Biochemistry and Molecular Biology Education

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Journal:	*Biochemistry and Molecular Biology Education*
Manuscript ID	BAMBED-16-0119
Wiley - Manuscript type:	Article
Date Submitted by the Author:	22-Dec-2016
Complete List of Authors:	Prieto, Daniel; Facultad de Ciencias - Universidad de la República, Sección Biología Celular; Institut Pasteur Montevideo, Aparicio, Gonzalo; Facultad de Ciencias - Universidad de la República, Sección Biología Celular; Institut Pasteur Montevideo, Sotelo-Silveira, Jose; Instituto de Investigaciones Biologicas Clemente Estable, Departamento de Genomica
Keywords:	medical education, skill development including cognitive skills, laboratory exercises, cellular biology

Cell migration analysis: a low-cost laboratory experiment for cell and developmental biology courses using keratocytes from fish scales.

Daniel Prieto 1, 2, 4*; Gonzalo Aparicio 1,2* ; Jose R. Sotelo-Silveira 1,3*

1. Sección Biología Celular, Facultad de Ciencias, Universidad de la República, Montevideo, Uruguay

2. Institut Pasteur de Montevideo, Montevideo, Uruguay

3. Departamento de Genómica, Instituto de Investigaciones Biologicas Clemente Estable, Montevideo, Uruguay

4. Daniel Prieto's current address is Recombinant Proteins Unit, Institut Pasteur Montevideo, Montevideo, Uruguay

* Corresponding authors

Running title: Cell migration lab exercise using fish keratocytes.

Keywords: Education, developmental biology, cell biology, laboratory exercise, cell culture

Abbreviations:

CBBR: Coomassie brilliant blue R 250; DMEM: Dulbecco's modified Eagle's medium; EGTA: ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; FBS: Fetal bovine serum; FITC: Fluorescein isothyocyanate; PBS: Phosphate-buffered saline; PFK: Primary fish keratocytes; TRITC: Rhodamine isothyocyanate.

Abstract

Cell and developmental processes are complex, and profoundly dependent on spatial relationships that change over time. Innovative educational or teaching strategies are always needed to foster deep comprehension of these processes and their dynamic features. However, laboratory exercises in cell and developmental biology at the undergraduate level frequently lack the time dimension. In this paper, we provide a laboratory exercise focused in cell migration, aiming to stimulate thinking in time and space dimension through a simplification of more complex processes occurring in cell biology or developmental biology. The use of open-source tools for the analysis, as well as the whole package of raw results (available at http://github.com/danielprieto/keratocyte) make it suitable for its implementation in courses with very diverse budgets.

Aiming to facilitate the student transition from science-students to science-practitioners we propose an exercise of scientific thinking, and an evaluation method. This in turn is communicated here to facilitate finding common caveats and weaknesses in the process of producing simple scientific communication describing the results achieved.

Introduction

Cell migration is a major topic in cell and developmental biology and has been intensively studied by many groups and through a plethora of approaches. Its investigation has led to the understanding of the basic mechanisms underlying dynamics in complex processes such as cell path-finding.

Students are naturally motivated by the observation of dynamic processes and the performance of hands-on real experiments. Moreover, the understanding of developmental processes requires thinking on spatial relationships changing over time. Although the relevance of 4-dimensional thinking is evident for processes such as morphogenesis, developmental biology instructors still have the challenging task of circumventing the intrinsic difficulty of learning in four dimensions [1]. However, cost-related issues and repetitions due to highly populated groups often limit instructors efforts cell culture in undergraduate laboratory experiments, neglecting their role in understanding principles instead of only learning them [2].

It is the relevance of this topic, plus the notorious value of a good understanding of the pros and cons of a cell culture system model and the need for low cost alternatives which have moved us to design this practical lab module with a simple, robust procedure without the need of complex cell culture conditions. A system that combines the aforementioned characteristics is the culture of primary fish keratocytes, hereby intended to be a pivot between facts and concepts [3].

Primary cultures of fish epithelial keratocytes (PFK) have been long used as a model system for the analysis of cell migration and has been described to be an ideal model for cell shape determination [4]. Furthermore, they have been described as being among the most rapidly moving eukaryotic cells [5], making them suitable for teaching lab sessions.

The aim of this work is to provide a laboratory exercise for cell and developmental biology courses that integrates cell migration dynamics, combining basic cell culture methods, videomicroscopy, drug-treatments (loss-of-function approach), and finally an introduction to image

analysis with ImageJ. We consider of paramount importance, especially for low budget teaching environments to minimize expenses. With that in mind we supply all the raw data and and lab handouts, allowing even an data analysis-only mode. Thus, we have designed a multi-level proposal ranging from the simplest data analysis form to the more elaborate complete lab protocol. Finally, an exercise of scientific thinking [6], a written communication in the scientific report format to evaluate overall performance, and an evaluation method are proposed.

Experimental Procedures

Part 1: *Establishment of a primary explant culture from a fish scale using the simplified sandwich method.*

Material preparation (for 1 culture)

The sandwich method is a simplification of Kolega's keratocyte culture method [7] consisting of a pair of coverslips (24x24 mm recommended) dry-heat sterilized or alternatively submerged in 95% ethanol and flambéed. A glass ring for placing the coverslips sterilized with the same procedure as described above is needed. Disposable Petri dishes or alternatively dry-heat sterilized glass Petri dishes. Dulbecco's PBS (autoclaved or 0.22 μm filter-sterilized). Culture media (F15, DMEM or any standard minimal medium), consider 100 μL per culture supplemented with 10% FBS (if available) with antibiotics. We suggest a mixture of penicillin 100 U/mL and Streptomycin 100 μg/mL.

Clove oil (as anesthetic) which can be purchased at any spice- or drug store at a negligible price, 70% ethanol for surface cleaning, Filter paper pieces (2cm x 2cm), dry-heat sterilized.

For the substrate dependence analysis, coverslips were coated by 15 minutes incubation either with 0.1% poly-L-lysine solution (Sigma-Aldrich) or with collagen. Type-I collagen was obtained from rat-tail tendon and lattices were formed as previously described [8]. Briefly, cleanly dissected tendons were immersed in 0.5% acetic acid with gentle agitation during 30 min and

centrifuged for 30 min at 400g to pellet cellular debris, and the supernatant stored at 4 °C. Polymerization was carried out onto the coverslip by adding 1/5 vol 5X PBS and incubating at 37 °C 15 min.

Method

Anesthetize a goldfish (*Carassius auratus*) by adding 100 ppm clove oil on fish tank water [9], if compatible with local animal care regulations; MS-222 can be considered as an established alternative. With the aid of a forceps (Dumont #4 or 5 recommended) remove a scale while keeping the fish humid taking special care with the gills. Note that up to 16 scales can be removed from the same fish as originally reported by Kolega [7] without compromising fish health. Wash the scale briefly by dipping it into PBS. Place the scale on the coverslip and cover the scale with the second coverslip taking care not to overlap the coverslips perfectly (Fig. 1), add 100 µL of supplemented culture medium with antibiotics and place lid over the Petri dish. Once the medium is added, coverslips will slide over each other due to surface tension making a perfect sandwich. Place the culture into a humid chamber (plastic Tupperware with a piece of wet cotton inside) and incubate overnight at room temperature. Put the fish back into fresh fish water with a few droplets of 2% methylene blue stock to prevent fungal infections, antimicrobial effects of the remnant Eugenio will also prevent infections. After 8 hrs the scale is removed from the culture to promote cell disaggregation, as high density cultures promote collective migration and the formation of clusters [10].

Part 2: *Imaging*

A good alternative to a culture-room inverted phase-contrast microscope is to place a phase contrast condenser and its corresponding 20X objective into a direct classroom microscope, which will do a quite acceptable phase contrast microscope, giving the opportunity to try a phase contrast

device at a very low cost. Moreover, the use of a direct microscope will allow the use of a homemade drug treatment chamber as described below.

A low-budget microscope digital camera was used. A trinocular microscope is not needed as affordable ocular-fitting cameras are available. For observation, the sandwich is removed from the Petri dish and placed over a microscope slide. Before microscopic observation, treating the cells with 85% PBS containing 2.5mM EGTA for approximately 5 min is suggested in order to obtain individually crawling cells [11]. The suggested procedure is to take images every 30 seconds.

Take pictures of a stage micrometer at each magnification which will be used later on for calibration purposes. The extraction of important parameters such as cell size, shape, migration velocity and directionality is achieved by using the ImageJ scale bar functions and the plugins *Manual Tracking* and *Chemotaxis and Cell Migration Tool*. Handouts are provided as Supplemental Materials.

Part 3: *Cytochalasin D treatment* (optional)

Minimal culture media (F15, DMEM or any standard minimal medium supplemented with 10% FBS (if available), with antibiotics and 4 μM Cytochalasin D.

Method

Remove culture medium by capillarity through approximation of a filter paper to the sides of the sandwich. Replace with cytochalasin D supplemented medium and incubate 10 – 20 min at room temperature and remove this medium with the procedure described above. For recovery, replace with normal culture medium and incubate 2 h at room temperature.

Part 4: *Cytoskeleton staining and fluorescent imaging* (optional)

Staining fixated cultures with Coomassie Brilliant Blue R 250 allows a general approach to cytoskeletal arrangement analysis. For this purpose, a modification of the protocols by Pena and

Mochizuki [12,13] is suggested. Briefly, cells are fixed 3% buffered glutaraldehyde for 10 min, and washed away with three washes with distilled water. Staining is performed by covering the coverslips with a droplet of staining solution (0.2% CBBR in 47.5% ethanol, 7% acetic acid) for 10 min. Wash by immersion in water 3 times for 5 min and air dry. Mount with synthetic Canada balsam.

For fluorescence microscopy analysis cells are fixed by immersion in 4% buffered formaldehyde (in PBS) for 15 min, and washed 3 times with PBS. F-actin and nucleic acids are stained by incubating with 0.05 µg/mL rhodamine-conjugated phalloidin and 1 µg/mL Hoechst 33342, 4 µg/mL methyl green [14] for 15 min. The lipophilic dye DiO C18 (5µM) can be used as a counterstain. Cells are washed 3 times in PBS for 5 min each. Coverslips are mounted on a slide with 50% glycerol in PBS and sealed with nail polish. Cells are imaged with a standard epifluorescence microscope with Hoechst and TRITC filters. A FITC cube is also required if DiO staining is performed, and a Cy5 filter set for methyl green.

Student cohort

A total of 11 students throughout 2 years (two independent assays) were evaluated retrospectively. All reports from final year undergraduate students from a one-semester developmental biology course were analyzed as described below.

Performance evaluation

Student performances summarizing the lab session were quantitatively evaluated with a scale elaborated on the basis of highlights on writing a scientific paper [15]. Briefly, five points were assigned to the introduction section; one point was assigned to each point that should be included in the introduction (background information, justification, objectives of the work, guidance to the reader, and a summary of the work). Two points were assigned to materials and methods

section, one for completeness of the information (experimental procedures should be reproducible for anyone who reads the paper); and another for the correspondence with the order of the experiments shown in the results section. Six points were assigned to results and discussion on the basis of ease of readability interpreted as self-descriptive results (headings should be as compact and information-rich as possible, 1 point); rational organization of the scheme (an ordered story leading from the more general to the more particular results, 2 points); figures and tables correctly formatted and with adequate self-descriptive captions (3 points). One extra point was added where an appropriate conclusion was put forth. Finally we decided to stress the importance of citation of previous work by assigning 5 points to references section discriminated as 2 points for the introduction where background experimental data should be cited (0-1 point for a poor bibliographic review, 2 for a complete one); 1 point for citations under the methods section, and 2 extra points for citations made within the discussion. Incorrect citations were penalized with 2 points. A second-chance policy is suggested when two or more items within a section scored 0, thus being able to give more support to the students

Results and Discussion

Parts 1 and 2: *Establishment of a primary explant culture from a fish scale using the simplified sandwich method and imaging*

The modified sandwich culture of fish scale keratocytes allows a low-cost setup for teaching cell migration analysis through a hands-on approach (Fig. 1). Keratocyte cultures are checked at 16-24 hours of establishment. Imaging through a phase-contrast microscope allowed the following of cell shape changes and migration (Fig. 2 and supplemental video 1). The establishment of a simple cell culture is an opportunity to discuss cell culture as a model system itself. Aseptic technique can be introduced; biosafety levels, laminar flow hood types and their uses may also be discussed. Moreover, this practical exercise is an opportunity to show how a non-invasively established primary cell culture system can be used for drug-based studies under the light of the 3R's principle [16], being this a replacement choice.

Cell migration analysis of imaged cells

Migration parameters were extracted by tracking individual cell paths. Apparently unbiased paths (Fig. 2A), reveal to have a directional preference towards opposite of the scale (origin) when represented on a migration plot normalized to a common origin of migration (Fig. 2B).

This part of the procedure is an opportunity for discussing phase contrast principles, and the use of optical contrast tools for *in vivo* imaging. A first remarkable point is the importance of the *in abstracto* analysis of data by comparing the information that can be obtained through the analysis of the individual tracks. A second relevant issue is the importance of scale bars when communicating scientific data. Average migration speed and total euclidean migrated distances were determined

(Table 1), and can be contrasted with previously described data. At this point, the paper by Csucs and collaborators may be discussed [17].

Substrate-dependence analysis

Cultured PFK migration parameters as described in parts 1 and 2 were extracted from cells cultures over different substrates, namely glass (as in part 1), collagen and ploy-L-lysine. Increases in migrated distances as well as in velocities were observed when comparing glass-only substrate to glass coated with collagen or the synthetic matrix poly-L-lysine (Fig. 3A).

Part 3: *Cytochalasin D treatment* (optional)

All migration activity was abolished immediately upon cytochalasin treatment (Fig. 3B). Moreover, cell shape was disrupted as the typical crescent shape turns into a spherical one, depicting loss of adhesion to the substrate as observed in supplemental video 2. If performed, this experiment allows a more refined dissection of the underlying mechanisms of cell movement and migration through a loss-of-function approach. Valuable discussions may arise on experimental design, the need for control experiments and function recovery. Moreover, it can trigger a discussion on several different drugs affecting different cytoskeletal elements and their relevance in disease treatments. After observation and imaging, fixation of the cells is suggested for staining and further analysis of the cytoskeletal mechanisms underlying these phenomena.

Part 4: *Cytoskeleton staining and fluorescent imaging*

Further analysis of the involvement of the cytoskeleton on cell shape and migration is ideally performed at 16-24 h as cultured goldfish keratocytes display notorious lamellipodia (Fig. 3B, upper panels). Coomassie staining of cellular proteins exhibit a great number of filamentous protein bundles in migrating PFKs (Fig. 3B, lower panels). This staining however, does not allow

any discrimination of which cytoskeletal element could be involved. Rhodamine-labeled phalloidin reveal an organized pattern of filamentous actin (Fig. 4).

Optional part 4 provides a different insight into the sub-cellular mechanisms of shape establishment and cell movement. While the preceding experiment provides a functional approach, this part involves descriptive cytoskeletal data. The analysis of CBBR stained keratocytes initiates the discussion on cytoskeletal filaments and their involvement in cell shape and migration. As this technique does not discriminate among cytoskeletal elements, it can be taken as a chance for discussing alternative experimental designs to clarify this issue. The second part, with fluorescent labeling of filamentous actin becomes a chance for discussing fluorescence microscopy and target-specific cytochemical detection techniques. An ideal scenario would allow the students to perform all four parts in order to provide convergent data. Whether this part is chosen to be done, we suggest performing the staining in untreated and cytochalasin D-treated cultures for assessing the effect of the drug on microfilaments. From the comparison it follows that cell shape is disrupted (Fig. 3B, upper panels), which relies on cytoskeletal organization as shown by CBBR staining (Fig. 3B, lower panels), and that filamentous actin is severely affected upon cytochalasin treatment (Fig. 4), and that the disruption of actin cytoskeleton abolishes PFK migration.

Student performance

Students were encouraged to extract as many information as possible from these series of experiments, which allowed them to being creative when presenting the results in a professional scientific paper format. This approach requires that the students systematize information with an

extra effort in reflection also in reviewing the literature [18], and provides a training instance in metareasoning [6] by considering and combining information to solve a new problem. The analysis of dynamic data, the determination of eventual directionality of cell migration, and the measurement of migration speed has shown in our experience, importance in developing analytical skills. To sum up, an integrative interpretation of real data with a model [19] is proposed with a discussion of the model presented by Fuhs and collaborators [20]. Therein, three main forces contributing to keratocyte motility are proposed; myosin as a molecular motor, actin polymerization at the leading edge, and actin depolymerization.

In our experience, four major pitfalls were identified at the evaluation, being the absence of a summary of the work in the introduction section; incomplete materials and methods section; the absence of adequate conclusions; and an poor discussion lacking references. A remarkable point was the absence of referenced literature within the whole report, albeit not frequently occurred.

Despite being exposed to scientific literature through seminars for paper discussion, the major weaknesses we have identified suggest that focusing in understanding the results of the articles, the students remarkably overlooked the structure of scientific literature. These unexpected results indicate a more detailed analysis on the construction of the discussed papers may be necessary, thus strengthening the students fine understanding of the current structure of scientific reporting.

The major strength we see from this proposal is that every step is committed to student formation, even the evaluation.

In conclusion, PFK are a robust, and low-budget alternative for cell biology instruction using a cell-culture model. ImageJ is an open-source tool that has a great potential for implementation in classrooms as it provides a cross-platform software suitable for image processing and analysis and figure preparation at professional level.

The evaluation scale proposed herein allowed identification of weaknesses in results reporting in our student cohort, and could be extended to other lab reports for advanced students.

A systematic analysis of the structure and building of scientific reports should be included in the discussion seminars in order to strengthen student performance, at the time of reporting their own results.

Acknowledgements

The authors would like to thank the students for the great time shared.

Disclosures

The authors declare no conflict of interest.

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TABLES

Table 1. Migration parameters of PFK on different substrates.

Track Number	Accumulated distance [µm]	Euclidean distance [µm]	Velocity [µm/sec]	substrate
2	14,98	13,33	0,06	glass
3	6,61	1,80	0,03	glass
4	9,06	6,93	0,04	glass
5	30,82	7,12	0,09	glass
7	26,43	24,80	0,11	glass
8	33,56	32,19	0,14	glass
1	8,14	3,26	0,03	poly-L-lysine
2	27,34	15,53	0,11	poly-L-lysine
3	30,23	24,27	0,14	poly-L-lysine
4	20,05	18,31	0,10	poly-L-lysine
5	50,29	41,72	0,21	poly-L-lysine
6	46,98	37,39	0,20	poly-L-lysine
1	37,37	37,14	0,10	collagen
2	24,82	19,51	0,10	collagen
3	29,36	27,67	0,16	collagen
4	36,44	31,62	0,20	collagen
5	33,15	31,99	0,18	collagen
6	31,88	21,45	0,12	collagen
7	41,28	38,78	0,15	collagen
8	27,67	26,57	0,10	collagen
9	38,21	30,20	0,14	collagen
10	28,22	20,69	0,10	collagen
11	22,88	18,70	0,08	collagen

LEGENDS

Figure 1. Basic Setup. (A) A coverglass is put over a glass ring on a Petri dish, and fish scale is then put on the coverglass. (B) A second coverglass is put on top slightly displaced to allow the adding of culture medium.

Figure 2. Cultured primary fish keratocytes migration and cell shape changes over time analyzed through a set of pictures taken every 30 seconds allows analysis and discussion. (A) Overlayed migration paths for comparative purposes. The path covered by the cell in the field is depicted in cyan (on the online version). (B) A series of tracks are plotted with a normalized origin to analyze preferential pathways as in chemotaxis assays. Bar 20 microns.

Figure 3. Migration and cytoskeleton. (A) Box-plots of migrated euclidean distances in microns (left panel), and migration velocities in microns per second (right panel). (B) Cytoskeletal proteins evidenced through a simple protein staining with Coomassie Blue which reveals the presence of protein bundles (untreated), and the disruption of their organization upon cytochalasin-D treatment (CytD). Bar: 30 microns (upper panels); 10 microns (lower panels).

Figure 4. Analysis of cytoskeletal on keratocyte shape and migration by F-Actin staining with phalloidin (upper panel, red on the online version), counterstained with the lipophilic DiO C18 (middle panel, green on the online version) and Hoechst 33342 for DNA (lower panel, cyan on the online version). Bar: 20 microns.

Supplemental video 1. PFKs migration in culture.

Supplemental video 2. Disruption of shape and migration upon addition of cytochalasin-D to cultured PFKs.

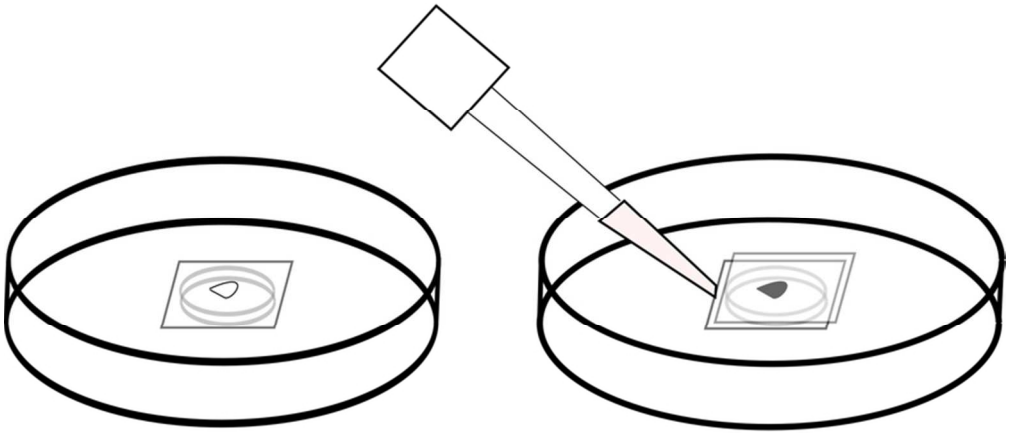

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Fig. 1
72x31mm (300 x 300 DPI)

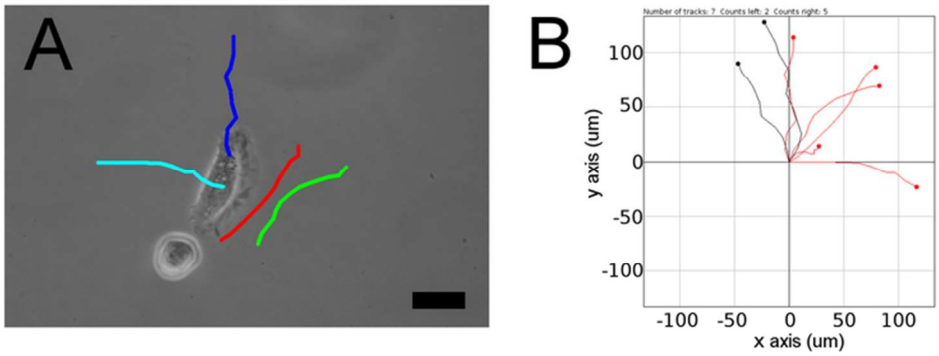

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Fig. 2

66x24mm (300 x 300 DPI)

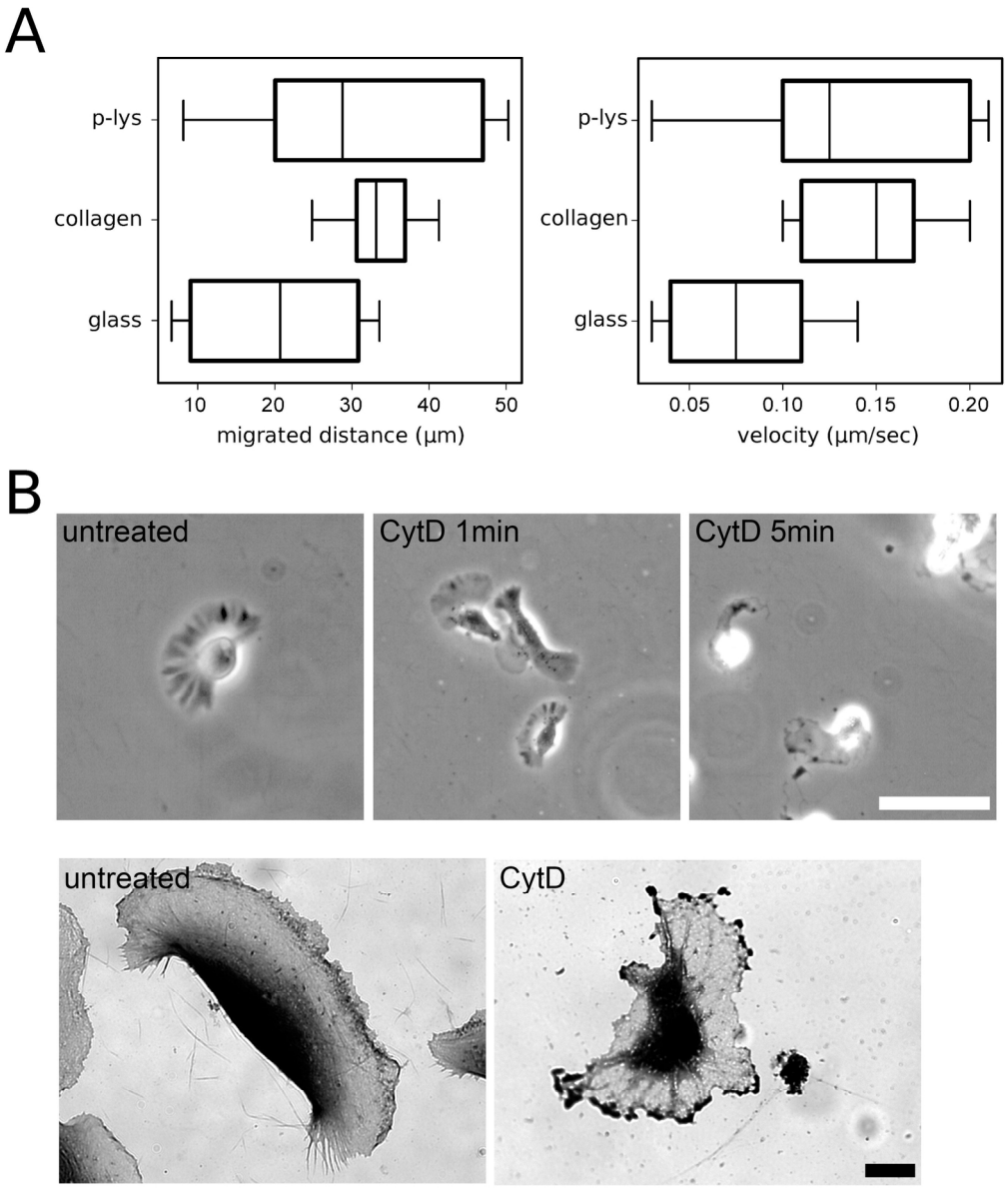

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Fig. 3
166x196mm (300 x 300 DPI)

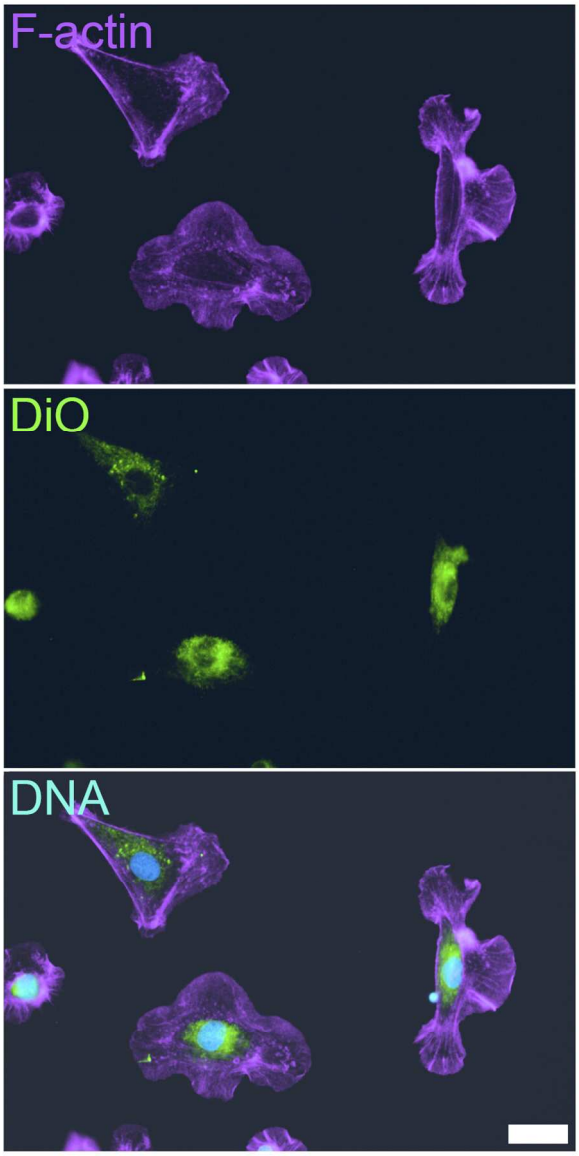

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Fig. 4
84x160mm (300 x 300 DPI)