Version 1
Jul 21, 2022

Staining of cells with GolgiTracker for Golgi flow cytometry analysis V.1

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dx.doi.org/10.17504/protocols.io.e6nvwk1d2vmk/v1

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ABSTRACT

We describe a method that allows the staining of intact Golgi using GolgiTracker for subsequent flow cytometry analysis. The analysis can provide a quantitative indication of the level of enrichment of the organelle immunoprecipitation (% of $GOLGI^+$ beads) and can be used as a quality control check for the purity of the immunoprecipitated Golgi. Since only a small fraction of the immunoprecipitated material is needed for flow cytometry, this technique can be coupled with other assays, like western blotting or proteomics.
The staining can be performed directly on the cells, or, alternatively, after the Golgi has been isolated from the cells (For our Golgi immunoprecipitation protocol, see dx.doi.org/10.17504/protocols.io.6qpvrdjrogmk/v1). In our experience, the first method (which is described in this protocol) results in a cleaner signal. It should be noted that the staining is performed in KPBS, the buffer is used as optimal for organelles isolation. This buffer is compatible with the GolgiTracker staining, however other staining, particularly those using antibodies, might require a buffer containing Mg^{2+} or Ca^{2+}. While this protocol is focused on the staining and detection of Golgi, it can be easily modified to analyse different organelles by changing the staining procedure and adjusting the parameters of the cytometer.

DOI

dx.doi.org/10.17504/protocols.io.e6nvwk1d2vmk/v1

PROTOCOL CITATION

Enrico Bagnoli, Rotimi Fasimoye, Dario R Alessi 2022. Staining of cells with GolgiTracker for Golgi flow cytometry analysis. **protocols.io** https://dx.doi.org/10.17504/protocols.io.e6nvwk1d2vmk/v1

KEYWORDS

GolgiTracker, GolgiTag, Organelle IP, Flow cytometry

CREATED

Mar 15, 2022

LAST MODIFIED

Jul 21, 2022

PROTOCOL INTEGER ID

59486

GUIDELINES

For a detailed method on how to introduce GolgiTAG to cells and immunoprecipitate intact Golgi, see dx.doi.org/10.17504/protocols.io.6qpvrdjrogmk/v1.

MATERIALS TEXT

- Cells expressing GolgiTag (or ControlTag), generated as described in dx.doi.org/10.17504/protocols.io.6qpvrdjrogmk/v1
- KPBS buffer: 136mM KCL, 10 mM KH2PO4. Adjust to pH 7.25 with KOH. (Note On the day of use, add Roche cOmplete protease inhibitor cocktail tablet (REF# 11873580001) and Roche PhosSTOP tablet (REF# 04906837001)
- BODIPY™ FL C_5-Ceramide (*N*-(4,4-Difluoro-5,7-Dimethyl-4-Bora-3a,4a-Diaza-*s*-Indacene-3-Pentanoyl)Sphingosine) (="GolgiTracker") Thermo Fisher Scientific, Cat #D3521
- Flow cytometer (BD LSR Fortessa or similar)
- FACS tubes

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Materials and reagents

1 Cell culture

10m

1.1 Grow HEK293 cells stably expressing GolgiTag or ControlTag (generated as described in dx.doi.org/10.17504/protocols.io.6qpvrdjrogmk/v1) in 10cm^2 Petri dish until near confluent.

Note: For each experimental conditions, two plates will be needed: one to be stained with GolgiTracker, and a control plate (not stained with GolgiTracker).

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Staining on cells + Immunoprecipitation 2h

2 Staining of cultured cells with GolgiTracker

2.1 Prepare a [M]**500 micromolar (µM)** stock of GolgiTracker in DMSO and dissolve thoroughly by vortexing.

2.2 Add the GolgiTracker to the cells to a final concentration of [M]**5 micromolar (µM)** (i.e. 1:100 dilution). Add DMSO vehicle (1% v/v) to control cells.

> Our method has been optimised in HEK293 cells. For different cell types, concentrations and time of incubation might need further optimization.

2.3 30m

Incubate at **37 °C** for **00:30:00**

2.4 30m

Discard media and add fresh media and incubate for further **00:30:00** at **37 °C**

3 Isolation of intact Golgi using GolgiTag immunoprecipitation method 1h

3.1 Perform Golgi immunoprecipitation as described in dx.doi.org/10.17504/protocols.io.6qpvrdjrogmk/v1

3.2 At the end (Step 56 in dx.doi.org/10.17504/protocols.io.6qpvrdjrogmk/v1), add **500 µL KPBS** to the beads (instead of adding lysis buffer) and gently resuspend by pipetting up and down.

Flow cytometry

4 Preparation of samples for flow cytometry.

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4.1 Prepare and label the appropriate amount of FACS tube. For example:
1. GolgiTAG +Tracker
2. GolgiTAG -Tracker
3. HA-Empty +Tracker
4. HA-Empty -Tracker

4.2 Dilute samples. We used a dilution of 1:100 (or 1:200) from the final IPs. So from the **500 µL KPBS and Beads** take **40 µL** and resuspend in a FACS tube with **400 µL KPBS** on **On ice**

Samples are still very concentrated so higher dilutions can also be used, particularly if this protocol is combined with other assays from the same IPs.

5 Flow cytometry analysis

1h

The following part is different depending on the cytometer used. The parameters reported refer to the BD LSR Fortessa that we use in our lab.

5.1 Check the waste container of the cytometer is not full. Check the buffer container of the cytometer is not empty. Refill or empty accordingly.

5.2 Turn on machine following relative SOPs. If first user of the day, also perform the starting procedure.

5.3 Prepare working windows and gates. The tracker used can be analysed in the FITC channel.

We normally run the samples using 3 windows:
1. Forward scatter (FSC) vs Side scatter (SSC)
2. Histogram of FITC channel
3. FITC vs FSC

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The first window allows to visualize the beads while the other two are indications of the enrichment of the IP.

5.4 If the samples are run for the first time, adjust voltages for the Forward and Side scatter and the FITC channel. Otherwise use the parameters already optimized before.

For the LSR Fortessa the voltages used are:
SSC: 231
FSC: 566
FITC: 400
For the scatters, we visualize using a logarithmic scale.

To get the best signal from the FITC channel, we normally run the sample with the highest and lowest signal to make sure we operate within the linear range of the cytometer. Similarly for the scatters, all evens should be inside the window and not on the edges of the chart (see Figure)

If optimizing the scatter for the first time, remember the beads are approximately **1 µm in diameter**, thus much smaller than a cell.

5.5 When FSC, SSC and FITC voltages are fine, start running the samples.
For each sample, record at least 20k events, but if possible 50k is a better option.

5.6 At the end, clean the machine and save the data (FCS 3.0 files)

Analysis 1h

6 Data analysis using FlowJo. 1h

The following steps are just an indication of the analysis performed with FlowJo. If new to the software we suggest to follow one of the many free tutorials available online.

6.1 Open the software and drag the samples to be analyzed in the main window

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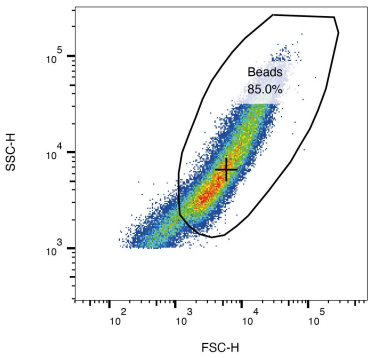
6.2 Gate the beads from the SSC and FSC plot as shown in the figure below.

For small samples like organelles IP, using the height (instead of area or width) is a better option when gating.



Example of gating to exclude broken beads and debris and include only beads and beads with organelle attached.

6.3 From these population calculate the % of GolgiTracker^{+} beads either from the FITC vs FSC plot or from the FITC histogram.

It is also useful to calculate the mean FITC intensity, mean SSC and FSC values for the gated beads. The SSC and FSC (indirect measure of complexity and size of the samples) should have higher values in the golgi-IPs compared to the mock-IPs, indication of the binding of the organelles to the beads. Similarly, the FITC intensity should be high only in the samples with the GolgiTag and the tracker.

6.4 In the workspace, create tables and graphs of the relevant samples.

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6.5 Export tables and images in the preferred format.

We export tables in an excel compatible format and images either in PNG or powerpoint.

6.6 Perform statistical analysis using Prism software or equivalent.

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