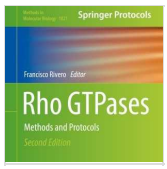

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3.2 Production of a Cleared Cell Lysate

Book Chapter

In 1 collection

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ABSTRACT

Small, monomeric guanine triphosphate hydrolases (GTPases) are ubiquitous cellular integrators of signaling. A signal activates the GTPase, which then binds to an effector molecule to relay a signal inside the cell. The GTPase effector trap flow cytometry assay (G-Trap) utilizes bead-based protein immobilization and dual-color flow cytometry to rapidly and quantitatively measure GTPase activity status in cell or tissue lysates. Beginning with commercial cytoplex bead sets that are color-coded with graded fluorescence intensities of a red (700 nm) wavelength, the bead sets are derivatized to display glutathione on the surface through a detailed protocol described here. A different glutathione-*S*-transferase-effector protein (GST-effector protein) can then be attached to the surface of each set. For the assay, users can incubate bead sets individually or in a multiplex format with lysates for rapid, selective capture of active, GTP-bound GTPases from a single sample. After that, flow cytometry is used to identify the bead-borne GTPase based on red bead intensity, and the amount of active GTPase per bead is detected using monoclonal antibodies conjugated to a green fluorophore or via labeled secondary antibodies. Three examples are provided to illustrate the efficacy of the effector-functionalized beads for measuring the activation of at least five GTPases in a single lysate from fewer than 50,000 cells.

Section 3.2 'Production of a Cleared Cell Lysate' from 'Small-Volume Flow Cytometry-Based Multiplex Analysis of the Activity of Small GTPases' https://www.protocols.io/view/small-volume-flow-cytometry-based-multiplex-analys-bpssmnee

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Small-Volume Flow Cytometry-Based Multiplex Analysis of the Activity of Small GTPases

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Small-Volume Flow Cytometry-Based Multiplex Analysis of the Activity of Small GTPases

GUIDELINES

Introduction

Members of the Ras-related superfamily of small, monomeric GTPases, including Rho, Ras, and Rab subfamilies, serve as critical integrators of cellular functions from cell division and survival to membrane trafficking [1-6]. Genetic diseases and infectious agents such as viruses and bacteria are known to co-opt the signaling functions of GTPases, making GTPases attractive diagnostic and therapeutic targets [7-15]. Current methods for measuring the activation status of small GTPases rely on glutathione bead-based effector pull-down/immunoblot assays, and ELISA-based effector-binding assay kits. The significant shortcomings of these methods are that they are labor intensive and require large sample sizes, purified effector proteins, or expensive kits. Additionally, sample processing times are critical because of the lability of the GTP-bound state due to hydrolysis. Here we describe the GTPase activity assay platform (G-Trap) [16], a multiplex , bead-based effector-binding assay that can rapidly monitor the activation status of multiple GTPases from a single-cell lysate [16, 17].

The GST-effector proteins consisting of the minimal GTPase-binding domains (RBD) for the studies are PAK-1 RBD (a Rac1 and Cdc42 effector), Raf-1 RBD (a Ras effector), Rhotekin-RBD (a Rho effector), RalGDS-RBD (a RAP1 effector protein), and RILP-RBD (a Rab7 effector) [16, 17]. Beads for each target effector (10,000/target) are mixed and added to cell lysates typically generated from 50,000 cells. The beads are incubated with cell lysates for 1 h at 4 °C, centrifuged, and resuspended in 50 µL of buffer (1:20 final antibody dilution). Monoclonal antibodies for each target GTPase are pooled and added to the multiplex bead suspension and incubated for 1 h at 4 °C. A secondary antibody tagged with Alexa 488 dye is then used to label bead-associated antibodies fluorescently. The samples are then analyzed on a flow cytometer where the red fluorescence identifies the specific effector bead and is used to gate the green fluorescence and quantify the amount of each target, GTP-bound GTPase. We demonstrate the functionality of the assay in three tests involving the activation of multiple GTPases. The first example measures the signaling cascade of GTPases that are activated to allow β_3 integrin-mediated cellular entry of Sin Nombre virus (SNV) in the course of a productive infection [16, 17]. The second example measures GTPase activity downstream of signaling of protease-activated receptors (PARs), after exposure to thrombin found in the plasma samples drawn from patients with hantavirus cardiopulmonary syndrome (HCPS) [18]. The

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third example measures GTPase activation due to bacterial factors [12, 19] present in a plasma sample from a septic patient. Control reagents used for these illustrative examples are given in Table 1.

A	B	C	D	E
GTPase	**Effector**	**Activator; final concentration; incubation time**	**Inhibitor; final concentration; incubation time**	**SNV particle titer; incubation time**
RhoA	Rhotekin RBD	Calpeptin; 1 µM; 30 min		10,000/cell; 3, 10, 20, 30, 60 min
Rac1	PAK-1 PBD	EGF; 10 nM; 15 min	NSC23766; 100 µM; 30 min	10,000/cell; 3, 10, 20, 30, 60 min
Rap1	Ral-GDS RBD	8-Cpt-2me-cAMP; 50 µM; 30 min	GGTI 298; 10 µM; 30 min	10,000/cell; 3, 10, 20, 30, 60 min
R-Ras	Raf-1 RBD		FTI-277; 100 nM; 30 min	
H-Ras	Raf-1 RBD		FTI-277; 100 nM; 30 min	
Rab7	RILP RBD	EGF; 10 nM; 15 min		10,000/cell; 3, 10, 20, 30, 60 min

Table 1 Reagents, concentrations, and conditions used for illustrative experiments [16]

It is well established that flow cytometry is an ideal platform for measuring multiple analytes, simultaneously using cytoplex bead populations encoded with fluorescent dyes of graded intensities, with which the bead is uniquely identified (*see* Fig. 1) [17, 20]. Flow cytometry is capable of exciting at multiple absorption bands and detecting fluorescence at different emission wavelengths, and it is then possible to detect various analytes, simultaneously from a single sample [21-25]. The commercially available cytoplex beads used by us have up to 12 different intensity levels that can be used as unique bead identifiers. Furthermore, the beads are currently available in two sizes that can be resolved by flow cytometry forward light scatter. Thus, up to 24 different analytes can be measured. Our experience to date suggests that a maximum of six targets at a time is optimal for reproducible assay results [17].

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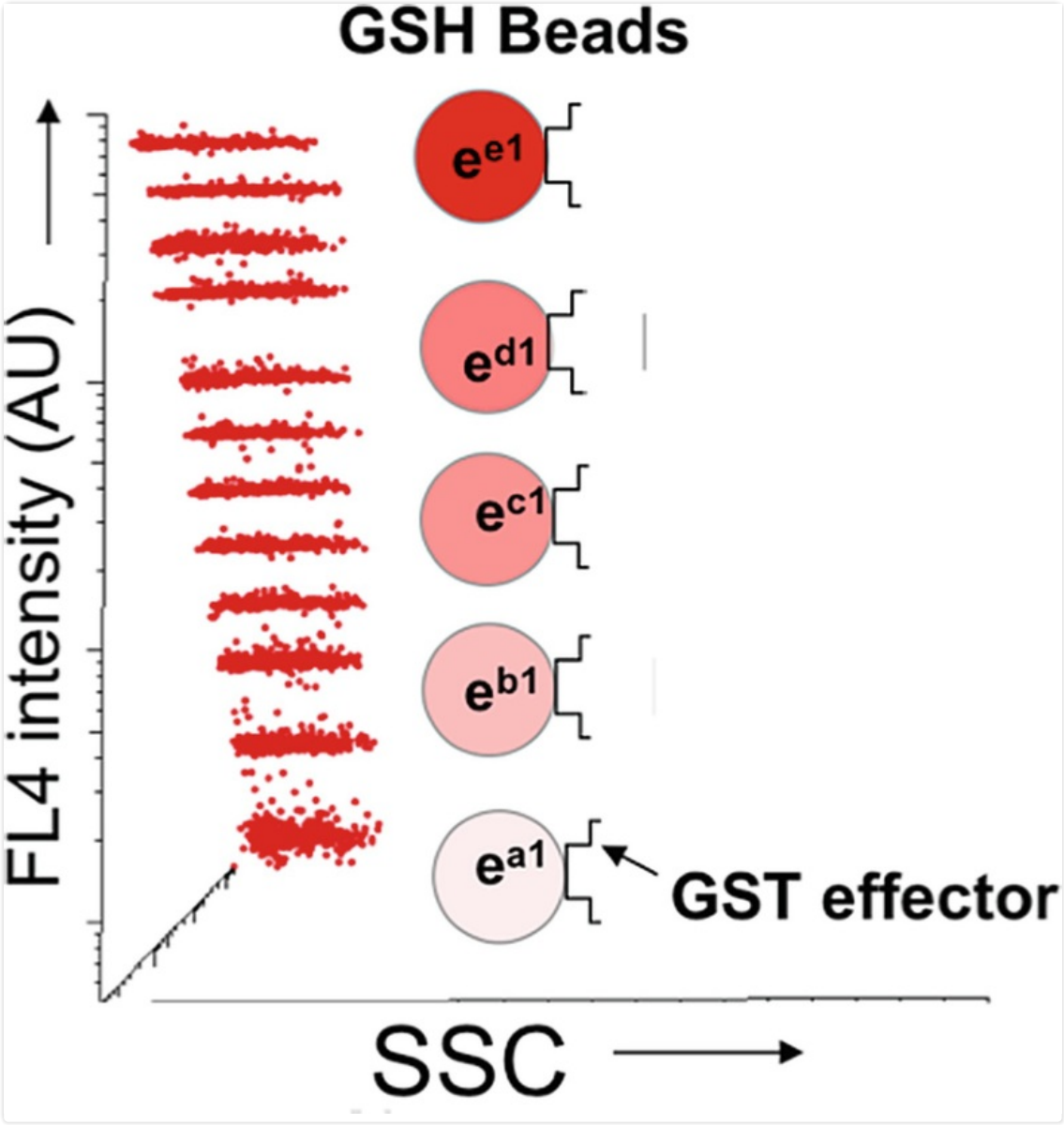

Fig. 1
The GTPase effector trap flow cytometry assay (G-Trap). The plot of red fluorescence (FL4) versus side scatter (SSC) of a set of 12 of Cyto-Plex™ beads dyed with 12 discrete levels of 700 nm fluorescence. In the G-Trap assay, the letters *a*, *b*, *c*, *d*, and *e* identify and link effectors, their cognate GTPases, and fluorescently labeled antibodies FL1 (520 nm emission) or FL2 (580 nm emission) used for readout. In single or multiplex format, glutathione bead populations coated with effectors are used to capture specific active GTPases. In multiplex format, the effector and GTPase identities are defined by the intensity level of red fluorescence encoded on each bead

Notes

1.The site density of glutathione sites on beads governs the magnitude of the fluorescence signal from GST proteins bound to the beads. A low site density of GST sites on beads can yield variable data or poor binding results [28]. Derivatization of the carboxyl Cyto-Plex ™ beads to glutathione requires an intermediate step of functionalizing to amino groups. Optimizing the synthesis of amino groups is essential. Derivatizing the amino-terminated groups with a fluorescent probe such as NHS-Alexa 488 tests optimum conversion of carboxyl to amino groups. For this purpose, it is useful to use inexpensive carboxyl-functionalized beads such as those from Spherotech (*see* **Note 3**). Glutathione derivatization is tested with 25 nM GST-GFP for 30 min as described in Subheading 3.1, **step 13**.

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2. All buffers contain 0.01% Tween-20, which is compatible with most biological molecules.

3. To test the conversion efficiency of carboxyl beads to amino beads, we derivatized 10 µL of generic carboxyl beads (Spherotech) to amino beads. We use commercial amino beads of similar size with known amino group site density for comparison, with our synthesis. The two amino bead sets are then reacted with NHS-Alexa488 in parallel. Approximately 0.1 mg of NHS-Alexa488 is dissolved in 20 µL of dry DMSO to give about 5 mg/mL, which is stored at −80 °C. Ten thousand synthesized amino beads and ten thousand commercial amino beads are put in 20 µL of pH 8.4 buffer, 2 µL of NHS-Alexa488 solution is added, the suspension is mixed, and reagents are allowed to react for 30 min in the dark. The beads are washed twice with pH 7 buffer, diluted to 50 µL of buffer, and analyzed by flow cytometry. We determine nonspecific binding of NHS-Alexa488 to beads by mixing carboxyl beads with the fluorophore. In our setting the fluorescence from the nonspecific attachment of NHS-Alexa488 to carboxyl beads was 20% of the conjugated fluorophores. Our amino beads were comparable to the commercial beads.

4. Bubbling nitrogen slowly through 400 µL of suspension in a 1.6 mL microfuge tube is not easy. We use a narrow nitrogen line and very low nitrogen pressure, and notice that the angle of the tube of suspended beads matters: tipping the slowly bubbling microfuge tube from horizontal to upright can stop bubbling, probably due to increased hydrostatic pressure. Another technique to prevent bubbling is to use a soft nitrogen tubing line, which can be pushed against the bottom of the centrifuge tube to stop bubbling. Tween-20 gives an observable bubble running up the microfuge tube, and we estimate that the volume of air above the suspension is displaced about ten times during the 2 min of bubbling.

5. Blocking of nonspecific binding sites for primary and secondary antibodies with BSA is critical for limiting nonspecific binding. It is also important to test new antibodies in single-target format before using in a multiplex format. In our experience new antibody batches from “trusted sources” can be highly nonspecific, and could bind to all bead surfaces regardless of effector functionalization, and either raise the background intensity for all beads in the multiplex assay or at worst degrade the readout of all the beads in a multiplex configuration.

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References

1. Jaffe AB, Hall A (2005) Rho GTPases: biochemistry and biology. Annu Rev Cell Dev Biol 21:247–269 CrossRef Google Scholar
2. Ridley A (2000) Rho GTPases. Integrating integrin signaling. J Cell Biol 150:F107–F109 CrossRef PubMed PubMedCentral Google Scholar
3. Bar-Sagi D, Hall A (2000) Ras and Rho GTPases: a family reunion. Cell 103:227–238 CrossRef PubMed Google Scholar
4. Van Aelst L, D'Souza-Schorey C (1997) Rho GTPases and signaling networks. Genes Dev 11:2295–2322 CrossRef PubMed Google Scholar
5. Nobes C, Hall A (1994) Regulation and function of the Rho subfamily of small GTPases. Curr Opin Genet Dev 4:77–81 CrossRef PubMed Google Scholar
6. Vega FM, Ridley AJ (2007) SnapShot: Rho family GTPases. Cell 129:1430 CrossRef PubMed Google Scholar
7. Hong L, Kenney SR, Phillips GK, Simpson D, Schroeder CE, Noth J, Romero E, Swanson S, Waller A, Strouse JJ, Carter M, Chigaev A, Ursu O, Oprea T, Hjelle B, Golden JE, Aube J, Hudson LG, Buranda T, Sklar LA, Wandinger-Ness A (2013) Characterization of a cdc42 protein inhibitor and its use as a molecular probe. J Biol Chem 288:8531–8543 CrossRef PubMed PubMedCentral Google Scholar
8. Agola JO, Hong L, Surviladze Z, Ursu O, Waller A, Strouse JJ, Simpson DS, Schroeder CE, Oprea TI, Golden JE, Aube J, Buranda T, Sklar LA, Wandinger-Ness A (2012) A competitive nucleotide binding inhibitor: in vitro characterization of Rab7 GTPase inhibition. ACS Chem Biol 7:1095–1108 CrossRef PubMed PubMedCentral Google Scholar
9. Friesland A, Zhao Y, Chen YH, Wang L, Zhou H, Lu Q (2013) Small molecule targeting Cdc42-intersectin interaction disrupts Golgi organization and suppresses cell motility. Proc Natl Acad Sci U S A 110:1261–1266 CrossRef PubMed PubMedCentral Google Scholar
10. Vega FM, Ridley AJ (2008) Rho GTPases in cancer cell biology. FEBS Lett 582:2093–2101 CrossRef Google

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Scholar
11. Boyer L, Lemichez E (2015) Switching Rho GTPase activation into effective antibacterial defenses requires the caspase-1/IL-1β signaling axis. Small GTPases 6:186–188 CrossRef PubMed PubMedCentral Google Scholar
12. Lemichez E, Aktories K (2013) Hijacking of Rho GTPases during bacterial infection. Exp Cell Res 319:2329–2336 CrossRef PubMed Google Scholar
13. Agola JO, Sivalingam D, Cimino DF, Simons PC, Buranda T, Sklar LA, Wandinger-Ness A (2015) Quantitative bead-based flow cytometry for assaying Rab7 GTPase interaction with the Rab-interacting lysosomal protein (RILP) effector protein. Methods Mol Biol 1298:331–354 CrossRef PubMed PubMedCentral Google Scholar
14. Guo Y, Kenney SR, Cook L, Adams SF, Rutledge T, Romero E, Oprea TI, Sklar LA, Bedrick E, Wiggins CL, Kang H, Lomo L, Muller CY, Wandinger-Ness A, Hudson LG (2015) A novel pharmacologic activity of ketorolac for therapeutic benefit in ovarian cancer patients. Clin Cancer Res 21:5064–5072 CrossRef PubMed PubMedCentral Google Scholar
15. Guo Y, Kenney SR, Muller CY, Adams S, Rutledge T, Romero E, Murray-Krezan C, Prekeris R, Sklar LA, Hudson LG, and Wandinger-Ness A (2015) R-Ketorolac targets Cdc42 and Rac1 and alters ovarian cancer cell behaviors critical for invasion and metastasis. Mol Cancer Ther 14:2215–2227 CrossRef PubMed PubMedCentral Google Scholar
16. Buranda T, Basuray S, Swanson S, Bondu-Hawkins V, Agola J, Wandinger-Ness A (2013) Rapid parallel flow cytometry assays of active GTPases using effector beads. Anal Biochem 144:149–157 CrossRef Google Scholar
17. Bondu V, Wu C, Cao W, Simons PC, Gillette JM, Zhu J, Erb L, Zhang XF, Buranda T (2017) Low affinity binding in cis to P2Y2R mediates force-dependent integrin activation during hantavirus infection. Mol Biol Cell 28:2887–2903 CrossRef PubMed PubMedCentral Google Scholar
18. Bondu V, Schrader R, Gawinowicz MA, McGuire P, Lawrence DA, Hjelle B, Buranda T (2015) Elevated cytokines, thrombin and PAI-1 in severe HCPS patients due to Sin Nombre virus. Virus 7:559–589 CrossRef Google Scholar
19. Boquet P, Lemichez E (2003) Bacterial virulence factors targeting Rho GTPases: parasitism or symbiosis? Trends Cell Biol 13:238–246 CrossRef PubMed Google Scholar
20. Curpan RF, Simons PC, Zhai D, Young SM, Carter MB, Bologa CG, Oprea TI, Satterthwait AC, Reed JC, Edwards BS, Sklar LA (2011) High-throughput screen for the chemical inhibitors of antiapoptotic bcl-2 family proteins by multiplex flow cytometry. Assay Drug Dev Technol 9:465–474 CrossRef PubMed PubMedCentral Google Scholar
21. Buican TN, Hoffmann GW (1985) Immunofluorescent flow cytometry in N dimensions. The multiplex labeling approach. Cell Biophys 7:129–156 CrossRef PubMed Google Scholar
22. Camp CH Jr, Yegnanarayanan S, Eftekhar AA, Adibi A (2011) Label-free flow cytometry using multiplex coherent anti-Stokes Raman scattering (MCARS) for the analysis of biological specimens. Opt Lett 36:2309–2311 CrossRef PubMed Google Scholar
23. Davies R, Vogelsang P, Jonsson R, Appel S (2016) An optimized multiplex flow cytometry protocol for the analysis of intracellular signaling in peripheral blood mononuclear cells. J Immunol Methods 436:58–63 CrossRef PubMed Google Scholar
24. Merat SJ, van de Berg D, Bru C, Yasuda E, Breij E, Koostra N, Prins M, Molenkamp R, Bakker AQ, de Jong MD, Spits H, Schinkel J, Beaumont T (2017) Multiplex flow cytometry-based assay to study the breadth of antibody responses against E1E2 glycoproteins of hepatitis C virus. J Immunol Methods 454:15–26. https://doi.org/10.1016/j.jim.2017.07.015 CrossRef PubMed Google Scholar
25. Saunders MJ, Graves SW, Sklar LA, Oprea TI, Edwards BS (2010) High-throughput multiplex flow cytometry screening for botulinum neurotoxin type a light chain protease inhibitors. Assay Drug Dev Technol 8:37–46 CrossRef PubMed PubMedCentral Google Scholar
26. Tessema M, Simons PC, Cimino DF, Sanchez L, Waller A, Posner RG, Wandinger-Ness A, Prossnitz ER, Sklar LA (2006) Glutathione-S-transferase-green fluorescent protein fusion protein reveals slow dissociation from high site density beads and measures free GSH. Cytometry A 69:326–334 CrossRef PubMed Google Scholar
27. Schwartz SL, Tessema M, Buranda T, Pylypenko O, Rak A, Simons PC, Surviladze Z, Sklar LA, Wandinger-Ness A (2008) Flow cytometry for real-time measurement of guanine nucleotide binding and exchange by Ras-like GTPases. Anal Biochem 381:258–266 CrossRef PubMed PubMedCentral Google Scholar
28. Surviladze Z, Young SM, Sklar LA (2012) High-throughput flow cytometry bead-based multiplex assay for identification of Rho GTPase inhibitors. Methods Mol Biol 827:253–270 CrossRef PubMed PubMedCentral Google Scholar
29. Buranda T, Swanson S, Bondu V, Schaefer L, Maclean J, Mo ZZ, Wycoff K, Belle A, Hjelle B (2014) Equilibrium and kinetics of Sin Nombre hantavirus binding at DAF/CD55 functionalized bead surfaces. Virus 6:1091–1111 CrossRef Google Scholar
30. Buranda T, Wu Y, Sklar LA (2011) Quantum dots for quantitative flow cytometry. Methods Mol Biol 699:67–84 CrossRef PubMed PubMedCentral Google Scholar
31. Buranda T, Jones GM, Nolan JP, Keij J, Lopez GP, Sklar LA (1999) Ligand receptor dynamics at streptavidin-

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coated particle surfaces: a flow cytometric and spectrofluorimetric study. J Phys Chem B 29:3399–3410 CrossRef Google Scholar
32. Shen B, Delaney MK, Du X (2012) Inside-out, outside-in, and inside-outside-in: G protein signaling in integrin-mediated cell adhesion, spreading, and retraction. Curr Opin Cell Biol 24:600–606 CrossRef PubMed PubMedCentral Google Scholar
33. Springer TA, Dustin ML (2012) Integrin inside-out signaling and the immunological synapse. Curr Opin Cell Biol 24:107–115 CrossRef PubMed Google Scholar
34. Nordenfelt P, Elliott HL, Springer TA (2016) Coordinated integrin activation by actin-dependent force during T-cell migration. Nat Commun 7:13119 CrossRef PubMed PubMedCentral Google Scholar
35. Schurpf T, Springer TA (2011) Regulation of integrin affinity on cell surfaces. EMBO J 30:4712–4727 CrossRef PubMed PubMedCentral Google Scholar
36. Shattil SJ (1999) Signaling through platelet integrin $\alpha_{IIb}\beta_3$: inside-out, outside-in, and sideways. Thromb Haemost 82:318–325 CrossRef PubMed Google Scholar
37. Kim C, Ye F, Ginsberg MH (2011) Regulation of integrin activation. Annu Rev Cell Dev Biol 27:321–345 CrossRef PubMed Google Scholar
38. Grove J, Marsh M (2011) Host-pathogen interactions: the cell biology of receptor-mediated virus entry. J Cell Biol 195:1071–1082 CrossRef PubMed PubMedCentral Google Scholar
39. Marsh M, Helenius A (2006) Virus entry: open sesame. Cell 124:729–740 CrossRef PubMed Google Scholar
40. Bos JL (2005) Linking Rap to cell adhesion. Curr Opin Cell Biol 17:123–128 CrossRef PubMed Google Scholar
41. Burridge K, Wennerberg K (2004) Rho and Rac take center stage. Cell 116:167–179 CrossRef PubMed Google Scholar
42. Feng Y, Press B, Wandinger-Ness A (1995) Rab 7: an important regulator of late endocytic membrane traffic. J Cell Biol 131:1435–1452 CrossRef PubMed Google Scholar
43. hen B, Zhao X, O'Brien KA, Stojanovic-Terpo A, Delaney MK, Kim K, Cho J, Lam SC, Du X (2013) A directional switch of integrin signalling and a new anti-thrombotic strategy. Nature 503:131–135 CrossRef PubMed PubMedCentral Google Scholar
44. Arachiche A, Mumaw MM, de la Fuente M, Nieman MT (2013) Protease-activated receptor 1 (PAR1) and PAR4 heterodimers are required for PAR1-enhanced cleavage of PAR4 by α-thrombin. J Biol Chem 288:32553–32562 CrossRef PubMed PubMedCentral Google Scholar
45. Coughlin SR (1999) How the protease thrombin talks to cells. Proc Natl Acad Sci U S A 96:11023–11027 CrossRef PubMed PubMedCentral Google Scholar
46. Coughlin SR (2000) Thrombin signalling and protease-activated receptors. Nature 407:258–264 CrossRef PubMed Google Scholar
47. Bilodeau ML, Hamm HE (2007) Regulation of protease-activated receptor (PAR) 1 and PAR4 signaling in human platelets by compartmentalized cyclic nucleotide actions. J Pharmacol Exp Ther 322:778–788 CrossRef PubMed Google Scholar
48. Voss B, McLaughlin JN, Holinstat M, Zent R, Hamm HE (2007) PAR1, but not PAR4, activates human platelets through a $G_{i/o}$/phosphoinositide-3 kinase signaling axis. Mol Pharmacol 71:1399–1406 CrossRef PubMed Google Scholar
49. McBane RD 2nd, Hassinger NL, Mruk JS, Grill DE, Chesebro JH (2005) Direct thrombin inhibitors are not equally effective in vivo against arterial thrombosis: in vivo evaluation of DuP714 and argatroban in a porcine angioplasty model and comparison to r-hirudin. Thromb Res 116:525–532 CrossRef PubMed Google Scholar
50. Wen W, Young SE, Duvernay MT, Schulte ML, Nance KD, Melancon BJ, Engers J, Locuson CW 2nd, Wood MR, Daniels JS, Wu W, Lindsley CW, Hamm HE, Stauffer SR (2014) Substituted indoles as selective protease activated receptor 4 (PAR-4) antagonists: discovery and SAR of ML354. Bioorg Med Chem Lett 24:4708–4713 CrossRef PubMed PubMedCentral Google Scholar
51. Diabate M, Munro P, Garcia E, Jacquel A, Michel G, Obba S, Gonçalves D, Luci C, Marchetti S, Demon D, Degos C, Bechah Y, Mege JL, Lamkanfi M, Auberger P, Gorvel JP, Stuart LM, Landraud L, Lemichez E, Boyer L (2015) *Escherichia coli* α-hemolysin counteracts the anti-virulence innate immune response triggered by the Rho GTPase activating toxin CNF1 during bacteremia. PLoS Pathog 11:e1004732 CrossRef PubMed PubMedCentral Google Scholar
52. Cheung AL, Bayer AS, Zhang G, Gresham H, Xiong YQ (2004) Regulation of virulence determinants in vitro and in vivo in *Staphylococcus aureus*. FEMS Immunol Med Microbiol 40:1–9 CrossRef PubMed Google Scholar
53. Aktories K, Barbieri JT (2005) Bacterial cytotoxins: targeting eukaryotic switches. Nat Rev Microbiol 3:397–410 CrossRef PubMed Google Scholar
54. Iliev AI, Djannatian JR, Nau R, Mitchell TJ, Wouters FS (2007) Cholesterol-dependent actin remodeling via RhoA and Rac1 activation by the *Streptococcus pneumoniae* toxin pneumolysin. Proc Natl Acad Sci U S A 104:2897–2902 CrossRef PubMed PubMedCentral Google Scholar

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55. Nguyen CT, Le NT, Tran TD, Kim EH, Park SS, Luong TT, Chung KT, Pyo S, Rhee DK (2014) *Streptococcus pneumoniae* ClpL modulates adherence to A549 human lung cells through Rap1/Rac1 activation. Infect Immun 82:3802–3810 CrossRef PubMed PubMedCentral Google Scholar
56. Feistritzer C, Riewald M (2005) Endothelial barrier protection by activated protein C through PAR1-dependent sphingosine 1-phosphate receptor-1 crossactivation. Blood 105:3178–3184 CrossRef PubMed Google Scholar
57. Feistritzer C, Schuepbach RA, Mosnier LO, Bush LA, Di Cera E, Griffin JH, Riewald M (2006) Protective signaling by activated protein C is mechanistically linked to protein C activation on endothelial cells. J Biol Chem 281:20077–20084 CrossRef PubMed Google Scholar
58. Bae JS, Yang L, Manithody C, Rezaie AR (2007) The ligand occupancy of endothelial protein C receptor switches the protease-activated receptor 1-dependent signaling specificity of thrombin from a permeability-enhancing to a barrier-protective response in endothelial cells. Blood 110:3909–3916 CrossRef PubMed PubMedCentral Google Scholar

MATERIALS TEXT

2.1 Microspheres, Supplies, and Equipment

1. Cyto-Plex™ far-red fluorescent carboxylated microspheres (beads), uniform 4–5 µm in diameter, 12 sets with 12 discrete dye levels, at 10^8 beads/mL, 1 mL of each set (Thermo Fisher Scientific): We use the 5.4 µm sized beads.
2. Carboxyl polystyrene beads, 5% w/v, 10 mL (Spherotech): Our batch was 5.28 µm.
3. Amino polystyrene beads, 5% w/v, 10 mL (Spherotech): Our batch was 3.57 µm.
4. Quantum™ FITC MESF (Molecules of Equivalent Soluble Fluorochrome) beads, five sets of commercial beads in which each subset is functionalized with discrete titers of fluorescein conjugates (Bangs Labs).
5. Refrigerated microcentrifuge with swinging bucket rotor and 0.65 mL microcentrifuge tubes.
6. Flow cytometer with a far-red laser, such as an Accuri C6.
7. pH meter.
8. Rotator, nutator.
9. Nitrogen-bubbling apparatus.

2.2 Synthesis of Glutathione Beads

1. 1% (v/v) Tween-20 stock.
2. pH 6 buffer: 0.1 M 2-(4-morpholino)-ethane sulfonic acid (MES), pH 6.0, 0.15 M NaCl, 0.01% (v/v) Tween-20.
3. 1-Ethyl-3-(dimethylaminopropyl) carbodiimide hydrochloride (EDAC).
4. Sulfo-N-hydroxysuccinimide (sNHS).
5. Rinse solution: 0.15 M NaCl, 0.01% (v/v) Tween-20, with no pH buffer.
6. pH 8.4 buffer: 0.1 M $NaHCO_3$, pH 8.4, 0.01% (v/v) Tween-20.
7. 2 M 1,6-diaminohexane (hexamethylenediamine), pH 8.4.
8. pH 7 buffer: 0.1 M Sodium phosphate, pH 7.0, 0.01% (v/v) Tween-20.
9. Bifunctional crosslinker: 0.2 M Sulfosuccinimidyl 4-[N-maleimidomethyl] cyclohexane-1-carboxylate (sSMCC) in dimethyl sulfoxide (DMSO). Store at −80 °C.
10. 0.2 M Reduced glutathione, pH 7.0: Store in 50 µL aliquots at −20 °C.
11. 5 mg/mL Alexa Fluor 488 NHS ester (Thermo Fisher Scientific) in DMSO: Store at −80 °C
12. A fusion protein such as glutathione-*S*-transferase-green fluorescent protein (GST-GFP) to measure glutathione on the GST-functionalized beads, or your laboratory's GST fusion protein and a fluorescent detection agent that binds to it.

2.3 Cell Culture

The G-Trap assay is used to measure GTP loading of multiple GTPase targets, found in cell lysates [16, 17]. The reader may cultivate cells using applicable standard procedures for their target cells, and treat cells with known activators and inhibitors (*see* Table 1) [16, 17] to establish the applicability of the assay in the setting defined by the reader.

2.4 GST-Effector Protein Production

Following GST-effector chimeras are used for the studies described here:
1. p21 activated kinase protein-binding domain (PAK-1 PBD), a Rac1 effector (MilliporeSigma).
2. Raf-1 (v-raf-1 murine leukemia viral oncogene homolog 1) RBD, a Ras effector protein (MilliporeSigma).
3. Rhotekin-RBD, a Rho effector protein (Cytoskeleton).
4. Ral-GDS RBD, a Rap-1 effector protein (Thermo Fisher Scientific).
5. GST-RILP, prepared as previously described [16].

Citation: Peter Simons, Virginie Bondu, Angela Wandinger-Ness, Tione Buranda (09/02/2021). 3.2 Production of a Cleared Cell Lysate. https://dx.doi.org/10.17504/protocols.io.bptrmnm6

2.5 GTPase Assay

1. Antibodies: Monoclonal rabbit anti-Rap1 (Santa Cruz Biotechnology); monoclonal mouse antibodies anti-Rho (A, B, C) clone 55, anti-Rac1, anti-Rab7, and the secondary antibody goat anti-mouse IgG (H + L) conjugated to Alexa Fluor 488 (MilliporeSigma); monoclonal mouse anti-Ras antibody (Abcam).
2. Activators and inhibitors: Rap1 activator 8-Cpt-2me-cAMP (50 µM) (R&D Systems); Rac1 inhibitor NSC23766 (100 µM) and Rho activator calpeptin (1 µM) (MilliporeSigma); recombinant epidermal growth factor (EGF) (10 nM) (Thermo Fisher Scientific); mP6, a myristoylated hexapeptide (myr-FEERA-OH), custom synthesized at New England Peptide, H-Ras and K-Ras inhibitor, FTI 277 (100 nM) (Tocris); Rap1 inhibitor (GGTI 298) (10 µM).
3. 2× RIPA buffer: 100 mM Tris–HCl, pH 7.4, 300 mM NaCl, 2 mM ethylenediaminetetraacetic acid (EDTA), 2 mM NaF, 2 mM Na_3VO_4, 2% (v/v) NP-40, 0.5% (w/v) sodium deoxycholate. Just before adding to the culture medium supplement with 2 mM phenylmethylsulfonyl fluoride (PMSF) and 2× protease inhibitors (Halt™ Protease and Phosphatase Cocktail # 78442, ThermoScientific).
4. HHB buffer: 7.98 g/L HEPES (Na salt), 6.43 g/L NaCl, 0.75 g/L KCl, 0.095 g/L $MgCl_2$, and 1.802 g/L glucose.
5. HPSMT buffer (an intracellular mimic): 30 mM HEPES, pH 7.4, 140 mM KCl, 12 mM NaCl, 0.8 mM $MgCl_2$, 0.01% (v/v) Tween-20.
6. Blocking buffer 1: 0.1% Bovine serum albumin (BSA) in HPSMT.
7. Blocking buffer 2: 5% BSA in HPSMT.
8. 10 mM EDTA and 0.2% sodium azide for use at 1:10 dilution in storage buffer.

SAFETY WARNINGS

Please refer to Safety Data Sheets (SDS) for health and environmental hazards.

3.2 Production of a Cleared Cell Lysate

1 Two days before an assay seed a 48-well plate with 20,000 target cells in 100 µL of culture medium per well, resulting in about 50,000 cells the next day. The rate of cell proliferation might vary based on cell type and conditions. The critical target is 50,000 cells at the start of an experiment.

2

Remove the culture medium and replace with **100 µl serum-free medium** **Overnight** . Specific inhibitors of signaling can be added to the cells for the desired amount of time depending on the requirements of the reader's assay to establish proper inhibition before stimulation.

3

After stimulating the cells (*see* Section 3.5 in Protocol "Applications of G-Trap Assay" for examples), chill the plate in an ice/water bath. Add **100 µl cold 2× RIPA buffer** to each well with a 1 mL pipette and triturate the mixture gently to achieve homogenous lysis of the cells.

4

Transfer the lysate into a 0.65 mL microfuge tube and centrifuge at **5000 x g, 00:02:00 , (between 3,000-5,000 x g)** . The 200 µL of cleared lysate is enough for triplicate assays using 50 µL for each test.

Citation: Peter Simons, Virginie Bondu, Angela Wandinger-Ness, Tione Buranda (09/02/2021). 3.2 Production of a Cleared Cell Lysate. https://dx.doi.org/10.17504/protocols.io.bptrmnm6