CMC Biosensors Initiative
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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 respository 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 NcoI / XhoI as a single cassette, and the NcoI site was blunt ended. The pBabe vectors were cut EcoRI/XhoI, and the EcoRI site was blunt ended. The insert was then ligated blunt/XhoI into the pBabe backbone.

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

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

The binding domain (RBD) can be cut out with NcoI/BamHI. NotI/XhoI 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 NcoI site, and then 6xHis tag plus GSG linker:
ccatggcacaccatcaccaccatcacggtagtggc

<u>RBD:</u>

ATCCTGGAGGACCTCAATATGCTCTACATCCGGCAGATGGCACTCAGCCTGGAGGACACA

GAGCTGCAGAGGAAACTAGATCATGAGATCCGGATGAGGGATGGGGCCTGCAAGCTGCTG

GCAGCCTGCTCCCAGCGAGAGCAGGCTCTGGAAGCCACCAAGAGCCTGCTGGTGTGCAAC

AGCCGTATTCTCAGCTACATGGGTGAGCTGCAGCGGCGAAAGGAGGCCCAGGTGCTGGAG

AAGACA

<u>GSG linker:</u>

GGATCCGGA

<u>CFP:</u>
AT GGTGAGCAAG GGCGAGGAGC TGTTCACCGG GGTGGTGCCC

ATCCTGGTCG AGCTGGACGG CGACGTAAAC GGCCACAAGT TCAGCGTGTC
CGGCGAGGGC
GAGGGCGATG CCACCTACGG CAAGCTGACC CTGAAGTTCA TCTGCACCAC
CGGCAAGCTG
CCCGTGCCCT GGCCCACCCT CGTGACCACC CTGACCTGGG GCGTGCAGTG
CTTCAGCCGC
TACCCCGACC ACATGAAGCA GCACGACTTC TTCAAGTCCG CCATGCCCGA AGGCTACGTC
CAGGAGCGCA CCATCTTCTT CAAGGACGAC GGCAACTACA AGACCCGCGC
CGAGGTGAAG
TTCGAGGGCG ACACCCTGGT GAACCGCATC GAGCTGAAGG GCATCGACTT
CAAGGAGGAC
GGCAACATCC TGGGGCACAA GCTGGAGTAC AACTACATCA GCCACAACGT CTATATCACC
GCCGACAAGC AGAAGAACGG CATCAAGGCC AACTTCAAGA TCCGCCACAA
CATCGAGGAC
GGCAGCGTGC AGCTCGCCGA CCACTACCAG CAGAACACCC CCATCGGCGA
CGGCCCCGTG
CTGCTGCCCG ACAACCACTA CCTGAGCACC CAGTCCGCCC TGAGCAAAGA
CCCCAACGAG
AAGCGCGATC ACATGGTCCT GCTGGAGTTC GTGACCGCCG CCGGGATCAC
TCTCGGCATG
GACGAGCTGT ACAAG

linker cassette:
ggatctacttctggttctggtaaacctggttctggtgaaggttctactaaaggt

Link into NotI-Citrine:
ggatctGCGGCCGCA

Citrine:

atggtgagca agggcgagga gctgttcacc ggggtggtgc ccatcctggt cgagctggac
ggcgacgtaa acggccacaa gttcagcgtg tccggcgagg gcgagggcga tgccacctac
ggcaagctga ccctgaagtt catctgcacc accggcaagc tgcccgtgcc ctggcccacc
ctcgtgacca ccttcggcta cggcctgatg tgcttcgccc gctaccccga ccacatgaag
cagcacgact tcttcaagtc cgccatgccc gaaggctacg tccaggagcg caccatcttc
ttcaaggacg acggcaacta caagacccgc gccgaggtga agttcgaggg cgacaccctg
gtgaaccgca tcgagctgaa gggcatcgac ttcaaggagg acggcaacat cctggggcac
aagctggagt acaactacaa cagccacaac gtctatatca tggccgacaa gcagaagaac
ggcatcaagg tgaacttcaa gatccgccac aacatcgagg acggcagcgt gcagctcgcc
gaccactacc agcagaacac ccccatcggc gacggccccg tgctgctgcc cgacaaccac
tacctgagct accagtccgc cctgagcaaa gaccccaacg agaagcgcga tcacatggtc
ctgctggagt tcgtgaccgc cgccgggatc actctcggca tggacgagct gtacaag

GG Linker:
ggggga

RhoAwt:
ATGGCTGCCATCCGGAAGAAACTGGTGATTGTTGGTGATGGAGCCTGTGGAAAGACATGCTT
GCTCATAG

TCTTCAGCAAGGACCAGTTCCCAGAGGTGTATGTGCCCACAGTGTTTGAGAACTATGTGGCA
GATATCGA
GGTGGATGGAAAGCAGGTAGAGTTGGCTTTGTGGGACACAGCTGGGCAGGAAGATTATGAT
CGCCTGAGG
CCCCTCTCCTACCCAGATACCGATGTTATACTGATGTGTTTTTCCATCGACAGCCCTGATAGT
TTAGAAA
ACATCCCAGAAAAGTGGACCCCAGAAGTCAAGCATTTCTGTCCCAACGTGCCCATCATCCTG
GTTGGGAA
TAAGAAGGATCTTCGGAATGATGAGCACACAAGGCGGGAGCTAGCCAAGATGAAGCAGGAG
CCGGTGAAA
CCTGAAGAAGGCAGAGATATGGCAAACAGGATTGGCGCTTTTGGGTACATGGAGTGTTCAGC
AAAGACCA
AAGATGGAGTGAGAGAGGTTTTTGAAATGGCTACGAGAGCTGCTCTGCAAGCTAGACGTGG
GAAGAAAAA
ATCTGGGTGCCTTGTCTTGTGAAAC

Stop and XhoI into pTriEX-4:
TAACTCGAG

References

Hodgson L, Nalbant P, Shen F, Hahn K. Imaging and photobleach correction of Mero-CBD, sensor of endogenous Cdc42 activation. Methods Enzymol., 2006;406:140-156. PubMed | CMC Update article.

Pertz O, Hodgson L, Klemke R, Hahn, KM. Spatio-temporal dynamics of RhoA activity in migrating cells. Nature, 2006;440:1069-1072. PubMed | CMC Update article.