). When successfully transfected with cadherins, L cells exhibit adhesion and aggregation

that is dependent on extracellular Ca^{2+} (Nagafuchi, Shirayoshi et al. 1987). As L cells are notoriously hard to transfect and measures of aggregation require high transfection efficiency, stable lines that express either the Cad-CFP (FRET donor) or Cad-YFP (FRET acceptor) were constructed. After one month of continuous selection and expansion from a single colony, 10 independent clones were picked for each construct. Western blotting analyses revealed 3 lines that stably express Cad-CFP and 6 lines that stably express Cad-YFP. A calcium-dependent aggregation assay was performed for all clones; representative results are shown in figure 3C. In addition, it was found that each fusion protein interacts with β-catenin via immunoprecipitation assays (figure 6D).

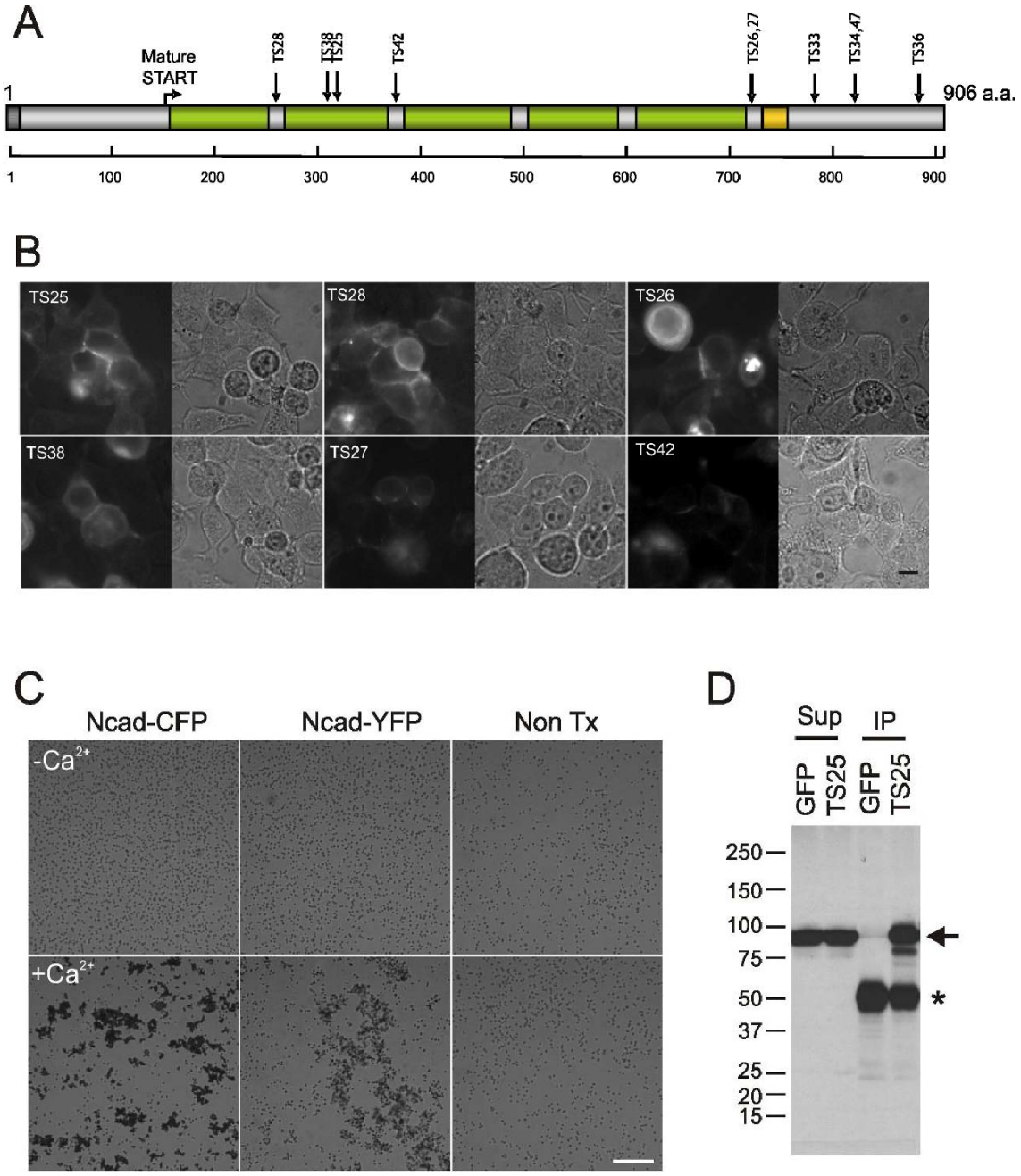


Figure 6. Functional assays of GFP-N-cadherin fusion proteins.
A. Schematic summary diagram of GFP insertion sites. The linear N-cadherin protein structure is colorized according to the following: dark grey reprents the signal peptide; green represents the extracellular domains; yellow represents the transmembrane domain. The segment beyond the transmembrane domain contains the cytoplasmic domain, which binds members in the catenin family. B. Adherens junction localization of 6 GFP-N-cadherin clones that have GFP inserted at the extracellular domain. C. Calcium-dependent homophilic interactions of the CFP or YFP fusion derived from the TS25 clone in L cells. Non Tx represents nontransfected cells. D. β-catenin interacts with the

GFP-N-cadherin fusion protein (arrow: 90 kDa -catenin, *: IgG heavy chain). Scale bars: in B: 5 µm; in C: 50 µm (From Dr. Chin-Yin Tai).

Monitoring cadherin interactions via acceptor bleaching (adFRET)

Acceptor bleaching or depletion is a simple and rapid method of measuring FRET that avoids some of the complications in other FRET measurement techniques, as it provides "*in situ*" (at every pixel of interest) generation of the reference state of the nonFRETing donor fluorophore. Simply put, the bleaching of the acceptor increases the donor fluorescence observed owing to dequenching of the signal (figure 7).

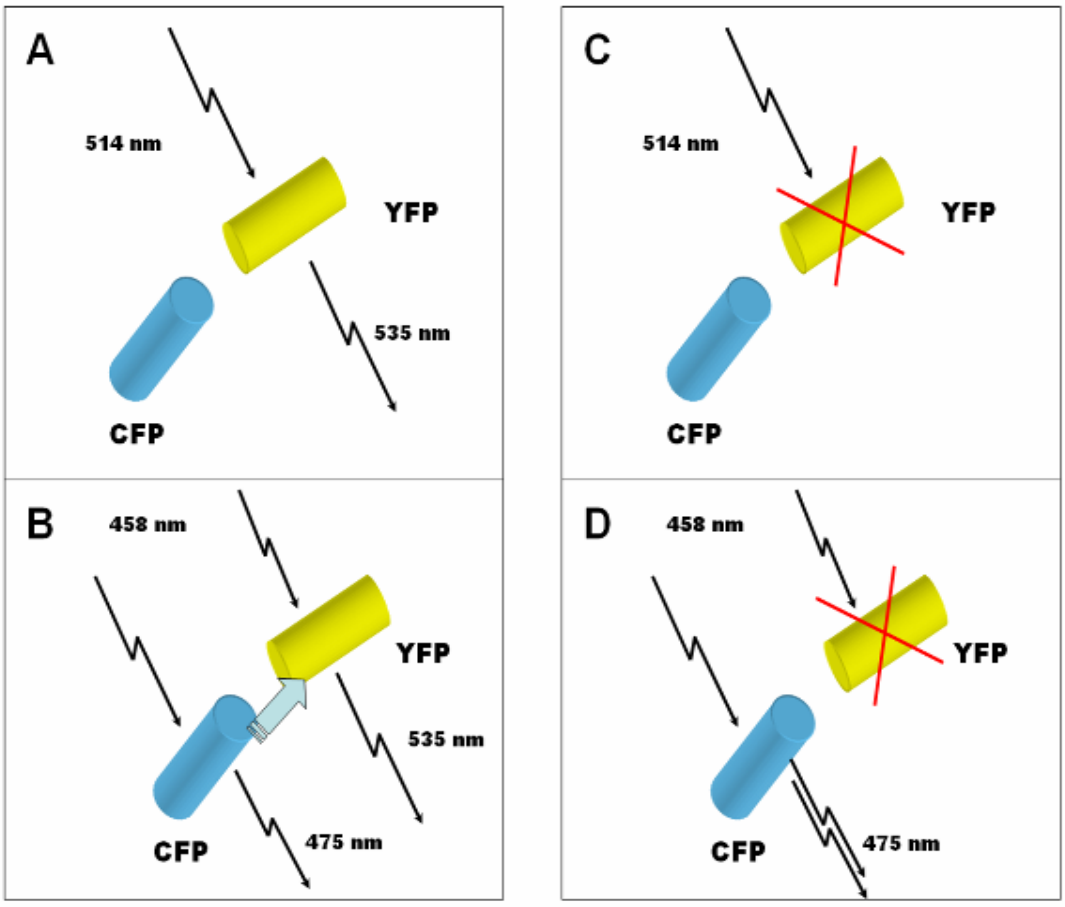


Figure 7. Overview of FRET and the acceptor bleach FRET experiment.

A. Cells expressing both donor and acceptor are illuminated with 514 nm laser light, exciting YFP (the FRET acceptor) and resulting in acceptor emission. B. The same cell is illuminated with 458 nm laser light, exciting primarily CFP, but also minimally exciting YFP. If donor and acceptor are in close enough proximity and oriented properly, FRET will occur, leading to increased acceptor emission. C. If the acceptor is depleted (through irreversible photobleaching), illumination with 514 nm light yields decreased acceptor emission (in practice, bleaching is rarely complete, leaving some intact acceptor molecules to fluoresce). D. When the acceptor is photobleached, FRET is no longer possible, so when the acceptor-depleted cell is illuminated with 458 nm light, the donor fluorescence is increased relative to B.

To determine whether the N-cadherin fusion proteins can detect cadherin-cadherin *cis* interactions, I doubly-transfected HEK-293 cells with ECFP- and EYFP-tagged N-cadherin (generated from GFP-Ncad clone TS25). I then imaged the cells using an LSM Meta laser scanning confocal microscope and unmixed the CFP and YFP spectra as described in the methods section (figure 8). Upon acceptor bleaching, modest yet detectable donor dequenching was observed, indicating detectable FRET between cadherins, presumably involved in *cis* interactions. Longer bleaching times and more thorough bleaching led to increased donor dequenching, as expected if the acceptor bleach and donor dequench are rooted in the same process (figure 8F).

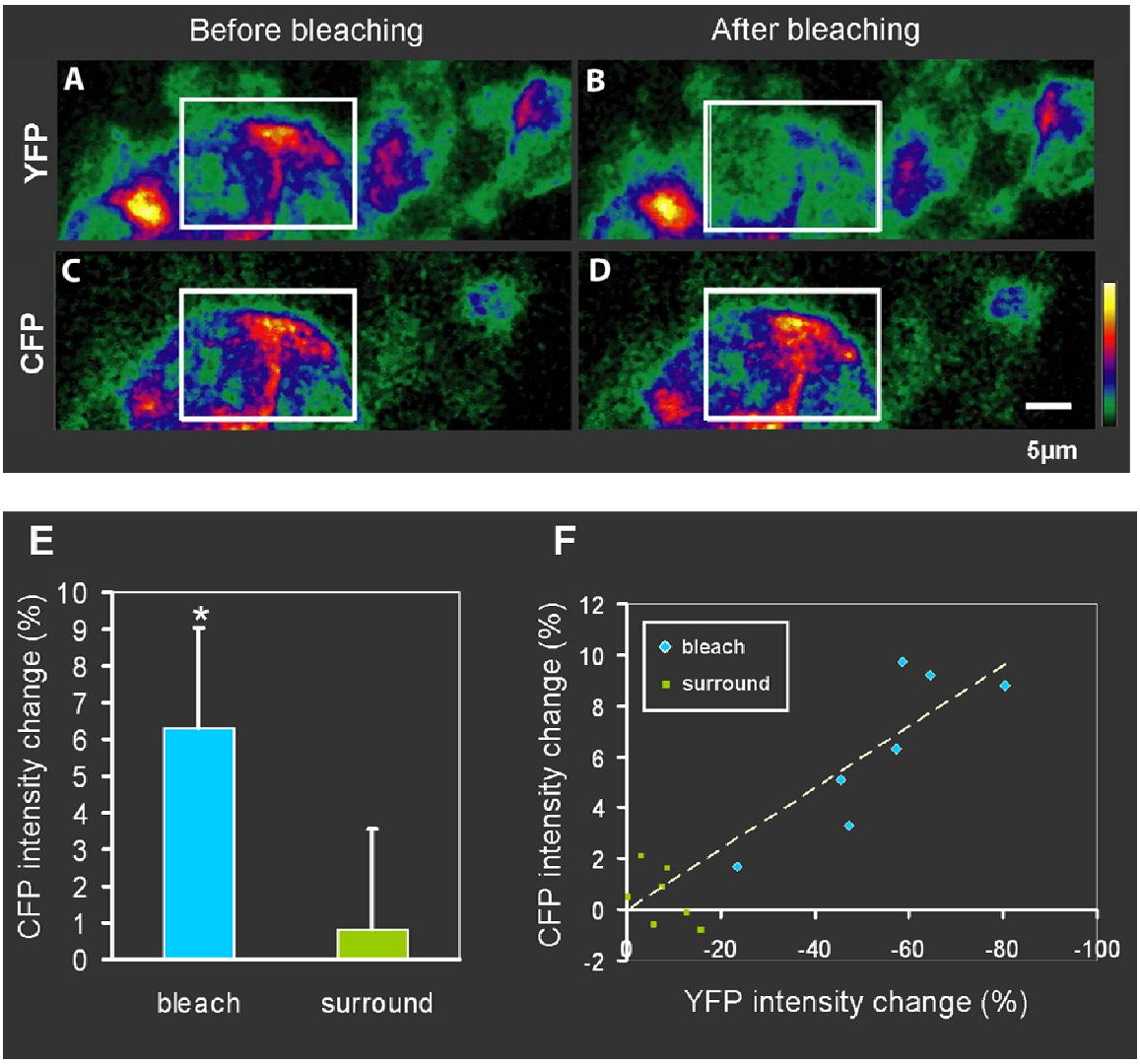
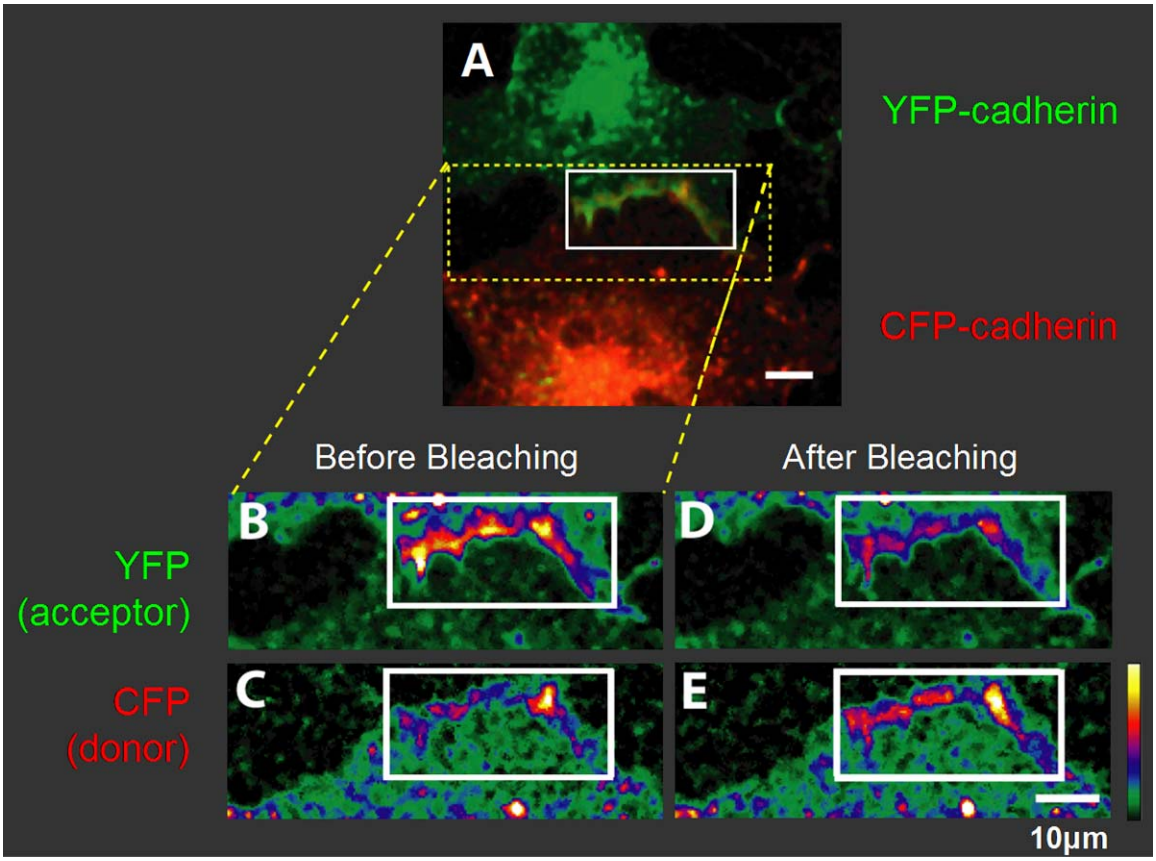


Figure 8. adFRET in HEK-293 cells expressing both CFP-N-cadherin and YFP-N-cadherin revealing cadherin *cis* interactions.
A. LSM Meta laser scanning confocal microscope generated YFP signal (linear unmixing of separately obtained YFP and CFP spectra was used to generate YFP-only signal). B. Post-acceptor-bleach YFP signal. C. LSM Meta laser scanning confocal microscope generated CFP signal. D. Post-acceptor-bleach CFP signal (white boxes represent the bleached area). E. Fluorescent intensities of bleached ROI and surrounding nonbleached areas were quantitated and the percent change of the donor signal was calculated. F. More thorough bleaching leads to increased donor dequenching, as expected if the YFP bleaching and the CFP dequenching are connected through and originating from the same process. Scale bar: 5 µm.

To investigate whether *trans* interactions (across cellular junctions) between labeled cadherins expressed in different cells could be detected by adFRET, I first replaced the ECFP and EYFP tags with Cerulean and Venus, respectively, to provide increased donor and acceptor brightness and improved signal-to-noise ratio (Rizzo, Springer et al. 2004; Nagai, 2002). I then transiently transfected COS-7 cells with Cerulean- *or* Venus-tagged N-cadherin (generated from GFP-Ncad clone TS25), and trypsinized and mixed the separately transfected cultures, before replating the cells in glass-bottomed dishes for imaging and analysis (figure 9). Upon acceptor bleaching, donor dequenching was comparable to that observed in the doubly transfected cells. A similar linear relationship was observed between the magnitude of acceptor bleaching and donor dequenching (figure 9G).



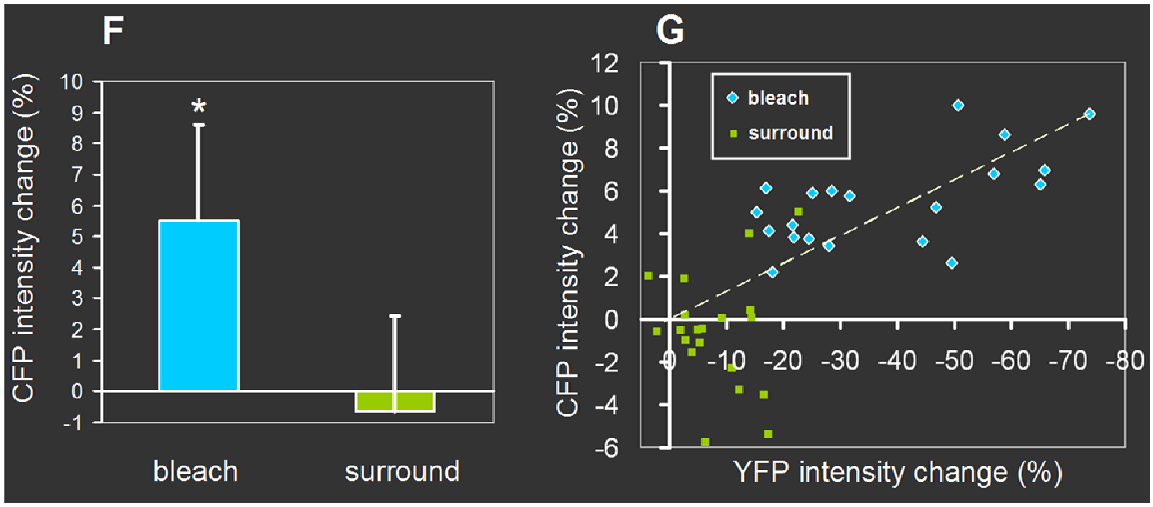
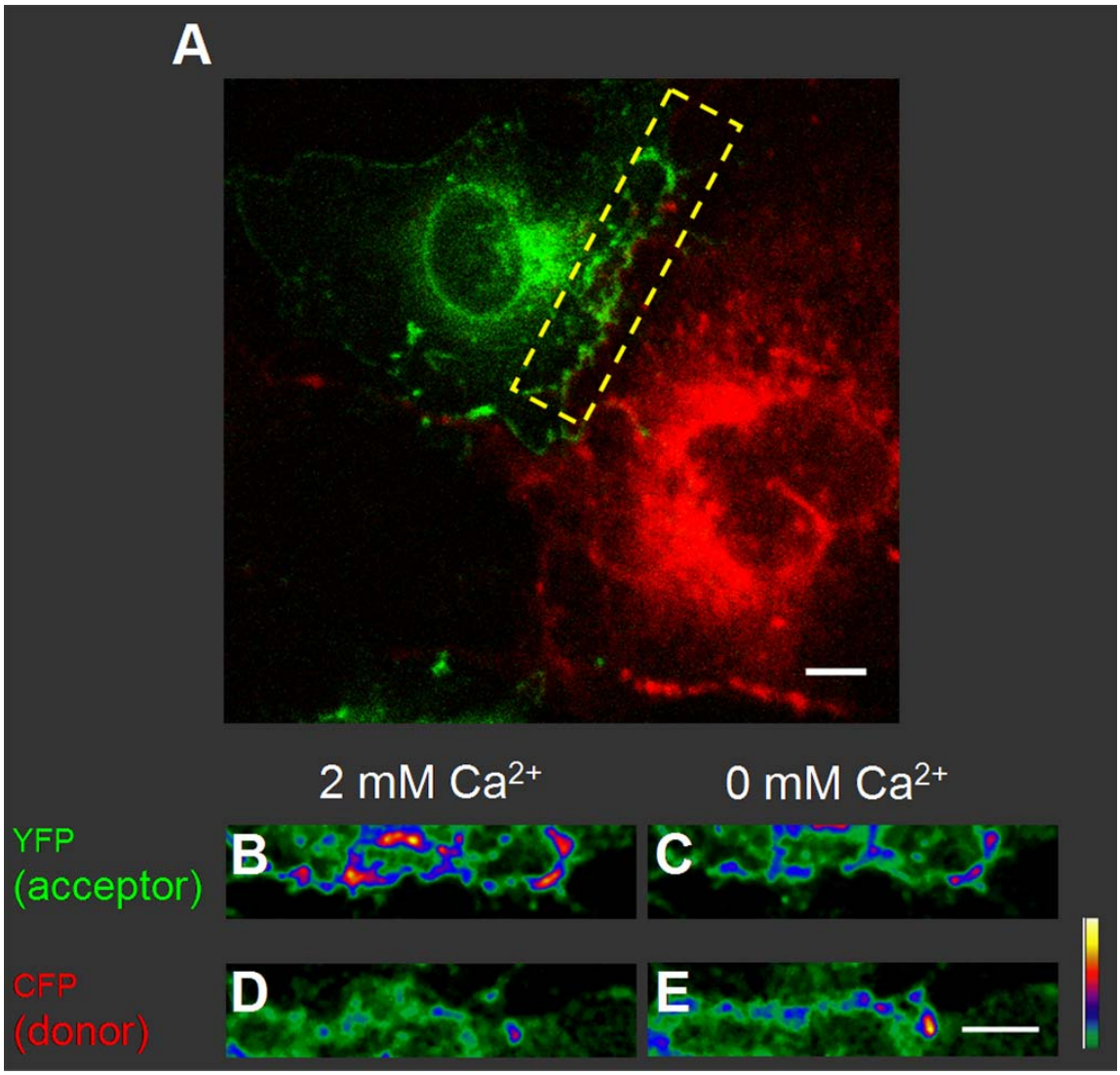


Figure 9. adFRET at cell junctions between COS7 cells separately expressing Cerulean-N-cadherin or Venus-N-cadherin.
COS7 cells were transfected with each construct (Venus- and Cerulean-Ncad) separately. After 8 hours, cells were trypsinized and replated together for another 24 to 30 hours. A. An example of the co-culture. Cells expressing Venus-Ncad are shown in green and cells expressing Cerulean-Ncad are shown in red. If two neighboring cells were found to express different fluorescent proteins and a clear junction was visible, then adFRET was

assayed at the junction/region of Venus/Cerulean overlap (dashed yellow outline shows region shown in B-E; solid white outline shows bleached area). B. LSM Meta laser scanning confocal microscope generated Venus signal (linear unmixing of separately obtained Venus and Cerulean spectra was used to generate Venus-only signal). C. LSM Meta laser scanning confocal microscope generated Cerulean signal. D. Post-acceptor-bleach Venus signal (white box represents bleached area). E. Post-acceptor-bleach Cerulean signal. F. Fluorescent intensities of bleached ROI (white box) and surrounding nonbleached areas were quantitated and the percent change of donor signal was calculated. G. More thorough bleaching leads to increased donor dequenching, as expected if the YFP bleaching and the CFP dequenching are connected through and originating from the same process. Scale bar: 10 μm.

Visualizing changes in cadherin-cadherin interactions induced by changes in extracellular Ca^{2+} concentration

As the Cerulean- and Venus-N-cadherin constructs exhibited detectable changes in FRET upon acceptor bleaching, I concluded that the FRET donor and acceptor pair must be in close proximity and in the correct orientation in the intact cell junction. I next manipulated the extracellular Ca^{2+} concentration. A decrease in the extracellular Ca^{2+} concentration should cause a cadherin conformational change and unbinding of the homophilic pairs. In the simplest experiment, FRET was monitored before and after extracellular Ca^{2+} was decreased by perfusion with new reduced Ca^{2+} media. We determined that the addition of calcium chelators was the most efficient means to rapidly quench Ca^{2+}. Co-cultured, singly-transfected COS-7 cells were first imaged in HBS containing 2mM Ca^{2+}, which was then washed off and replaced with HBS lacking Ca^{2+} and containing 2mM EGTA, to chelate any remaining Ca^{2+}. The cells were then imaged again and FRET donor and acceptor signals were compared to the baseline, Ca^{2+} image. We observed that reducing Ca^{2+} in the extracellular solution led to a significant loss of FRET signal at COS-7 cell junctions expressing FRET pairs (figure 10).


A
2 mM Ca2+
0 mM Ca2+
YFP
(acceptor)
CFP
(donor)
B
C
D
E

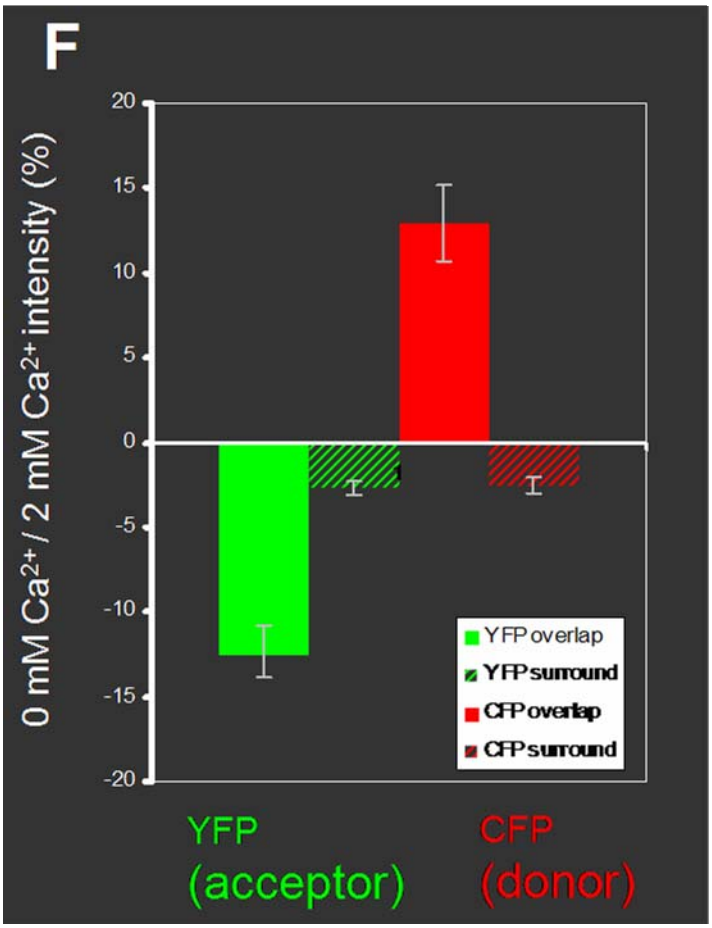


Figure 10. Monitoring changes in FRET induced by changes in extracellular Ca2+ concentration in COS7 cells.
COS7 cells were transfected with each construct (Venus- and Cerulean-Ncad) separately. After 8 hours, cells were trypsinized and replated together for another 24 to 30 hours. A. Representative image of a pair of cells; cells expressing Venus-Ncad are shown in green and cells expressing Cerulean-Ncad are shown in red (dashed yellow outline shows region shown in B-E). B. LSM Meta laser scanning confocal microscope generated Venus signal (linear unmixing of separately obtained Venus and Cerulean spectra was used to generate Venus-only signal). C. Venus signal after removal of extracellular Ca^{2+}. D. LSM Meta laser scanning confocal microscope generated Cerulean signal. E. Cerulean signal after removal of extracellular Ca^{2+}. F. Fluorescent intensities of area of Venus /Cerulean overlap and surrounding nonoverlapping areas were quantitated and the percent change of donor (Cerulean: red bars) and acceptor (Venus: green bars) signals was calculated. Scale bars: 10 μm.

Having observed that reducing Ca^{2+} in the extracellular solution leads to a significant loss of FRET signal at COS-7 cell junctions expressing FRET pairs, I next used repeated, fast scans of the junction between transfected cell pairs to monitor FRET changes dues to changes in extracellular Ca^{2+} during perfusion, allowing me to examine the dynamics of cadherin-cadherin interactions on a shorter time scale.

Baseline images of COS-7 co-cultures were taken during perfusion with media containing 2mM Ca^{2+}; after a baseline period of 5 scans, the perfusate was switched to media containing 0mM Ca^{2+} and EGTA; at the same time, additional EGTA was added to the dish. This caused a rapid loss of FRET, as indicated by the increase in Cerulean signal and concomitant decrease in Venus signal in areas with Cerulean/Venus overlap (figure 11). After further scanning, imaging was stopped (to allow for complete replacement of the residual HBS in perfusion tubing), and the perfusate was switched back to media containing Ca^{2+} (2mM). This can be observed as the discontinuity between scans 30 and 31 and reflects a period of ~1 minute. After perfusate in the pump had been exchanged, imaging was reconvened and a return of FRET was observed, reflecting the reassociation of cadherins in the presence of Ca^{2+}.

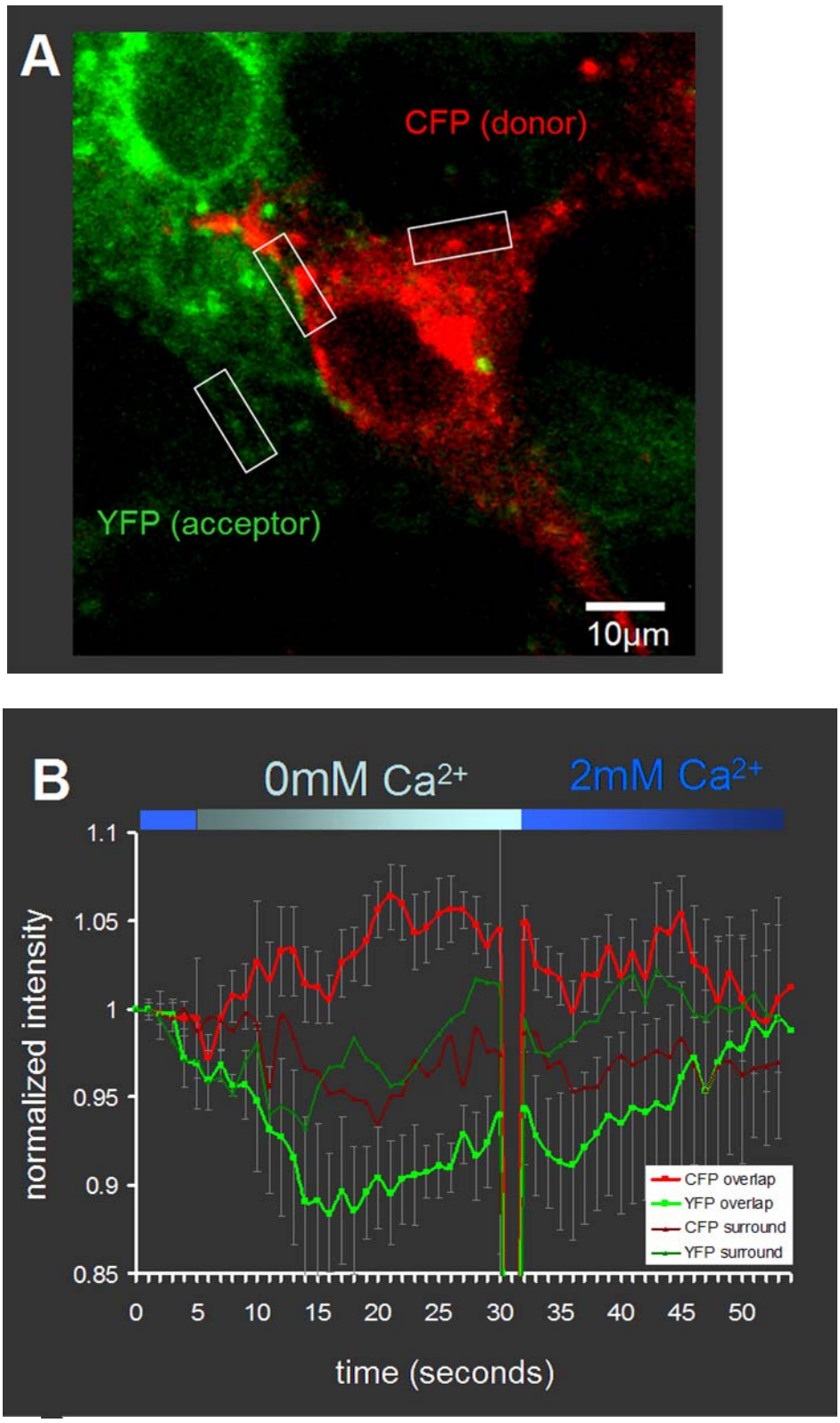


Figure 11. Monitoring changes in FRET induced by changes in extracellular Ca^{2+} concentration in COS7 cells.

COS7 cells were transfected with each construct (Venus- and Cerulean-Ncad) separately. After 8 hours, cells were trypsinized and replated together for another 24 to 30 hours. A. Representative image of the pair of cells imaged to generate the intensity profile in (B). Cells expressing Venus-Ncad are shown in green and cells expressing Cerulean-Ncad are shown in red. B. Cerulean and Venus signals were monitored over time as extracellular Ca^{2+} concentration was manipulated. Cell media was switched from 2 mM Ca^{2+} to 0 mM after scan #5, then back to 2 mM Ca^{2+} after scan #30, as described in the text. Bold traces are Cerulean (red trace) and Venus (green trace) signals measured at the cell junction where Cerulean/Venus overlap occurs (box 1 in A); dimmer traces (control) are measured in areas with no Cerulean/Venus overlap (boxes 2 and 3 in A). Colored bars above plot represent Ca^{2+} concentration used in perfusion; black bars = 2 mM Ca^{2+}, red bar = 0 mM Ca^{2+} + 2mM EGTA. Gap between red and black bar represents period of perfusate replacement in tubing. Scale bar: 10 μm.

These data show that the TS-25 based XFP-cadherin clones exhibit FRET when expressed in living cell junctions, and can be used to monitor and detect changes in cadherin conformational and adhesive state in response to changes in extracellular Ca^{2+} concentration. I believe they will be valuable tools in the effort to determine the cadherin response to synaptic activity and to elucidate a cadherin role in synaptic plasticity.