RhoA Single chain FRET Biosensor; - sequence information & helpful points

Introduction
Described here are helpful points, sequence information and caveats for the RhoA single chain FRET Biosensor described in Pertz O, Hodgson L, Klemke R, Hahn KM. Spatio-temporal dynamics of RhoA activity in migrating cells. Nature 2006;440:1069-1072. As stated in the paper, it is important to use appropriate expression levels, since too much biosensor overwhelms endogenous GDI, leading to constitutive activation of the biosensor. Use of retroviral transduction is described below.

Hahn lab biosensors (RhoA) are available at addgene, a nonprofit repository of constructs (http://www.addgene.org/Klaus_Hahn). Additional information on these biosensors can be found at the Hahn lab biosensor page http://www.med.unc.edu/pharm/faculty/labpages/hahnlab/protocols.html

Materials
Reagents:
1) pTriEX mammalian expression and cloning vector (pTriEX-RhoA/ amp resistance)
2) pBabe retroviral expression vector (pBabe-RhoA/ amp resistance)
3) pBabe-sin-tet-CMV-puro retroviral vector for Tet-On/Off system (pBabe-sin-tet-CMV-puro-RhoA/ amp resistance)

Procedure
The Full sequence for (1) follows below. To clone into (2) and (3), (1) was cut with Ncol / Xhol as a single cassette, and the Ncol site was blunt ended. The pBabe vectors were cut EcoRI/Xhol, and the EcoRI site was blunt ended. The insert was then ligated blunt/Xhol into the pBabe backbone.

Construct: start-RBD-GSG-CFP-1LINKER-Citrine-GG-RhoA-END

Restriction sites: Ncol/Xhol will cutout the full length biosensor from (1) in frame.

The binding domain (RBD) can be cut out with Ncol/BamHI. NotI/Xhol will cut out "Citrine-RhoA"

Expression considerations:
A) Transfecting (1): This will result in overexpression of the biosensor. Cells will most likely round up and die/ be unhealthy. You will have a very tough time measuring reasonable RhoA activity readouts this way, if you succeed at all.
B) Retroviral transduction without tet-control using (2): This is better than (A) and certainly will bring the expression to a lower level. Infect cells and wait 48 hrs prior to using them. Within the first 24 hrs, bright, high expressors will usually die off. In 36~48hrs, moderate to low expressors will survive and are more suitable for imaging.

C) Retroviral transduction with tet-control: This is preferred. Use either established Tet-Off lines, or produce a Tet-off cell line using Clontech pREV-Tet system. Following infection of cells with (3), doxycyclin (0.5~1.0 microg/ml) is added to repress the expression of the biosensor. Puromycin should be GRADUALLY applied to reach final concentration of 10microg/ml. Increase in 2 microg/ml increments and let the cells fully recover from each increase. Once cells are selected, they should be induced (see below) and FACS sorted to produce a tight population expressing similar biosensor levels, then should be put back on Dox to repress and maintained in culture (without Puro).

Inducing stable Tet-off cells: When optimally subconfluent, trypsinize and detach into 10ml (assuming 10cm TC dishes are used) of normal growth media without Dox. Spin at 300 RCF for 3 min, then carefully suction out the media supernatant. Resuspend the cells into 10ml growth media without Dox. Plate sparsely, usually 1 x 10^4 cells per 10cm TC dish. Let cells go 24hrs, observe briefly to confirm YFP fluorescence, then let them go another 16~24hrs before imaging experiments.

Notes re; photobleach correction
A Methods in Enzymology article describing photobleach corrections as applied to a Cdc42 biosensor but can be used for this biosensor as well (Hodgson et al., 2006). The Matlab routines referenced in this publication can be found at the Hahn lab web page.

pTriEX-RhoA bio1 sequence:
Start into pTriEX Ncol site, and then 6xHis tag plus GSG linker:
ccatggcacacccatcaccacccatcaggtgtggc

RBD:
ATCCTGGAGGACCTCAATATGCTCTACATCCGGGCAGATGCCACTCAGCCTGGAGGACACA
GAGCTGCAGAGGAAACTAGATCATGAGATCCGGATGGGGATGGGGCCTGCAAGCTGCTG
GCAGCCTGCTCCAGCGAGAGCAGGGCTCTGGAAAGCCACCAAGAGCCTGCTGGTGTGCAAC
AGCCGTATTTCCTCAGCTACATGGGTGAGCTGCAGCGGCGAAAGGAGGCCCAGGTGCTGGAGAAGACA
GSG linker:
GGATCCGGA

CFP:
ATGGTGAGCAAGGGCAGGAGCCTGTCACCGGGGTGGTGCCC
ATCCTGGTCTG AGCTGGACGG CGACGTAACG GGCCACAAAGT TCAGCGTGTC
CGGCGAGGGC
GAGGGCGATG CCACCTACGG CAAGCTGACC CTGAAGTTCA TCTGCACCAC
CGGCAAGCTG
CGGCTGCCCT GGCCCCCCT GTGACCTGGG GCCTGCAGTG
CTTCAGCCGC
TACCCCGGACC ACATGAAGCA GCAGCAGCTTC TTCAAGTCCG CCATGCGCCGA AGGCCGTCGTC
CAGGAGCCCA CCATCTTCTT CAAGGAGCAC GGCAACTACA AGACCCCGGC
CGAGGGTGAAG
TTCCAGGGCG ACACCGCTGTT GAACCGCATC GAGCTGAAGG GGCGCAGACTT
CAAGGAGCAC
GGCAACATCC TGGGGGACAA GCTGGAGTAC AACTACATCA GCCACAACGT CTATATCACC
GCCGACAAAGC AGAAGAAACGG CATCAAGGCC AACTTCAAGA TCGGCCACAA
CATCGAGGAC
GGCGATCGGC AGTCTGCAGA CCACTACCAG CAGAACACCC CCATCGGCAG
GGGGCGGCG
GTGCTGCCCG ACAACCACTA CCTAGCACC CAGTCCGCCG TGAGAAAGAG
CCCAACGGG
AAGCGCGATCT ACATGGTCCT GCTGGAGTTCC GTGACCGGCC CCAGGATCAC
TCTCAGCGATG
GAGCAGCTGTACT:

Link into NotI-Citrine:
ggatctGCGGCCGA

Citrine:

atggtgacagcagggcagcttcagcttcgctctgctaaggctgggtgc ccatctctgttgctcagctggac
gcgcaagttaa aacgccacaa gttcagcgtg tccggcgaag gcgaagcagga tspcggatcctgc
gccagctga ccgtgaaagctcagctgc accgca�gac gcgagttgc gcggaggtga gatgctrggg
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gtgctggtgagtttgctgccgacgctgctgctggagttgccagagcttcagcttcgcgaacagcttcctgccctgccgacgcaggtgaa
GG Linker:
gggggg

RhoAwt:
ATGGCCTCGCATCCCGGAAAGAAACTGGGATTTGTTGGTGATGGAGCGCTGTGGGAAAGACATGCT
GCTCATAG
TCTTCAGCAAGGACCAGTTCCAGAGGTTATGTGCCCACAGTGTTTGAGAACTATGTGGCAGATATCGAGGGTGGATGGAAAGCAGGTAGAGTTGGCTTTGTGGGACACAGCTGGGCAGGAAGATTATGATCGCCTGAGGCCCTCTCCTACCCAGATACCGATGTTATACTGATGTGTTTTTCCATCGACAGCCCTGATAGTTTAGAAAACATCCCAAGAAAGTGGAACCCCAGAAGTCAAGCATTTCTGTCCCAACGTGCCCATCATCCTGGTGTTGGGAAAAGGATCTTCGGAATGATGAGCACACAAGGCGGGAGCTAGCCAAGATGAAGCAGGAGCGGGTGAAAACCTGAAGAAGGCAGAGATATGGCAAACAGGATTGGCGCTTTTGGGTACATGGAGTGTTCAGCAAAGACCAAAAGATGGAGTGAGAGAGGTTTTTGAAATGGCTACGAGAGCTGCTCTGCAAGCTAGACGTGGGAAGAAAAATCTGGGTGCCTTGTGTTGTGAAAC

Stop and XhoI into pTriEX-4:
TAACTCGAG

References
