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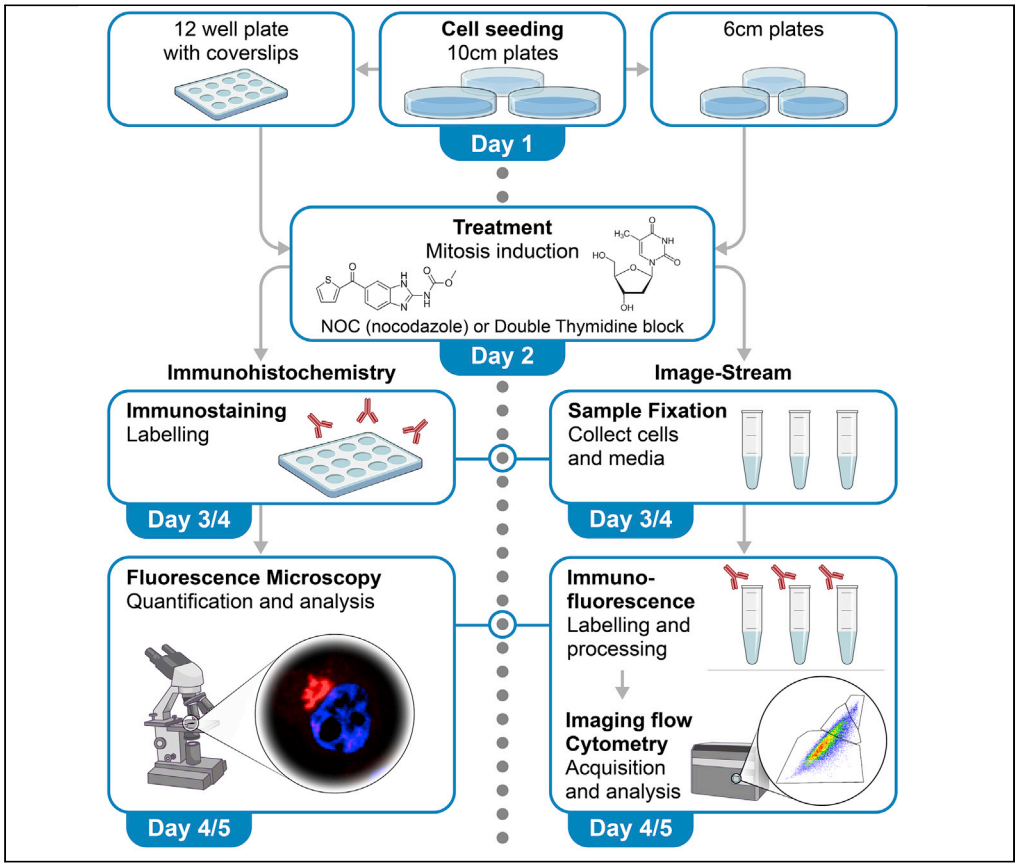
Applying imaging flow cytometry and immunofluorescence in studying the dynamic Golgi structure in cultured cells



The Golgi apparatus is subjected to fragmentation under several cellular processes such as mitosis. Here we describe two complementary approaches to analyze different Golgi morphological changes during its mitotic fragmentation, using classical immunofluorescence and imaging flow cytometry. Although fluorescent microscopy provides information on the exact Golgi architecture in distinct cells, the imaging flow cytometry combines the morphological data with the high-throughput quantification of flow cytometry. Taken together, both approaches provide robust and significant unbiased data analysis.

Inbal Wortzel, Ziv Porat, Rony Seger, Galia Maik-Rachline

inw2001@med.cornell.edu (I.W.)
galia.maik-rachline@weizmann.ac.il (G.M.-R.)

Highlights

Two complementary Golgi staining protocols to determine mitotic structural changes

Immunofluorescence protocol for Golgi staining in cells fixed on coverslips

Imaging flow cytometry protocol for Golgi staining in fixed suspension cells

Gating strategy for the three Golgi populations using imaging flow cytometry

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Applying imaging flow cytometry and immunofluorescence in studying the dynamic Golgi structure in cultured cells

Inbal Wortzel,[1,3,4,*] Ziv Porat,[2] Rony Seger,[1] and Galia Maik-Rachline[1,5,*]

[1]Department of Biological Regulation, Weizmann Institute of Science, Rehovot 7610001, Israel

[2]Department of Life Sciences Core Facilities, Weizmann Institute of Science, Rehovot 7610001, Israel

[3]Present address: Children's Cancer and Blood Foundation Laboratories, Departments of Pediatrics, and Cell and Developmental Biology, Drukier Institute for Children's Health, Meyer Cancer Center, Weill Cornell Medicine, New York, NY 10021, USA

[4]Technical contact

[5]Lead contact

*Correspondence: inw2001@med.cornell.edu (I.W.), galia.maik-rachline@weizmann.ac.il (G.M.-R.)
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SUMMARY

The Golgi apparatus is subjected to fragmentation under several cellular processes such as mitosis. Here we describe two complementary approaches to analyze different Golgi morphological changes during its mitotic fragmentation, using classical immunofluorescence and imaging flow cytometry. Although fluorescent microscopy provides information on the exact Golgi architecture in distinct cells, the imaging flow cytometry combines the morphological data with the high-throughput quantification of flow cytometry. Taken together, both approaches provide robust and significant unbiased data analysis.

For complete details on the use and execution of this protocol, please refer to Wortzel et al. (2021).

BEFORE YOU BEGIN

The Golgi apparatus is a dynamic organelle that undergoes fragmentation under various cellular processes, particularly mitosis (Lucocq and Warren, 1987; Presley et al., 1998; Sutterlin et al., 2002). Fragmented Golgi is characterized by distinct morphological changes ranging from the initial formation of separated chunks to a complete vesiculation of the organelle. In this protocol we combine the use of two powerful methods, immunofluorescence (IF) and imaging flow cytometry (IFC) using Image-Stream (ISX), to detect and quantify changes in the Golgi structure of a given cell population subjected to mitosis-inducing treatment, in a statistically robust manner. These two methods were previously used in our laboratory thus, for additional information please refer to (Zuba-Surma et al., 2007; Wortzel et al., 2015, 2017; Berti and Seger, 2017). It should be noted that results may slightly vary when comparing the two methods due to different fixation and inherent differences between each protocol. This protocol is optimized using HeLa cells since their Golgi structure is mostly intact and morphology changes in cycling cells. However, it has been successfully applied to other cell-lines such as HAP1, and primary human embryonic lung fibroblast, WI-38.

A list of all reagents required for this protocol are described in the key resources table section.

All reagents and buffers required should be prepared in advance.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-GM130 antibody (EP892Y) Rabbit (1:100)	Abcam	Cat#ab52649
Anti-GM130 mAb (Monoclonal) (1:100)	MBL	Cat#M179-3
Goat anti-Rabbit IgG (H+L) Cross-adsorbed Secondary antibody, Alexa Fluor 568 conjugate (1:400)	Thermo Fisher Scientific	Cat#A-11011
Goat anti-Mouse IgG (H+L) Cross-adsorbed Secondary antibody, Alexa Fluor 568 conjugate (1:400)	Thermo Fisher Scientific	Cat#A-11004
DAPI 4′,6-Diamidino-2-phenylindole dihydrochloride (1:250)	Sigma-Aldrich	Cat#D9542
Chemicals, peptides, and recombinant proteins		
DMEM (Dulbecco's Modified Eagle Medium)	Gibco	Cat#41965-039
Fetal Bovine Serum, certified, South America	Gibco	Cat#12657-029
PBS (Phosphate Buffered Saline)	Sigma-Aldrich	Cat#D8537
Trypsin EDTA Solution C (0.05% Trypsin)	Sartorius	Cat#03-053-1B
Penicillin-Streptomycin Solution	Sartorius	Cat#03-031-1B
L-Glutamine Solution	Sartorius	Cat#03-020-1B
Triton X-100	Sigma-Aldrich	Cat#X100
Albumin, Bovine Fraction V	MP Biomedicals	Cat#160069
Paraformaldehyde 32% solution	Electron Microscopy Sciences	Cat#15714
Nocodazole	Sigma-Aldrich	Cat#M1404
Thymidine	Sigma-Aldrich	Cat#T1895
Methanol Absolute AR	Bio-Lab	Cat#136805
Experimental models: Cell lines		
HeLa cells	ATCC	Cat#CCL-2
Software and algorithms		
ZEISS Observer.Z1 Confocal Spinning Disk microscope system with camera-Q IMAGING ROLERA	Carl Zeiss	N/A
Imaging software - ZEN	Carl Zeiss	Version 2.5 system https://www.zeiss.com/microscopy/int/products/microscope-software/zen.html#downloads
ImageStreamX mark II	Luminex	N/A
Acquisition software - INSPIRE	Amnis/part of Luminex.	Version 200.1.681.0 https://www.luminexcorp.com/imagestreamx-mk-ii/#software
Analysis software - IDEAS	Amnis/part of Luminex.	Version 6.3 https://denovosoftware.com/full-access/download-landing/
Other		
60 × 15 mm Tissue Culture Dish	Falcon	Cat#353004
12 well cell culture plates. Flat Bottom with Lid	Costar	Cat#3513
Microscope Cover Slips diameter 18 mm #1	Menzel Glaser Gmbh	CB00180RA120MNZ
Elvanol Mounting Solution	Waterborne Inc	Cat#M102
Orbital-Mixer/Intelli-Mixer	Thomas Scientific	N/A

MATERIALS AND EQUIPMENT

Complete DMEM

Reagent	Final concentration	Amount
DMEM	n/a	440 mL
Heat-inactivated FBS	10% (v/v)	50 mL
Pen/Strep solution (10,000 units/mL)	1% (v/v)	5 mL

(*Continued on next page*)

Continued		
Reagent	Final concentration	Amount
L-Glutamine solution (200 mM)	1% (v/v)	5 mL
Total	**n/a**	**500 mL**
Prepare sterile and filter through 0.22 um membrane. Store at 4°C.		

Blocking-permeabilization solution for IF and IFC		
Reagent	Final concentration	Amount
BSA	2%	1 gr
Triton X-100 (10% solution)	0.1% (v/v)	0.5 mL
PBS	n/a	45.5 mL
Total	**n/a**	**50 mL**
Prepare freshly on the day of staining for optimal results.		

4% Paraformaldehyde solution (PFA)		
Reagent	Final concentration	Amount
Paraformaldehyde 32% vial	4%	10 mL
PBS	n/a	70 mL
Total	**n/a**	**80 mL**
Store at 4°C for 2 weeks or at −20°C for a long-time storage.		

Nocodazole stock

Add 1.33 mL DMSO to 2 mg nocodazole powder to achieve a stock concentration of 1.5 mg/mL (5 mM). Vortex to mix well and store as small aliquots at −20°C. Avoid freeze-thaw cycles.

Thymidine stock

Add 8.25 mL DMSO to 1 gr thymidine powder to achieve a stock concentration of 121 mg/mL (0.5 M). Vortex to mix well and store as small aliquots at −20°C. Avoid freeze-thaw cycles.

STEP-BY-STEP METHOD DETAILS

Cell culture and mitosis induction

Timing: 2 days for nocodazole—3 days for thymidine treatment

In this section we describe how to culture HeLa cells for IF or ISX and treat them to induce mitosis.

Note: All reagents used to treat cells such as complete medium (10% fetal calf serum in DMEM and supplements; termed here complete medium) or phosphate buffered saline (PBS) are warmed at 37°C prior to use.

Note: Microscope cover slips are autoclaved prior to use to ensure sterilization.

1. For IF, seed 2–4 × 10^5 HeLa cells per well in a 12-well culture dish containing one 18 mm diameter sterile microscope cover slip and complete medium in each well.
2. For ISX, seed 5 × 10^5 HeLa cells in a 60 mm plate in complete medium.
3. Place cells at 37°C humidified atmosphere with 95% air and 5% CO_2 incubator. Cells allowed to attach for approximately 18–20 h.

Note: Optimal cells confluency is critical. Cell number and complete medium type were optimized for HeLa cells. When other cells are used, each line should be optimized individually to

reach 70%–80% confluency the day following seeding in its optimal complete medium. This is because mitosis-inducing treatment may cause cells detachment. Detachment may also occur when siRNA or overexpression transfection are applied. A lower cell density may result in either insufficient number of cells following treatment, or insufficient number of transfected cells.

4. Cells synchronization at prometaphase using nocodazole:
   a. Block mitotic progression (end of prometaphase) by adding nocodazole directly into the complete medium of the relevant wells (IF)/plates (ISX) at a final concentration of 0.3 μM. Incubate for 16 h.
   b. Control cells are left untreated, containing complete medium only.
5. Alternatively, cells can be synchronized by a double-thymidine block:
   a. Treat cells by directly adding thymidine to a final concentration of 2 mM into the complete medium of the relevant wells/plates. Incubate for 16 h.
   b. Aspirate complete medium containing thymidine, and rinse plates twice with PBS by applying and immediately aspirating it.
   c. Add complete medium for 8 h. This step completes one thymidine block cycle.
   d. Repeat the thymidine treatment for an additional cycle of 16 h.
   e. Wash twice with PBS as mentioned above and apply fresh complete medium. This marks time 0.
   f. Continue growing the cells under regular conditions.

Note: For the double-thymidine block, save an unwashed plate. This will represent cells at the G1/S border.

Optional: When smaller plates or wells are used and total volume is reduced, it is recommended to aspirate medium and add a fresh complete medium that already contains nocodazole or thymidine diluted to the desired concentration. This will ensure homogenous distribution of the reagents.

Determining Golgi structure in synchronized HeLa cells by IF

In this section we describe the protocol for immunostaining HeLa for Golgi marker followed by imaging acquisition using fluorescence microscopy.

Cell fixation by paraformaldehyde for IF

Timing: 40 min

6. Carefully aspirate the complete medium from each well.
7. Wash cells once with PBS to remove any residual medium. Immediately aspirate PBS.
8. Fix cells with 4% paraformaldehyde (PFA) in PBS, 1 mL per well and incubate for 20 min at room temperature (22–25°C, RT).

Note: Since cells might be more susceptible to detachment due to previous treatments, it is recommended to carefully apply all solutions and washes on the edge of the well and not directly on cells.

9. Remove PFA and add PBS for 5 min at RT. Repeat wash with PBS for a total of three times to remove all residual PFA.

Pause point: Cells can be kept following fixation in PBS at 4°C for up to 2 weeks before proceeding to the next step.

Immunofluorescence staining for IF

Timing: 3 h

10. Permeabilization / Blocking: permeabilize cells with Triton X-100 and block with bovine serum albumin (BSA) by applying 1 mL of blocking-permeabilization solution per well. Incubate for 5 min at 23°C.

    Optional: This step may be separated to a permeabilization step (0.1% Triton X-100 in PBS, 5 min at 23°C), which is followed by a blocking step (2% BSA in PBS, 15 min at 23°C). This may be required when harsher conditions are needed in order to permeabilize the cell membrane, or for better blocking of non-specific binding according to the antibody used.

11. Primary antibody labeling: Prepare the primary antibody solution. Dilute the GM130 antibody at a ratio of 1:100 in PBS for a total volume of 25 μL per sample.

    Note: We use GM130 antibody as an endogenous Golgi marker to detect mitotic Golgi dynamics, however other Abs (p58, Giantin and GRASP65) were equally successful (Shaul and Seger, 2006; Wortzel et al., 2017).

    Note: Both GM130 antibodies mentioned in the KRT worked equally well and are diluted similarly. They should be preferred based on the relevant species.

    Optional: To increase antibody specificity it is recommended to dilute the antibody in 2% BSA in PBS.

12. Once blocking is completed, remove the solution and wash cells once with PBS.
    a. Prepare parafilm on a clean bench surface and apply a drop of 25 μL diluted antibody for each slide.
    b. Carefully invert each cover slip on the liquid drop, while making sure that the cells are facing the antibody dilution.
    c. Cover the parafilm with an aluminum foil covered box. Attach wet Kimwipes on top of the box. This will ensure a humidified cover preventing the coverslips from drying out.
    d. Incubate for 1 h (at RT).
13. Transfer coverslips back to the 12-wells plate and wash cells three times with PBS, 5 min per wash at RT as explained above to remove the residual antibody.
14. Secondary antibody labeling: Prepare the secondary antibody solution by diluting the Alexa Fluor 568 conjugated antibody 1:400 together with 4′,6-diamidino-2-phenylindole (DAPI) for the nuclear staining at a dilution of 1:250 in PBS.
    a. Prepare parafilm on the clean bench surface and apply a drop of 25 μL diluted secondary antibody for each slide as described in step 12.
    b. Invert coverslips on the secondary antibody drop and incubate for 1 h (at RT) using the humidified covered box.
    c. Secondary antibody should be applied in the dark to prevent photobleaching.
15. Transfer coverslips back to the 12 wells tissue culture plate and wash cells with PBS three times to remove any residual antibody as specified in step 13.
16. Prepare microscope slides marked with the specific cell type, treatment and antibody wave-length. Apply a drop of 10–20 μL Elvanol mounting solution per coverslip. Once washing is complete, carefully remove each slide from the well and absorb leftover PBS using Kimwipes.
17. Slowly invert each slide, cells side facing down, on the mounting reagent within an angle until fully placed on the slide. Carefully place the slides in a covered dark place and allow it to dry overnight in RT.

Optional: Transferring of the slides from the well plate to the paraffin back and forth, as described above in steps 12–15, allows the use of a smaller antibody volume. Alternatively, the primary or secondary antibody mix can also be applied directly into the coverslips in the well plate, but this requires much larger volumes. The latter, however, limits the chance of confusion between treatments and/or breaking/damaging the slides from the frequent removals.

Note: It should be noted that IF can be successfully applied to co-localize two different proteins within the same cells as well as staining of specific proteins within overexpressed cells. For this purpose, the two primary antibodies should be generated in different species (i.e., rabbit and mouse), while both secondary antibodies should be generated in the same third different species animal (i.e., goat) to prevent cross reactivity. Moreover, secondary antibodies should be conjugated to fluorescent probes with different, non-overlapping, wavelengths to ensure specificity of the signal.

Imaging by a spinning disk confocal microscopy and image analysis

Timing: 1–2 h

18. Image acquisition of the stained cells to analyze the endogenous Golgi is applied using fluorescence microscopy. We used the Observer.Z1 Confocal Spinning Disk Microscope (Zeiss, Jena, Germany) equipped with a ROLERA (Q-Imaging) 12-bit monochrome CCD camera. Turn on the microscope and the ZEN 2.5 system software on the computer.
19. Add the relevant channels on the software:
    a. DAPI - 405 nm laser. BP450/50 emission filter.
    b. AF568 - 532/561 nm, 529/62 emission filter.
20. Optimize image to avoid saturation for each channel, while maximizing the dynamic range of the camera. Capture images with a 63×/1.4 NA oil immersion objective according to the specific microscope software.
21. Capture images of at least 5 different fields from each slide for quantification to have a total of at least 100 cells.

Note: Counting should include at least 100 cells per treatment to have enough cells to reach statistical significance. Thus, if the confluency of the cells is low, more images per treatment should be acquired.

22. Acquired images are transferred to Photoshop software (only to view the image, while maintaining the original scientific information of the picture. Other image-handling programs can be used as well). Analysis and quantification of the fine Golgi structure according to the GM130 staining is then done manually for each cell within each frame (by eye). Intact Golgi appears as one concentrated GM130 spot (~2–3 μm) adjacent to the nucleus (stained with DAPI). Initial fragmentation appears as a little more spread (>4 μM), less intense GM130 staining. Partially fragmented ones (chunks) will be seen as 2–5 distinct spots, and fully fragmented Golgi will be seen as a diffused GM130 staining all over the cells.

Note: Automated programs for microscope data analysis such as Image J, ilastik, or CellProfiler are available and can be used for quantification either alone, or preferably when are used together. These are very good tools mainly when following just fragmented vs intact Golgi. However, due to the fluidity of the Golgi structure and the different stages of fragmentations that vary among cells and can all appear simultaneously within the same frame, manual counting can be considered. The automated programs are robust specifically when combined together and result with a good automated quantification of the Golgi, however, a specific knowledge is required for their use.

23. Count cells in each field and document number of cells with fragmented Golgi and number of cells with intact Golgi. Repeat counting in 5 different fields for a total of at least 100 cells.
24. Calculate percentage of cells containing fragmented vs intact Golgi out of the total cells counted.
25. Determine significance using student's T-test to determine difference between untreated to mitosis induced cells. Distinct stages of the fragmentation can be quantified as well.

Note: More than 100 cells should be counted and three independent experiments should be performed to determine significant results. Double-blinded counting can be applied to ensure accurate results.

Determining Golgi structure and mitotic phases in synchronized HeLa cells by ISX

In this section we describe the protocol for immunostaining HeLa for Golgi marker followed by imaging acquisition using the Image-Stream X instrument.

Methanol fixation for IFC

Timing: 30 min (with an overnight incubation)

26. Collect the complete medium from each 60 mm culture dish, and transfer into a 15 mL conical centrifuge tube. Place the tube on ice.

Note: Mitotic induction (either nocodazole or thymidine) may result (in some cell types) in the detachment of cells from the plate. Therefore, it is important to collect the cells suspended in the complete medium.

Note: Make sure to have additional plates for the single-color controls as well as an unstained sample for compensation analysis. In this layout, 3 additional plates are needed (for unstained, DNA stain only and for Golgi stain only).

27. Wash the culture dishes with PBS. Carefully aspirate the PBS and add 500 μL Trypsin C solution to each dish. Incubate the dishes in a 37°C incubator for 3–5 min to allow cells to detach from the plate.
28. Use the medium from step 26 to wash the detached cells from the dish. Collect the medium with the washed cells and return to the tube.
29. Spin down cells at RT for 5 min at 300 × *g*.
30. Aspirate the supernatant, tap to resuspend and add 5 mL of ice-cold PBS. Centrifuge again at RT for 5 min at 300 × *g*.
31. Aspirate the supernatant and then resuspend the cell pellet in 500 μL ice-cold PBS.
32. Set the vortex condition to a low mixing level relative to the vortex used. Gently vortex the cell suspension while slowly adding 1.7 mL ice-cold methanol into the tube in drop-by-drop manner.
33. Place sample at 4°C for an overnight incubation.

Pause point: Samples can be stored after fixation up to two weeks at 4°C.

Immunofluorescence staining for ISX

Timing: 3 h

34. Add 6 mL ice-cold PBS (4°C) to each tube and spin-down at RT for 5 min at 300 × *g*. Repeat once more to remove all residuals methanol.
35. Permeabilization / Blocking: Aspirate the supernatant and resuspend the cell pellet in 500 μL per tube of the following mixed solution; 0.1% Triton X-100 and 2% BSA in PBS, for 5 min at 23°C.

36. Immediately after, wash cells by adding 6 mL ice-cold PBS and spin-down at RT for 5 min at 300 × *g*.
37. Primary antibody labeling: Prepare the primary antibody solution. Dilute the GM130 antibody at a ratio of 1:100 in PBS for a total volume of 100 µL per sample. Aspirate the supernatant and resuspend the cell pellet in 100 µL primary antibody dilution. Mix in an orbital-Mixer for 1 h at 4°C, 99° turns.

Note: Both GM130 antibodies mentioned in the KRT worked equally well and are diluted similarly. Should be preferred based on the relevant species.

38. Add 6 mL ice-cold PBS (4°C) and spin-down at RT for 5 min at 300 × *g*.
39. Secondary antibody labeling: Prepare the secondary antibody solution containing Alexa Fluor 568 conjugated antibody (1:400) together with DAPI (1:250) for the nuclear staining in PBS.
40. Aspirate the supernatant in the tube and resuspend the cell pellet in 100 µL secondary antibody solution. Mix in an orbital-Mixer for 30–60 min at 4°C.

Note: Secondary antibody should be applied in the dark to prevent photobleaching.

41. Add 6 mL ice-cold PBS (4°C) and spin-down at RT for 5 min at 300 × *g*.
42. Aspirate the supernatant in the tube, resuspend the cell pellet in 200 µL PBS, and then transfer to a clean 1.5 mL Eppendorf centrifuge tube.

Image acquisition by ISX

Timing: 1–3 h

43. Turn on the imaging flow cytometer, server and acquisition computer. Log in to the acquisition computer.

Note: For a detailed explanation on how to use the instrument and analysis software, please refer to the user manual.

44. Start the acquisition software, INSPIRE (the version used was 200.1.681.0). Once loaded, click on 'STARTUP' and make sure that all the automatic calibrations and tests have passed (marked green).
45. Choose the magnification according to your cell size. We recommend the 60× magnification (NA=0.9, pixel size 0.33 mm), if possible, to allow the highest resolution.
46. Check the excitation/emission data for your dyes and turn on the relevant lasers. Refer to the user manual for the detailed spectra for the available acquisition channels. Set the lasers to maximal power. In the example we provide (Figure 1B), we used the 561 nm laser (200 mW) for the AF568 dye and 405 nm laser (120 mW) for the DAPI.
47. Turn on the relevant acquisition channels for your dyes. These should include the brightfield channels (#1 and #9 for a two-camera instrument) and side-scatter channel (#6 or #12, depending on your dye combination). In the example we provide, AF568 was acquired on channel 4 (595–640 nm) and DAPI was read on channel 7 (430–505 nm).
48. Load your first sample – start with the sample that contains all dyes, which is expected to produce the highest staining intensity. In this experiment, it would be the control sample.
49. Create a scatter plot of the Area vs. Aspect ratio of the bright-field channel (as set in step 47). Gate on single cells according to your population distribution.

Note: To include dividing cells in the telophase stage, increase the gate to include higher-area, low-aspect-ratio cells as well.

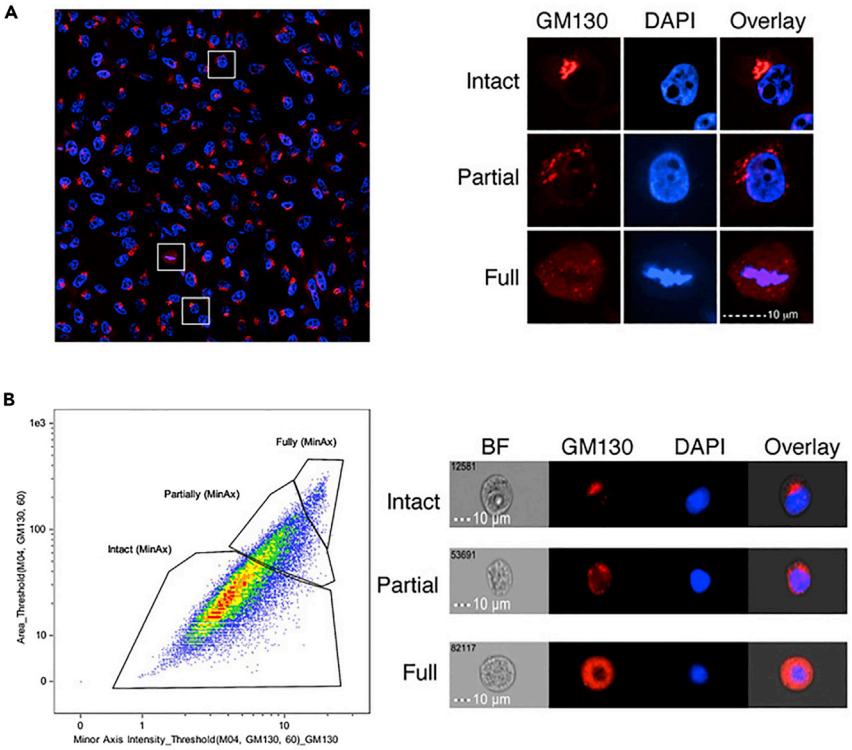

Figure 1. Examples of expected results for Golgi analysis by IF and IFC

HeLa cells were fixed and stained for GM130 (red) and DAPI (blue) as described in the protocol. Right columns show examples of the different stages of the Golgi (Top row - intact, middle row - partially fragmented, lower row – fully fragmented).

(A) Slides were imaged by a spinning-disc confocal microscope with 63× oil objective. Representative cells with intact, partial and fully fragmented Golgi are enlarged on the right.

(B) Cells were imaged by ISX using a 40× magnification and analyzed by IDEA 6.2. Cells positive for Golgi staining (Golgi +) were plotted for 'minor axis intensity' and 'area' of Golgi staining. Each Golgi population is gated on the graph, and representative cells are presented on the right. Scale bar 10 µm.

50. Create a histogram of Gradient_RMS of the bright-field channel (Usually channel 1). Draw a linear gate that includes values between 55 and 90, to collect only cells that are in focus.
51. Create a 'Raw Max Pixel' histogram for each fluorescent channel, taken from the 'single' gate.

Note: Make sure the values do not reach 4095 (the instrument uses a 12-bit camera), which indicates the camera is saturated. If they do, reduce the relevant laser intensity until the values are within the target range preferably less than 4000.

52. Collect ~30,000 cells from each sample, using the same setup of lasers and acquisition channels as set in step 47 for all samples. If a rare population is assessed, collect enough cells so that the final population contains at least 500 cells.
53. Monitor camera focus and cell position during the acquisition.

Note: If the autofocus fails to maintain its focus, it could be disabled by going to Instrument-> Advanced settings-> Autofocus and disabling the 'autofocus' option. Once the autofocus is disabled, the focus can be manually changed on the 'Focus' tab.

Note: If the cell position in the flow is not centralized (this has to be inspected visually, as the software does not alert in case of drift), it can be adjusted manually by moving its position on 'Centering'.

54. After recording your samples, acquire single stained controls with the compensation wizard, using the same laser settings as those used for the experimental samples.

Image analysis by IDEAS

Timing: 1–3 h

Set analysis template.

55. Start the IDEAS analysis software (preferably version 6.2 or higher).
56. Open the control experimental sample file and calculate the compensation using the single-stain files acquired, using the compensation wizard in IDEAS.
57. Draw a bi-variate plot of Area vs. Aspect ratio of the bright-field channel. Draw a gate around the single cells population, usually with low area and high aspect ratio. Verify the gate by visual inspection of the cells. If a cell-cycle analysis is required, make sure to include telophase cells, which are a close-to-doublet population (i.e., have higher area and lower aspect ratio).
58. Draw a bi-variate plot of RMS vs. Contrast of the bright-field image. Gate on focused cells, usually values higher than 55 for Gradient RMS and higher contrast values. Verify the gate by visual inspection of the cells.
59. Draw a bi-variate plot of Area vs. Centroid X of the bright-field channel. Gate on uncropped cells, which have already higher values of Centroid X.
60. Draw a bi-variate plot of the Intensity vs. Max Pixel values of the Golgi staining channel. Gate on cells with a positive Golgi staining (Golgi +), by comparing the staining to control samples labeled with all the dyes except the Golgi staining.
61. **Define populations**: Using 'Tag Objects', manually select a group of cells with an intact Golgi and another group featuring a fragmented Golgi (see Figure 1B for a typical morphology). Include around 100 cells per group.
62. **Define masks:** Calculate several masks that delineate the Golgi staining. Usually, this would be done using the 'Morphology' and 'Threshold' masks, and we recommend using several 'Threshold' values. In the example provided (Figure 1B), we used 'Threshold_60' (top 60% intensity pixels).
63. **Define feature**: Calculate all the features using the 'Add Multiple Features' option in the 'Features' panel. Select all the feature options under the 'Size', 'Location', 'Shape' and 'Texture' categories. It is undesirable to enable the 'Signal strength feature option, as it may differ between samples and between experiments. Choose the masks you just created, choose the Golgi staining channel under 'Image', and click on 'Add Features'.
64. **Discriminating statistics**: Add a new statistics table. Under 'Statistics', choose 'RD-Mean' and 'RD-Median' (RD value, or Fisher's discriminant, is defined as difference between the means or medians, divided by the sum of standard deviations, of the two populations). Choose as a reference population the intact Golgi cells chosen in step 61, and click 'Add' to calculate.
65. **Choose the best features**: Copy the values obtained to an Excel file and arrange them in a descending order according to the RD values.
66. Examine the ability of the features that are at the top of the list with the highest RD values to separate between the chosen populations, by plotting histograms / bi-variate plots of the selected features. Verify by visual inspections of the populations.
67. **Apply features**: Apply the selected features to the whole population and verify that they correspond to the different Golgi morphologies.
68. Save the analysis template.

Apply analysis template to samples.

69. Open the files recorded from treated samples using this template.
70. It is recommended to merge the treated and control samples into one file, as it makes it easier to determine the gating. To do so, in each file click on Tools -> Create Data File from Populations,

and then select the single, focused, uncropped, Golgi-positive population, and create a *.CIF file. Then, click on 'Merge CIF files', select the files you just created and use the template that was saved in the previous step.

71. Determine the gating that distinguishes between the different populations according to the controls. Verify by visual inspection of the populations.
72. Apply the gating on all the other experimental datasets, either in the merged file or by saving the template and applying it to all the other experimental samples.
73. If you have IDEAS version 6.3, quantification of the different Golgi morphologies could also be done by using the Machine Learning Wizard. In this case, use the populations chosen in step 61 to allow the wizard to create a classifier that combines several features weighted for the best separation between populations.

Potential applications.

74. You can follow the Golgi fragmentation during the different stages of the cell cycle and cell division by following the next steps:
    a. Cell cycle and division stages: Label DNA using a dye compatible with the ones you used to stain your samples. Common dyes and their corresponding channels (in brackets) include DAPI (Ch7), SyBR Green (Ch2), propidium iodide (Ch4) and Draq5 (Ch11). You can quantify the different stages according to the DNA intensity, plotted as a histogram on a linear scale.
    b. Cell division: using the DNA staining, you can determine the stages of cell division by DNA morphology (examples can be seen in (Filby et al., 2011); (Wortzel et al., 2015); (Vivante et al., 2021)), however adjustments may be needed according to particular cell type.
        i. Gate for G2/M based on DNA staining intensity.
        ii. Gate for G2, prophase, prometaphase and mitotic (metaphase-telophase) populations using 2 parameters: the bright detail intensity (BDI) of DNA staining (intensity of localized bright areas, subtracted for the local background), and the area of the 50% highest intensity pixels of the DNA staining (defined by the Threshold_50 mask).

Note: Non-dividing cells will have high area, low BDI, mitotic cells will have a low area and high BDI, and prophase will be in between.

        iii. Gate for cells in metaphase according to their lower width using the Width feature masked for the 50% top intensity pixels of the DNA staining (Threshold 50).
        iv. Cells with higher area and lower aspect ratio Brightfield (BF) images are defined as belonging to the telophase stage.
        v. The rest of the cells can be then gated using two spot features based on the mask of the 50% high intensity pixels of the DNA staining (Threshold 50). The features used are Spot Distance Min (shortest distance in microns between two spots within the mask) and Spot Area Min (the area of the smallest spot within the mask). Three populations can be defined: (1) High Spot Area Min and 0 Spot Distance Min are considered as metaphase and added to the previously gated cells. (2) Intermediate Spot Area Min and high Spot Distance Min are defined as cells during anaphase. (3) Cells with low Spot Area Min and low Spot Distance Min are defined as cells during Pro/ProM and added to the previously gated cells.

75. Once the Golgi fragmentation is quantified as detailed above, it could be combined with additional parameters or markers:
    a. Apoptosis: Apoptotic cells will have fragmented and condensed DNA. This can be quantified, for example, using the contrast of the bright-field image vs. the area of the top 30% intensity pixels of the DNA staining.
    b. Co-localization: The co-localization of the Golgi with other proteins or organelles can be quantified by fluorescently labeling them and calculating the similarity using similarity

feature (i.e., log transformed Pearson's Correlation Coefficient, a measure of the degree to which two images are linearly correlated within a masked region) or bright detail similarity (i.e., the intensity of localized bright spots whose radius is three pixels (if defined as number of pixel, important to clarify the magnification used on the Image-Stream) or less within the masked area in the image, with the local background around the spots removed). The pixel resolution is 0.3 μm for the 60× lens, and 0.5 μm for the 40× lens (Wortzel et al., 2015).

c. Cell classification: As you can utilize up to 10 fluorescent channels, you can label additional markers to increase your resolution of specific population identification.

EXPECTED OUTCOMES

IF and IFC, as described in this protocol, are both effective methods used to analyze the dynamic Golgi changes. Typically, in a non-synchronized cycling cell population, the majority of the cells contain an intact Golgi, while less than 5% should be mitotic, apoptotic or migrating cells with some sort of Golgi fragmentation. Inducing mitosis will result in a significantly higher number of dividing cells and therefore more Golgi fragmentation.

As described above, intact Golgi (at interphase) will be visualized as one spot in a juxtanuclear position. Initial fragmentation appears as a little more spread (>4 μM), less intense GM130 staining at the same position. Partially fragmented one (chunks) will be seen as 2–5 closely related spots. Fully fragmented Golgi will be observed as a small punctum that appears as a haze (based on resolution) dispersed throughout the cell (excluding the DNA area; Figure 1A). Mitotic treatment causes cells to become round, therefore they are often found in a different focal plane and as a result may be slightly unfocused. In IFC, following the identification of the 3 different Golgi populations described above, one can apply gating to the total population and quantify the percentage of cells in each group (Figure 1B left). Then specific cells can be chosen as representative images for each population (Figure 1B right). Similar to IF, in a non-synchronized cycling cell population, the majority of the cells should present an intact Golgi and will be mostly located at the lower axis values (Area / minor axis intensity). However, treatment will shift the population to the upper right higher values indicating fragmented Golgi (Wortzel et al., 2017, 2021).

LIMITATIONS

The methods described are very well-developed and usually provide reliable and sound results. Nonetheless, limitations do exist in several stages of the methods. One of the inherent problems include the two methods of fixation. Thus, not all proteins or antibodies are suitable for the paraformaldehyde fixation, in particular when staining large protein complexes. Since this fixation preserve the three-dimensional conformation and complexes of most proteins, some antigenic sites may be hindered. As for the methanol fixation, it may sometimes interfere with membranal structures, and thereby the structure of some organelles. Moreover, methanol often denatures some proteins, and thereby may prevent binding of some antibodies, although it may increase the recognition of many antibodies produced against peptides or denatured proteins. These problems are not seen with the methods and reagents described here, but may cause problems in other cells or with other antibodies.

Another point is the fact that IF may be problematic to quantitate, especially when analyzing the dynamic fluidity changes in the Golgi structure. Although several automated image analysis software programs are available, they are not all easily adapted by the average user, as these methods require establishing stable cell-lines or specialized imaging and/or image analysis programs that are not readily available. Even this can be problematic due to the large number of distinct Golgi structures during the fragmentation process. Therefore, the average user will resort to a manual counting of cells. Obviously, this type of counting may be user-biased, and can be overcome by a double-blind counting, and a large number of represented cell populations. Imaging flow cytometry (IFC), is suitable for a large-scale quantification, and by its nature, offers an unbiased quantification. However, using this method, it is very difficult to focus on the small differences between the fragmented structures that are sometimes important for understanding the various fragmentation stages.

Finally, the methods used may cause problems in analyzing signaling networks. Thus, if the fixation is not rapid enough, the addition of the various reagents or the transfer to cold buffer can by itself induce the phosphorylation and activation of some signaling proteins. In addition, the fact that the IFC requires adherent cell harvesting may by itself cause changes in structure or induction of signaling, especially, if not done quickly enough. However, when procedures described are followed accurately results are reliable.

TROUBLESHOOTING

Problem 1

A lower number of cells is observed on the coverslips following mitosis induction during image analysis of IF (steps 1 and 18).

Potential solution

Increase initial number of cells in nocodazole treated slides. Additionally, when applying PFA and PBS wash, pay attention to gently apply liquids on the edge of the well plate in order to avoid any direct contact of liquids on the cells.

Problem 2

A low number of cells is acquired by ISX (steps 2 and 48).

Potential solution

Cells were lost while they were being washed. Aspirate the samples carefully on the opposite side to where the pellet is formed. Do not vacuum all the way and leave residual supernatant to avoid losing cells. If the Vacuum is too strong, hand pipette to remove the solution slowly. In addition, you can increase the protein content of the washing buffer (e.g., 1% BSA).

Problem 3

The focus and location for cells in the stream are not stable on the ISX – the cells' focus is constantly changing, the flow rate is unstable, and / or the cell localization in the stream is variable and many cells are cropped (steps 44–49).

Potential solution

Return the sample, vortex it and load again. If the cell concentration is too high, you can dilute the samples. In case of a bubble or a partial clog, you can use the 'purge bubbles' script (under "Instrument" in the main menu.

Problem 4

A low percentage of cells are mitotic (steps 4 and 5).

Potential solution

Calibrate the concentration of nocodazole or the time applied.

Find mitotic peak for the double thymidine block by releasing the cells for 8 h then fixing the cells every 30 min until 11 h. Most cell lines enter mitosis 9–10 h from the release.

Mitotic induction was achieved but cells were lost due to washing – see problems 1 and 2.

Problem 5

Insufficient staining (steps 10–17 and 34–37).

Potential solution

Some antibodies may not yield a sufficient staining in either PFA (less likely) or methanol (more common) fixation. Before performing the full experiment, it is recommended to test that the antibodies

of choice are optimized for concentration and if they are suitable for the chosen fixation. It should be noted that we found no difference between the two fixation methods for all Golgi antibodies tested in our laboratory.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Galia Maik-Rachline (galia.maik-rachline@weizmann.ac.il).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate datasets or code.

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AUTHOR CONTRIBUTIONS

All authors wrote the protocol.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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