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3.4 Assay Runtime

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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 cytoflex 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 3.4 'Assay Runtime' 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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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), RaIGDS-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

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 μ M; 30 min		10,000/cell; 3, 10, 20, 30, 60 min
Rac1	PAK-1 PBD	EGF; 10 nM; 15 min	NSC23766; 100 μ M; 30 min	10,000/cell; 3, 10, 20, 30, 60 min
Rap1	Ral-GDS RBD	8-Cpt-2me-cAMP; 50 μ M; 30 min	GGTI 298; 10 μ M; 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].

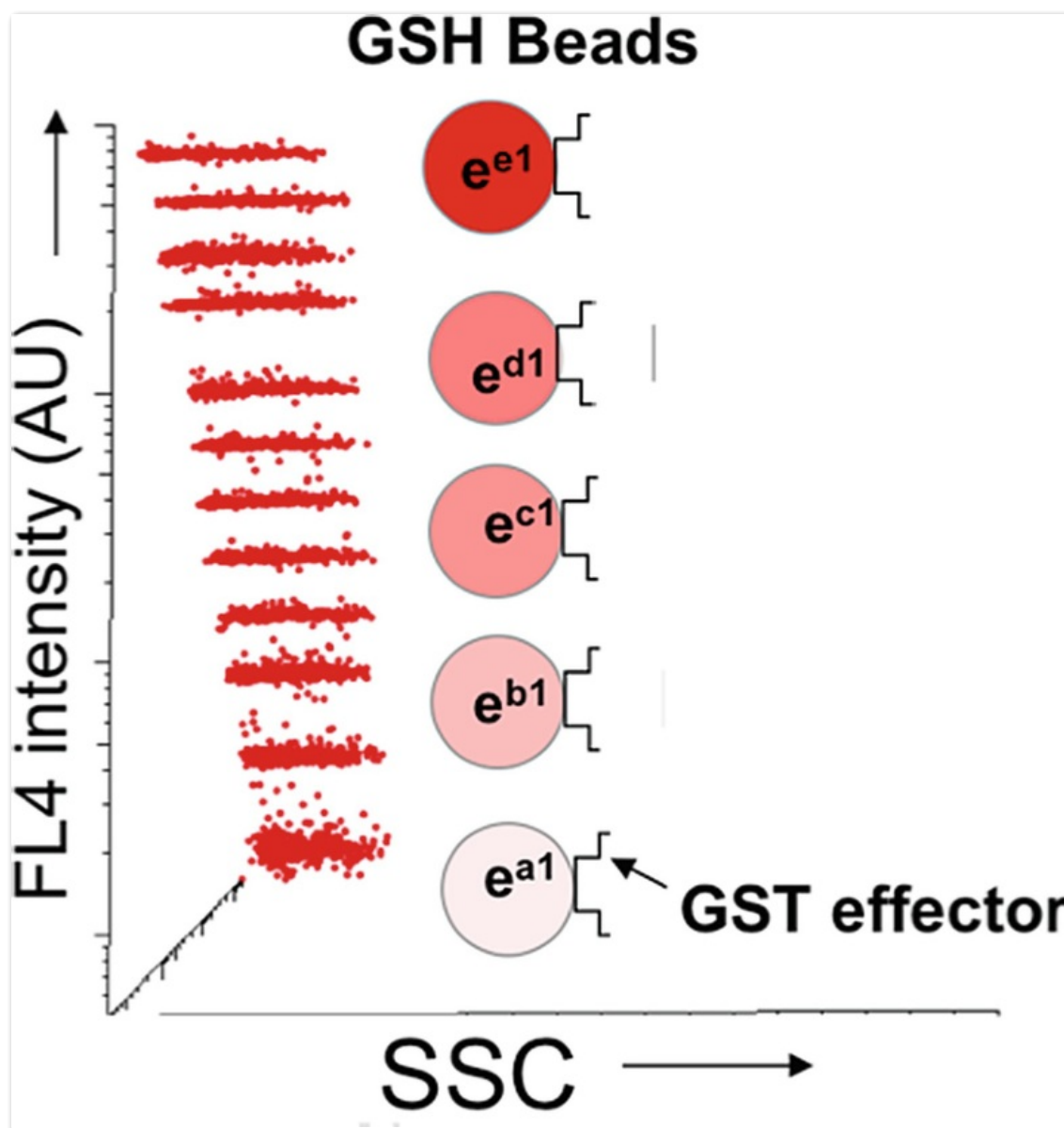


Fig. 1

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**.

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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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 10⁸ 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 NaHCO₃, 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].

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 μ M) (R&D Systems); Rac1 inhibitor NSC23766 (100 μ M) and Rho activator calpeptin (1 μ M) (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 μ M).
3. 2 \times 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 \times 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.

3.4 Assay Runtime

1h



Just before the addition of lysates, mix the five different effector beads, centrifuge at **3000 x g, 00:02:00**, and reduce the supernatant to about 5 μ L. Resuspend the beads in **65 μ L HPSMT**, giving approximately **0.8 Nanomolar (nM) each GST-effector**. Add **5 μ L suspension** to twelve 0.65 mL microcentrifuge tubes for 12 multiplex assays. Leftover beads can be used later to set up the cytometer.



1h

Add **50 μ L cleared lysate** to each of the 12 tubes, mix, and rotate for **01:00:00**. When prepared as in Section 3.2 (Protocol "[Production of a Cleared Cell Lysate](#)"), there is enough of each lysate for triplicate determinations.



Centrifuge the tubes at **5000 x g, 00:02:00**, reduce the supernatant to about 5 μ L, and resuspend the beads in the residual volume.



Wash the beads with **50 μ L blocking buffer #2** to block the beads' nonspecific antibody-binding sites, centrifuge at **3000 x g, 00:02:00**, and resuspend in **5 μ L residual buffer** (see Note 5).



1h

Add **50 µl primary antibodies** (1:20 dilution) against the 5 GTPases to each tube, mix, and rotate the tubes for **01:00:00**.

6 

1h

Wash the beads with **50 µl blocking buffer #2**, centrifuge at **3000 x g, 00:02:00**, and resuspend in **5 µl residual buffer**. Add **50 µl goat anti-mouse antibody fluorescently labeled with Alexa488** (1:100 dilution) to each tube, mix, and rotate the tubes for **01:00:00**.

7 

Centrifuge at **3000 x g, 00:02:00**, reduce the supernatants to 5 µL, and resuspend the beads in **5 µl residual buffer**.

8 Dilute the beads from each tube with **100 µl blocking buffer #1** just before each flow cytometric reading.