Towards an automated measurement of proteolysis *in-vivo*

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Abstract

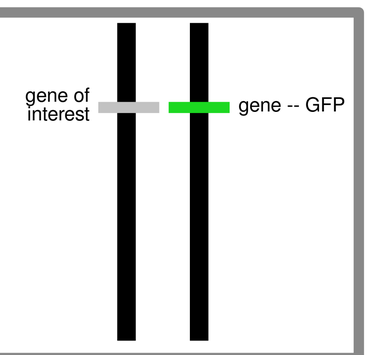
During the cell cycle key proteins present at specific times have to be degraded after they fulfilled their function. This protein degradation (proteolysis) is crucial because it provides unidirectionality to the cell cycle. *In vivo* studies of proteolysis can be realized using fluorescently-tagged proteins, which are created by homologous recombination and can be filmed by time-lapse microscopy.

We propose tools that allow partially automated extraction of fluorescence from time-lapse movies to quantify proteolysis. Degradation kinetics of various substrates of the Anaphase Promoting Complex (APC, a major proteolytic complex involved in mitosis) are then assayed to show the potential of the method.

Background

Genes of fluorescent (GFP-like) proteins were successfully introduced into the endogenous *loci* of genes regulated by proteolysis. This allows not only the determination of subcellular localization, but also the quantification of protein levels over time by time-lapse fluorescence microscopy.

Many softwares already exist to track and align cell tracks (CellProfiler[1], CellCognition[2],TimeLapseAnalyzer[3]), but are not designed to quantify fluorescence. Most of them rely on automatic classification of specific features (shape, variance, texture) to sort cells into predefined classes. This approach can lead to the detection of predefined cell phenotypes. However, the detected cells might not be representative of the original population. Furthermore, many of them of them rely on non-free libraries or are platform-dependent. Here, we propose a new software that implements a partially automated approach, trying to allow the user to supervise and correct each step of the analysis in order to collect representative data.



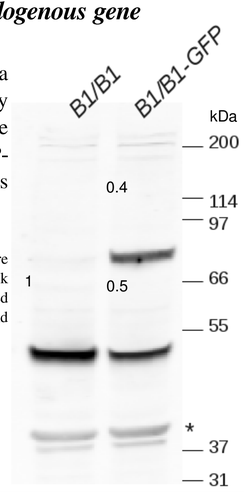
I. Monitoring fluorescence: a good proteolysis readout.

In order to determine degradation kinetics, it is important to establish a correlation between the actual protein level (input) and the measured pixel intensity.

1. GFP-tagged gene expression is similar to the untagged endogenous gene

Western Blot with a Cyclin B1 antibody shows a comparable protein level of the GFP-targeted protein versus the untagged protein.

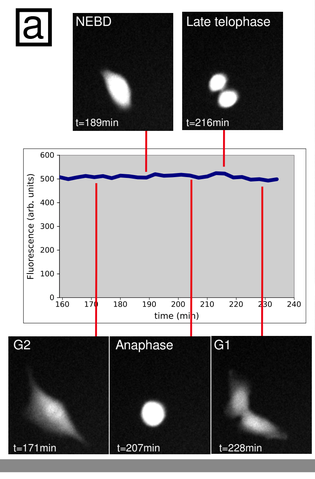
(relative band intensity are indicated next to the bands, asterisk indicates non specific binding used as loading control and normalization).

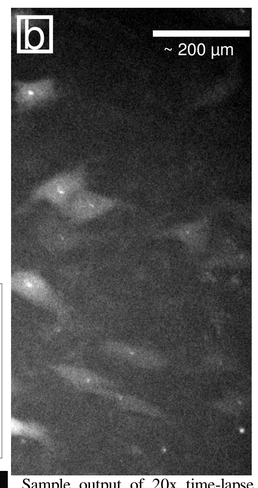


2. Time-lapse fluorescence microscopy can be used to quantify fluorescence over time

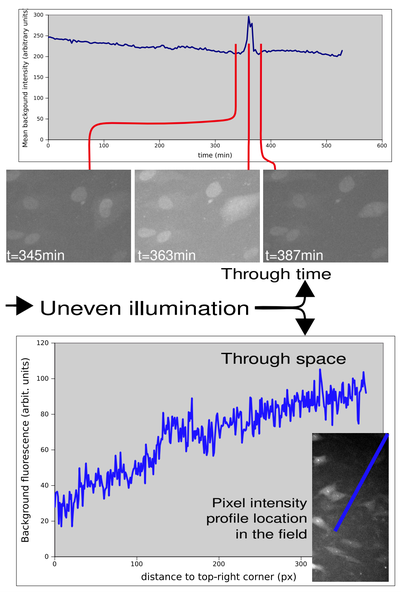
[a] The shape of cells changes during the cell cycle: they round up and detach from the support during mitosis. To make sure that changes in cell shape have no effect on the measured fluorescence, we recorded the fluorescence of a freely diffusible and constantly expressed cytoplasmic marker.

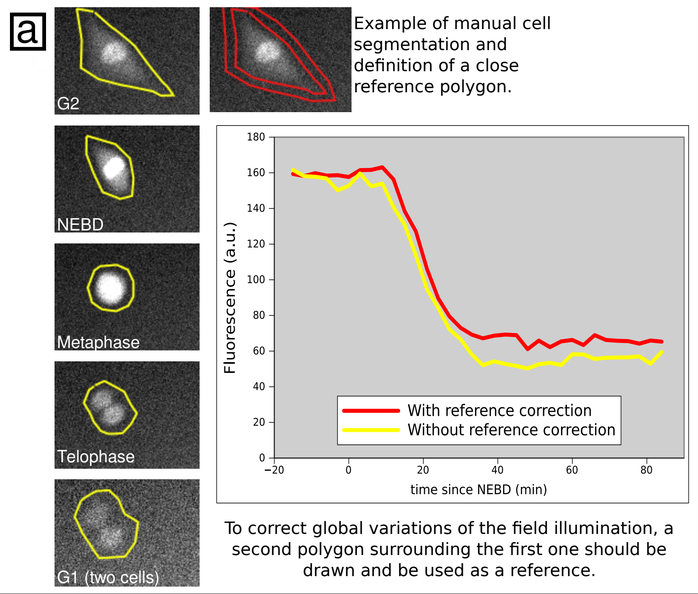
Time-lapse microscopes suffer several flaws. A major problem is the uneven illumination through space and time ([b]) that have to be corrected ([c]).





Sample output of 20x time-lapse microscopy - Cell line : AuroraA-Venus.





II. Automated fluorescence extraction can be achieved in different ways.

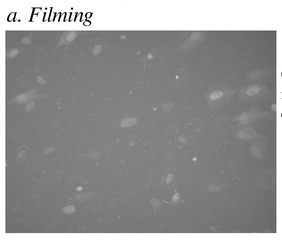
In vivo assays and derivation of quantitative data require either the user or the automated program to face several constraints:

Biological constraints:
- Moving cells
- Overlapping cells
- Mitosis (one gives two)

Experimental constraints:
- Representative sample size (>50 cells)
- Various cell lines with various fluorescent markers

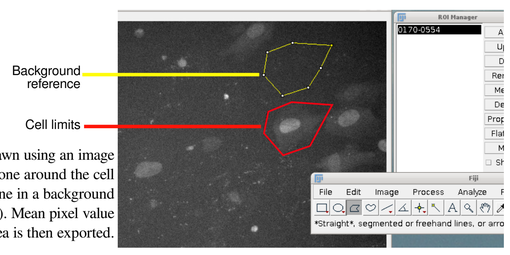
1. Standard quantification assay

a. Filming



Cell fluorescence is recorded for ~20hrs every 3-5mins.

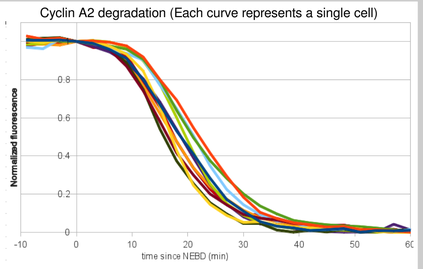
b. Cell segmentation



Two polygons are manually drawn using an image analysis software[4]. The first one around the cell (area of interest), the second one in a background region (background subtraction). Mean pixel value of the area is then exported.

c. Fluorescence extraction

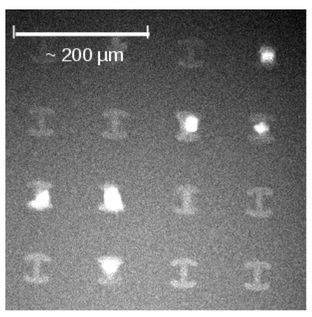
Background corrected data are normalized and aligned to a specific point (maximum of intensity, maximum of degradation, nuclear envelope break down (NEBD), etc.



2. Making things faster

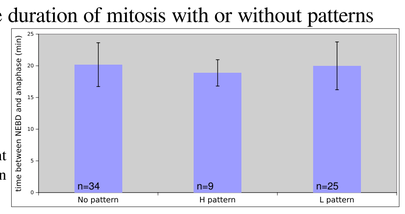
A. Preventing cells from moving

Chips coated with regularly spaced patterns of fibronectin can prevent cells from moving, since they will only attach to the coated surfaces.



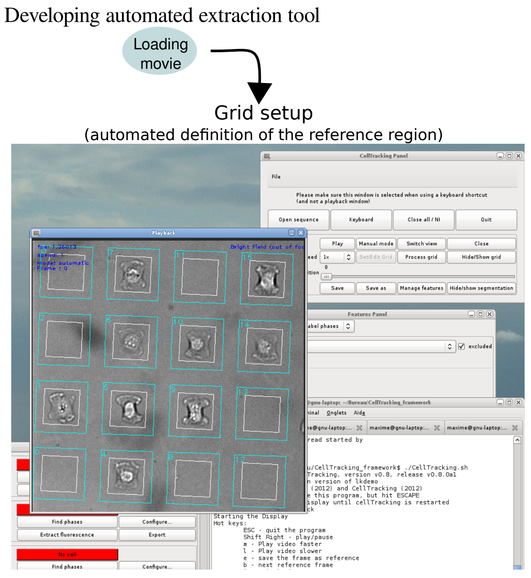
20x microscope visualization of fluorescent fibronectin patterns. Patterns are I-shaped, big bright dots on the patterns are attached cells visualized by a nuclear marker (PCNA).

- Comparing the duration of mitosis with or without patterns



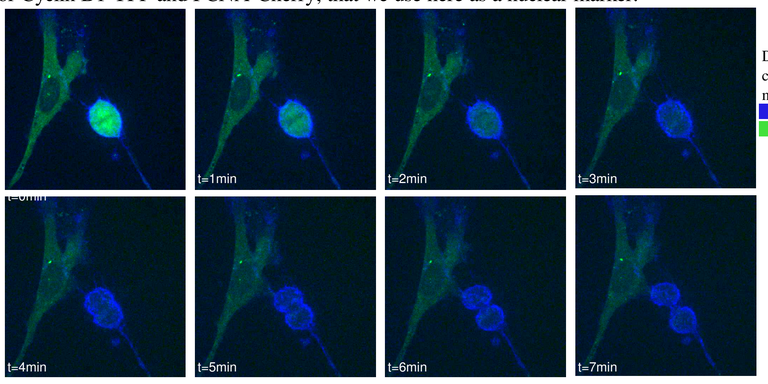
Error bars represent standard deviation

- Developing automated extraction tool

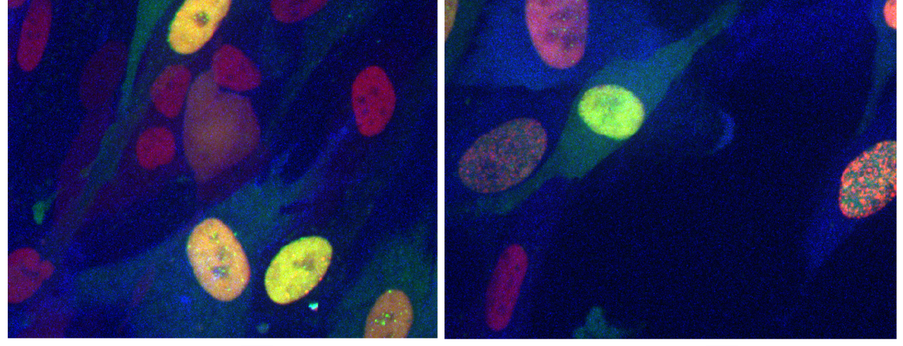


B. Cell lines with fluorescent cytoplasm/membrane allow automated quantification

In order to define cells limits and perform automatic fluorescence extration, we created four differents cell lines that stabely express a freely diffusible blue fluorescent protein (CFP) or a membrane-targeted CFP into the genome of already existing cell lines expressing either Cyclin-A2-YFP or Cyclin-B1-YFP and PCNA-Cherry, that we use here as a nuclear marker.



Degradation of cyclin B1 during mitosis
Plasmid membrane
Cyclin B1



CyclinA2-PCNA-CeruleanCAAX
Plasmid membrane
Nuclear marker (PCNA)
Cyclin A2

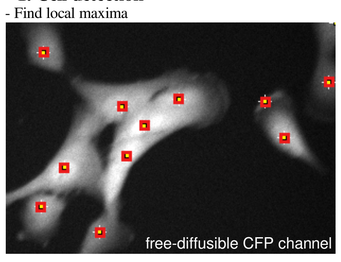
CyclinA2-PCNA-Cerulean
Cytoplasm
Nuclear marker (PCNA)
Cyclin A2

a. Easier segmentation and tracking

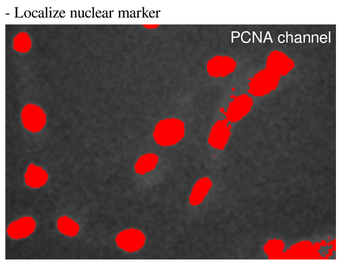
For each step of data analysis, different approaches were undertaken. We present the most efficient ones.

1. Cell detection

- Find local maxima

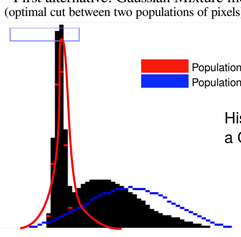


- Localize nuclear marker

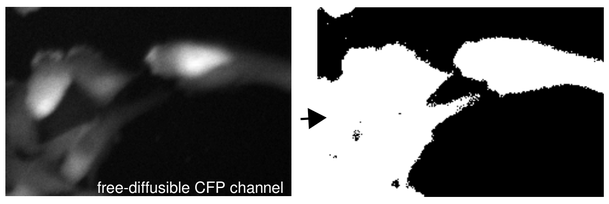


2. Segmentation

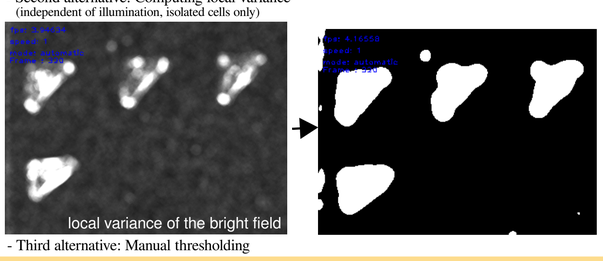
- First alternative: Gaussian Mixture models
(optimal cut between two populations of pixels)



Histogram and its modelization by a Gaussian Mixture Model

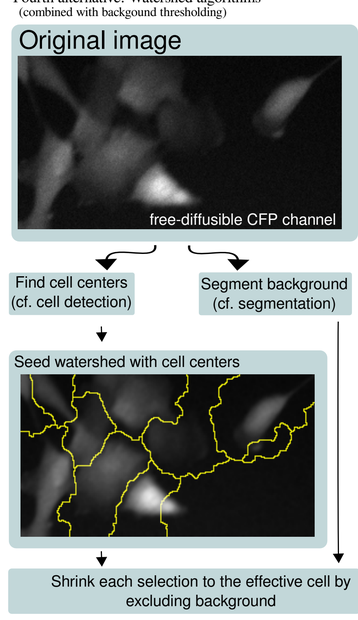


- Second alternative: Computing local variance
(independent of illumination, isolated cells only)



- Third alternative: Manual thresholding

-Fourth alternative: Watershed algorithms
(combined with backgound thresholding)

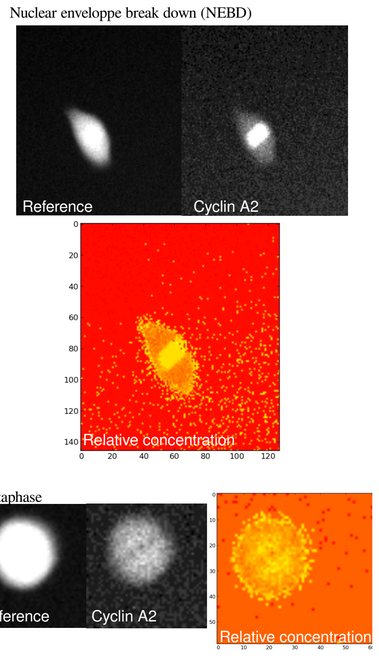


3. Tracking

We proposed in previous work a computationnally efficient algorithm to reconstruct cell tracks in noisy conditions of acquisition and with high particle density.

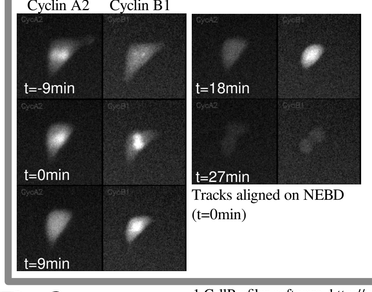
b. A tool for subcellular analysis

Uniform expression of a freely diffusible fluorescent protein can be used as a reference to compute relative concentration of other fluorescent proteins in the cell (e.g: Cyclin A2).



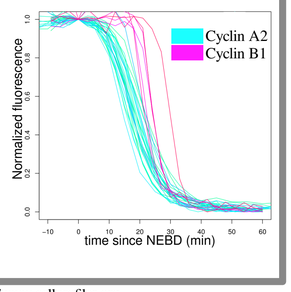
III. Assaying degradation kinetics of different APC substrates

- Sample pattern output



Tracks aligned on NEBD (t=0min)

- Aligned degradation curves



IV. Putting everything together: The CellTracking framework

Load movies
- Load different channels
- Precompute heavy filters
- ImageJ interface

Manually define polygons around cells
- Interpolated polygon between moves

Set grid on pattern

Automatic analysis*
- Cell finding
- Cell segmentation
- Cell Tracking

Tag cell phases
(prophase, anaphase...)
- Manual interface
- Automatic tagging*

Automatic background subtraction

Export
- aligned movies
- fluorescence

Save

The CellTracking framework is a free/open source software written in Python and released under the GPL license. It only uses free/open source libraries.

* : Feature not implemented yet

Conclusions and perspectives

Using the APC-dependent proteolysis as an example, we:

- ***set-up an analysis procedure allowing automated extraction of fluorescence from cells attached on regularly spaced patterns,***
- ***experimented with different cell detection and segmentation algorithms, and implemented some of them,***
- ***designed an extensible framework allowing partially automated processing under user control.***

→*We now expect to improve the automation provided by the software and the overall accuracy.*
→*Increased efficiency in data extraction would allow to collect easily representative samples to elaborate statistical models of mitosis.*
→*The freely diffusible marker can be used to assay local inequalities in protein concentration and degradation.*

References

1.CellProfiler software, http://www.cellprofiler.org
2. *CellCognition: time-resolved phenotype annotation in high-throughput live cell imaging*, Held M. *et al.*, Nature Methods 7, 747–754 (2010)
3. TimeLapseAnalyzer: multi-target analysis for live-cell imaging and time-lapse microscopy, Huth J. et al., Comput Methods Programs Biomed. 2011 Nov;104(2):227-34
4. Schindelin *et al.*, *Fiji: an open-source platform for biological-image analysis*, Nature Methods 9(7):676-682-2012

Acknowledgements

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