

Video Article

Studying Proteolysis of Cyclin B at the Single Cell Level in Whole Cell Populations

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Abstract

Equal distribution of chromosomes between the two daughter cells during cell division is a prerequisite for guaranteeing genetic stability¹. Inaccuracies during chromosome separation are a hallmark of malignancy and associated with progressive disease²⁻⁴. The spindle assembly checkpoint (SAC) is a mitotic surveillance mechanism that holds back cells at metaphase until every single chromosome has established a stable bipolar attachment to the mitotic spindle¹. The SAC exerts its function by interference with the activating APC/C subunit Cdc20 to block proteolysis of securin and cyclin B and thus chromosome separation and mitotic exit. Improper attachment of chromosomes prevents silencing of SAC signaling and causes continued inhibition of APC/C^{Cdc20} until the problem is solved to avoid chromosome missegregation, aneuploidy and malignant growths¹.

Most studies that addressed the influence of improper chromosomal attachment on APC/C-dependent proteolysis took advantage of spindle disruption using depolymerizing or microtubule-stabilizing drugs to interfere with chromosomal attachment to microtubules. Since interference with microtubule kinetics can affect the transport and localization of critical regulators, these procedures bear a risk of inducing artificial effects⁵.

To study how the SAC interferes with APC/C-dependent proteolysis of cyclin B during mitosis in unperturbed cell populations, we established a histone H2-GFP-based system which allowed the simultaneous monitoring of metaphase alignment of mitotic chromosomes and proteolysis of cyclin B⁶.

To depict proteolytic profiles, we generated a chimeric cyclin B reporter molecule with a C-terminal SNAP moiety⁶ (**Figure 1**). In a self-labeling reaction, the SNAP-moiety is able to form covalent bonds with alkylguanine-carriers (SNAP substrate)^{7,8} (**Figure 1**). SNAP substrate molecules are readily available and carry a broad spectrum of different fluorochromes. Chimeric cyclin B-SNAP molecules become labeled upon addition of the membrane-permeable SNAP substrate to the growth medium⁷ (**Figure 1**). Following the labeling reaction, the cyclin B-SNAP fluorescence intensity drops in a pulse-chase reaction-like manner and fluorescence intensities reflect levels of cyclin B degradation⁶ (**Figure 1**). Our system facilitates the monitoring of mitotic APC/C-dependent proteolysis in large numbers of cells (or several cell populations) in parallel. Thereby, the system may be a valuable tool to identify agents/small molecules that are able to interfere with proteolytic activity at the metaphase to anaphase transition. Moreover, as synthesis of cyclin B during mitosis has recently been suggested as an important mechanism in fostering a mitotic block in mice and humans by keeping cyclin B expression levels stable^{9,10}, this system enabled us to analyze cyclin B proteolysis as one element of a balanced equilibrium⁶.

Video Link

The video component of this article can be found at <https://www.jove.com/video/4239/>

Protocol

1. Seeding of U2OS-based Cyclin B-SNAP Reporter Cells (Clone 11 Cells⁶) on Microscope Chamber Slides

1. Trypsinize subconfluent SNAP reporter cells that were allowed to grow asynchronously in log phase for at least 48 hr.
2. Working with 8 well microscope chambers (constant distribution of cells).

For seeding of cells onto 8 well microscope chambers at a constant distribution across the entire surface of the microscope chamber, centrifuge 10,000 cells and resuspend in 350 µl of phenol red-free normal growth medium (supplemented with 10% fetal bovine serum, penicillin/streptomycin and sodium pyruvate). Transfer cell suspension to the microscope chamber (**Figure 2**).

Working with 8 well microscope chambers (maximum cell density in the center).

For seeding of cells at a higher density in the center of the microscope chamber, load the chamber with 300 μ l of phenol red-free normal growth medium. Add 5,000 cells carefully to the center of the microscope chamber (**Figure 2**).

Working with 96 well special optics plates (constant distribution of cells).

For seeding of cells onto 96 well plates at a constant distribution across the entire surface of the well, centrifuge 5,000 cells and resuspend in 300 μ l of phenol red-free normal growth medium. Transfer the cell suspension to a 96-well plate. Depending on the total number of cell-containing wells required, adjust the cell number and the total volume of the suspension medium (**Figure 2**).

Working with 96 well special optics plates (maximum cell density in the center).

For seeding of cells onto 96 well plates, carefully add 1,500 cells in 15 μ l of phenol red-free normal growth medium in a small drop to the center of each well to achieve restriction of cell growth to the center of the well (**Figure 2**).

3. Allow seeded cells to grow for at least 18 hr under standard cell culture conditions (37 °C, 100% air humidity, 5% CO₂).

2. Staining of Reporter Cells with SNAP Substrate

1. 30 min prior to the beginning of the staining procedure allow aliquots of phenol red-free normal growth medium to warm up to 37 °C.
2. For easy handling of the SNAP substrate (in our case TMR Star) dissolve TMR Star in DMSO to obtain a concentration in the stock solution of 400 μ M, which can be stored at -20 °C.
3. Prior to staining, dilute 0.5 μ l of TMR Star stock solution in 200 μ l of phenol red-free normal growth medium (37 °C) to obtain a final labeling concentration of 1 μ M.
4. Remove normal growth medium from the asynchronously growing cells and incubate in labeling medium for 25 min under standard culture conditions.
5. Remove labeling medium and wash cells four times with phenol red-free normal growth medium (37 °C). Incubate cells in 300 μ l of phenol red-free normal growth medium (37 °C) for 30 min. Prior to transporting to the microscope replace the medium with fresh phenol red-free normal growth medium (37 °C) to remove residual unbound SNAP substrate.
6. Transport cells to the microscope in a styrofoam box on a pre-warmed (37 °C) heat block to minimize temperature variation.

3. Measurement of Fluorescence Intensity

1. Two hours prior to the analysis adjust the air temperature of the climate chamber to 37 °C in dry mode in order to bring the entire microscope with all its components to the desired temperature. Pre-heating before setting the humidity is important to avoid condensation and subsequent damage to the microscope.
2. Adjust air humidity to 60% and CO₂ to 5% prior to the start of the analysis.
3. Start the Scan[^]R Acquisition software and define standard settings (see **Table 1**).
4. Define the positions of the wells to be analyzed.
5. Define the Δt (acquisition cycle time) and the absolute number of acquisition cycles.
6. If analysis of a higher number of wells is desired, select hardware autofocus, otherwise it is sufficient to use software autofocus alone.
7. Start the acquisition and supervise for the first two acquisition cycles. The microscope will focus on the histone H2-GFP signal, with subsequent acquisition of a first image in that channel before the filter is changed and the corresponding TMR Star image is acquired (**Figure 3**). This is then repeated for all positions within a well and for each of the wells to be examined, before repeating again the next cycle.

4. Analysis of Proteolytic Profiles using Scan[^]R

1. Start the Scan[^]R Analysis software and analyse the images with the cell nuclei, as visualized by histone H2-GFP, defined as the main object, using a threshold based on signal intensity and a watershed algorithm to assist in separating neighboring cells. A subobject consisting of a nucleus with cytoplasm should be created for TMRstar analysis. (Important properties of the main object are X and Y positions, time, and maximum and mean intensities of GFP for the main object and the total intensity divided by area for the TMRstar subobject.) This analysis process may take several hours due to large data quantities.
2. Change to trace mode to visualize the subobject mean TMR Star fluorescence intensity over time (cell traces), assigned to the analyzed main objects (**Figure 4**). The number of cells to be examined can be narrowed down by gating on those measurements lasting at least 140 cycles and with a high maximum intensity of H2-GFP. Looking at larger numbers of cells allows a first representative and objective view (**Figure 4**).
3. Select a cell trace of interest to visualize histone H2-GFP and TMR Star fluorescence simultaneously at the single cell level (**Figure 5A**).
4. Using the right mouse click on a cell of interest and generate an exportable picture gallery for illustration of histone H2-GFP and cyclin B-SNAP TMR Star for every single time point.
5. Change to the population mode of the Scan[^]R Analysis software and gate the region where the cell of interest is represented on the X vs. Y dot plot (**Figure 5B**).
6. Apply a new dot plot window to the gated region and visualize mean TMR Star fluorescence intensity over time (**Figure 5C**).
7. Export data (time and fluorescence intensity) to Microsoft Excel for further calculation.

5. Representative Results

Figure 5D and 5E depict cyclin B kinetics, represented by a TMR Star fluorescence intensity curve, of a cell that proceeds through a regular mitosis without signs of chromosomal misalignment (**Figure 5E**). Upon compaction of the cytoplasm following nuclear envelope breakdown (NEBD, as indicated by the red triangle), TMR Star fluorescence intensity shows an abrupt increase until the isomorphic window (brighter area in the diagram) is reached when the cell enters prometaphase⁶. Fluorescence intensity remains at a stable level as long as the cell proceeds

through prophase and metaphase and then starts to drop rapidly once all of the chromosomes have established a stable metaphase plate (**Figure 5D and 5E**). This drop precedes chromosome separation during anaphase (blue dot on the curve). In late mitosis the chromatin starts to decondense (blue bars) and the cell adopts interphase morphology while the fluorescence intensity curve approaches a plateau which is lower than the plateau before mitosis (**Figure 5D and 5E**).

Autofocus settings	Histone H2-GFP (main object acquisition settings)	Cyclin B-SNAP labeled with TMR Star (acquisition settings)	Acquisition cycle time Repetitions
Coarse autofocus +/-39 μ m 24 Layers Fine autofocus +/-5.4 μ m 14 Layers	GFP filter set: Exposure time: 100 msec Light intensity: 25%	TRITC filter set: Exposure time: 150 msec Light intensity: 33.3%	2 to 5 min.
GFP filter set: Exposure time: 12 msec Light intensity: 12.5%			Up to 48 hr of continued analysis (limited by reduced air humidity of 60%)

Table 1. Standard settings as used for analysis of cyclin B proteolysis. Histone H2-GFP was used as a reference structure to define the focus plane for measurement of cyclin B-SNAP fluorescence intensity. Light intensities during the focusing procedure are lower as compared to image acquisition settings to avoid cumulative phototoxicity.

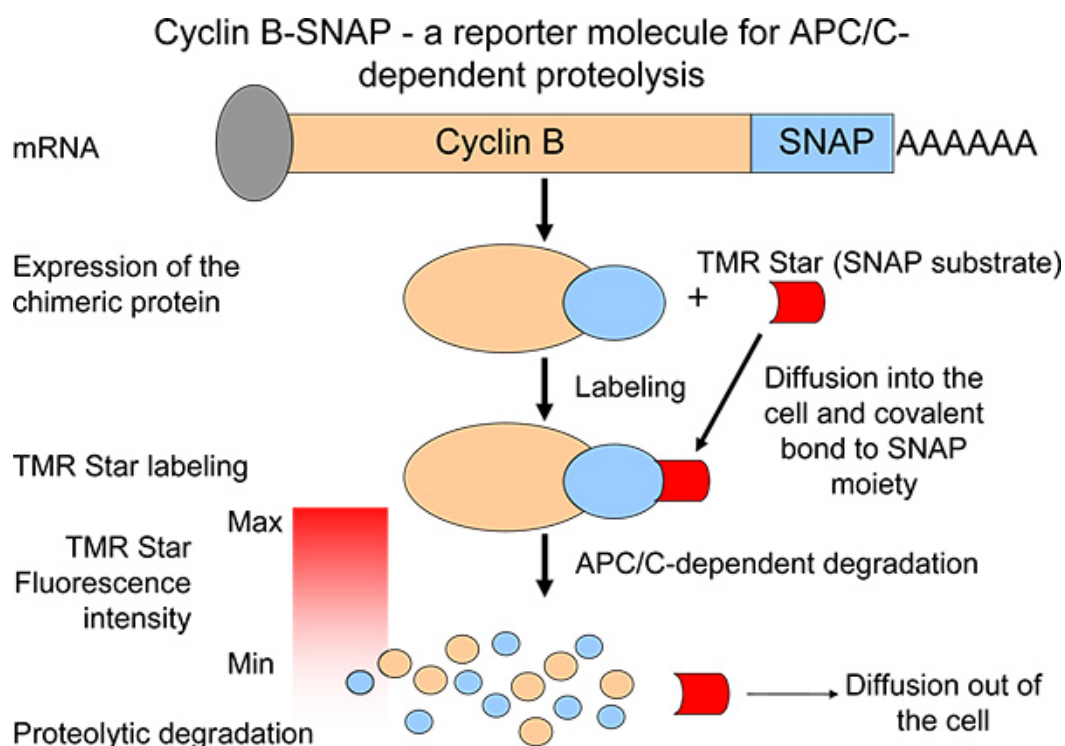


Figure 1. Schematic of the measurement of cyclin B proteolysis through expression of a chimeric cyclin B derivative with degradation characteristics similar to the endogenous protein. The decline in fluorescence intensity after pulse-chase labeling is a measure of APC/C-activity.

Techniques for seeding of cells

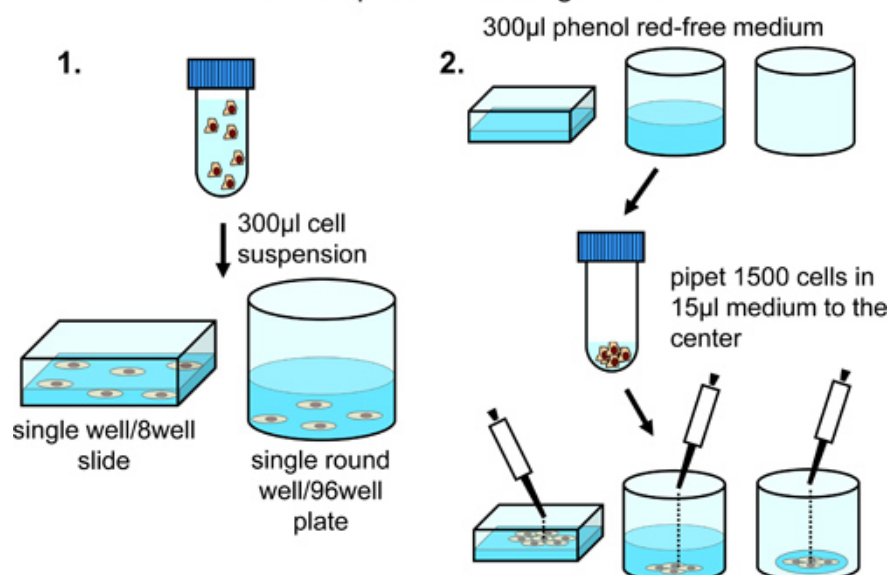


Figure 2. Analysis of cells on 8 well slides or 96 well plates. Regular distribution of the cells across the surface of the well is achieved by resuspension in a final volume of 300 µl and is recommended, if only a single or few positions are manually defined for analysis. Enrichment of cells in the center region is achieved by addition of cells to the center of pre-filled or empty wells. This technique is recommended in case of automated image acquisition in different wells.

Data acquisition

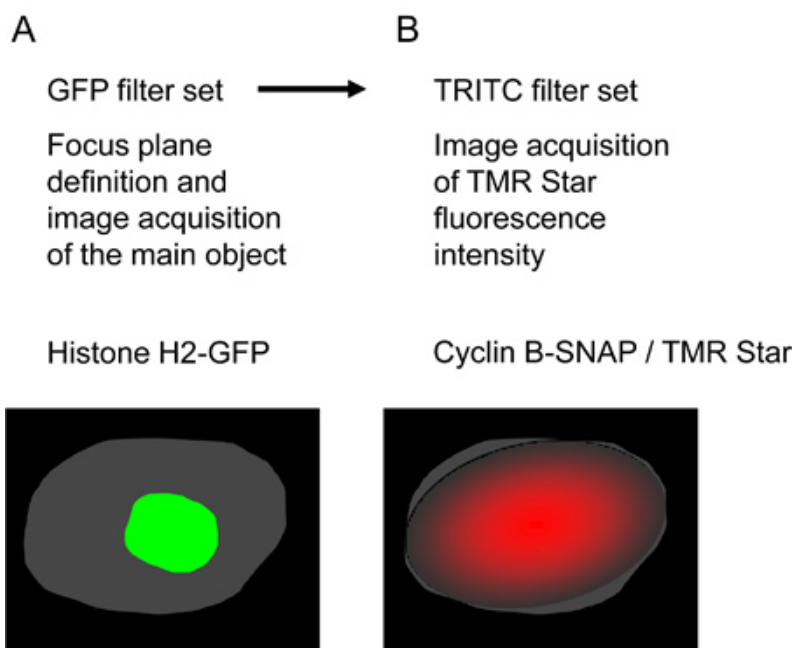


Figure 3. Sequence of data acquisition. A) Detection of histone H2-GFP is used for focus plane definition and monitoring of chromosomal alignment during mitosis. B) Fluorescence intensity of the TMR Star fluorescence intensity is a measure for the absolute amount of pulse-chase labeled cyclin B-SNAP.

Image analysis

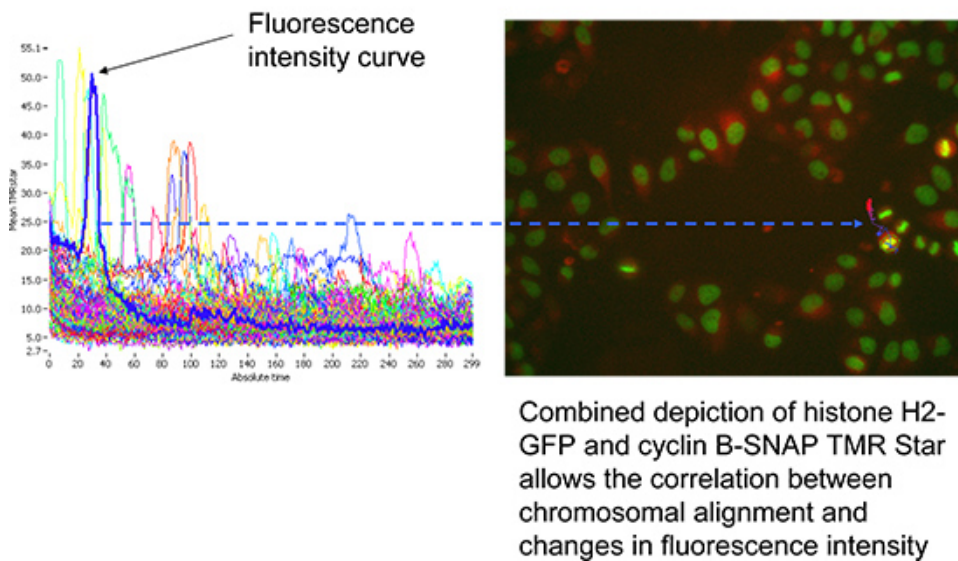


Figure 4. Representative Scan^R Analysis software-based depiction of mean TMR Star fluorescence intensities (total TMRstar intensity divided by area) over time. Selection of a representative cell trace (blue). The corresponding images (shown on the right) allow the simultaneous visualization of histone H2-GFP (green) and cyclin B-SNAP (red). [Click here to view larger figure.](#)

Data export and further calculation

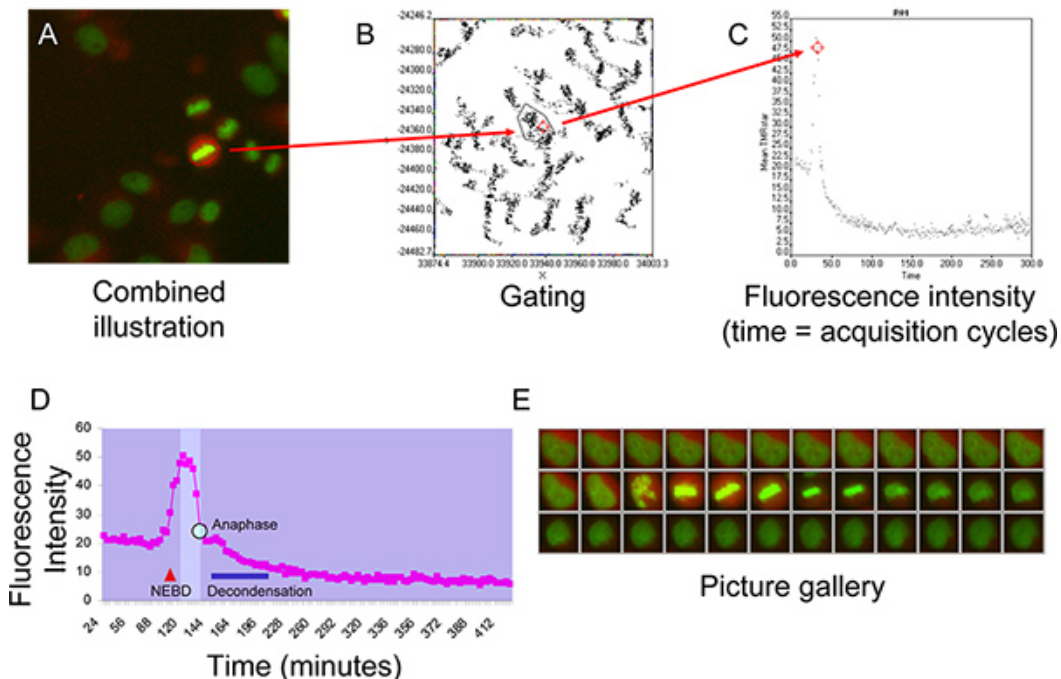


Figure 5. A+B) Example for gating of dots representing a cell of interest on the XY-dot plot. **C)** Visualization of the mean TMRstar fluorescence intensity (total TMRstar intensity divided by area) over time using the dot plot. **D+E)** Representative fluorescence intensity curve of cyclin B-SNAP with isomorphous window (brighter field on the diagram) between NEBD (nuclear envelope breakdown) and chromatin decondensation (blue bar) as generated in Microsoft Excel. Anaphase is indicated by the blue dot on the curve. [Click here to view larger figure.](#)

Movie 1. [Click here to view supplemental movie.](#)

Discussion

We present here a live-cell imaging-based approach facilitating the simultaneous monitoring of cyclin B proteolysis and chromosome alignment. This approach allows the study of mitotic control in unperturbed cell populations at the single cell level. Cyclin B degradation curves facilitate direct insights into the activity of the APC/C and thus indirectly reflect the status of the SAC⁶.

This approach, even though established based on the Scan^R software, can easily be reproduced on any comparable microscope station. Moreover, using our retroviral cyclin B-SNAP expression construct, a reporter cell line can easily be established by stable retroviral integration into any desired cell line. To make the system even more flexible, the chimeric SNAP proteins can be stained with various fluorochromes.

The implementation of histone H2-GFP, besides its requirement in monitoring chromosome alignment, facilitates fast and easy autofocusing. Autofocus and intensity settings also depend on the kind of cell and have to be established for each cell line. The acquisition of proteolytic profiles of large numbers of cell divisions using Scan^R technology furthermore allowed us to select for single mitoses, which showed chromosome alignment errors. This enabled us to define differences between the proteolytic profiles of aberrant and regular divisions⁶.

Mitoses displaying chromosomal misalignment can also be selected by analysis of raw image stacks, although this is less time-efficient. Cyclin B-SNAP fluorescence intensities can be quantified using freeware programs for image analysis such as ImageJ. Fluorescence intensity curves can be calculated based on the mean pixel intensity over time inside an area/gate defining the mitotic cell (technique described by Wolthuis *et al.*¹¹). Within an isovolumetric window, which has been described previously⁶, the shape and the volume of the cell remain nearly constant. This provides a rationale for using the same area/gate between nuclear envelope breakdown until early anaphase for measuring mean pixel intensities to manually estimate cyclin B proteolysis during prometaphase, metaphase and early anaphase.

Using the Scan^R-based approach, further mathematical analyses of degradation curves using specific software, such as Microsoft Excel, requires gating at the single cell level. Innovative gating strategies to allow automated data export of all analyzed cells might facilitate a more time-efficient analysis of whole cell populations. A fully automated approach covering all cell traces might lead to more representative results, further enhance precision of this technique and hence facilitate deeper insights into the regulation of mitotic exit.

Fast and efficient automated image acquisition enables our model to be used in large-scale screening analyses. Here, screens using shRNA libraries may lead to the identification of novel mitotic regulators. Moreover, the system should be useful in screening small molecules with regard to their potency in interfering with APC/C-dependent proteolysis in order to identify novel drugs for antimitotic therapy.

Cyclin B kinetics have been widely addressed by monitoring cyclin B-GFP fluorescence intensities. Here, we present a system that facilitates the delineation of cyclin B proteolysis from whole protein expression and therefore constitutes a valuable amendment to widely distributed techniques in the mitosis field⁶.

Disclosures

No conflicts of interest declared.

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