SOFTWARE TOOL ARTICLE

Quantification of cancer cell migration with an integrated experimental-computational pipeline [version 1; peer review: 2 approved with reservations]

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Abstract

We describe an integrated experimental-computational pipeline for quantifying cell migration *in vitro*. This pipeline is robust to image noise, open source, and user friendly. The experimental component uses the Oris cell migration assay (Platypus Technologies) to create migration regions. The computational component of the pipeline creates masks in Matlab (MathWorks) to cell-covered regions, uses a genetic algorithm to automatically select the migration region, and outputs a metric to quantify cell migration. In this work we demonstrate the utility of our pipeline by quantifying the effects of a drug (Taxol) and of the extracellular Anterior Gradient 2 (eAGR2) protein on the migration of MDA-MB-231 cells (a breast cancer cell line). In particular, we show that inhibiting eAGR2 reduces migration of MDA-MB-231 cells.

Keywords

Migration, Quantification, User-Friendly, Microscopy.

Open Peer Review

Approval Status ? ?

	1	2
version 1		
15 Aug 2018	view	view

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2. **Assaf Zaritsky** , Ben-Gurion University of the Negev, Be'er Sheva, Israel

Any reports and responses or comments on the article can be found at the end of the article.

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Competing interests: No competing interests were disclosed.

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Introduction

In order to understand and treat cancer, we need to study and ultimately control metastasis¹. A key aspect of metastasis is cell migration¹. Thus, assays that can reliably provide quantitative readouts of cell migration are an important component of cancer research. Here we describe an integrated computational pipeline to quantify cell migration using fluorescent microscopy.

The anterior gradient protein 2 (AGR2) has been shown to promote cell migration^{2,3}. High expression of AGR2 is correlated with aggressive forms of various adenocarcinomas including prostate³ and breast cancer^{4,5}. Therefore, AGR2 is a biomarker and therapeutic target that will provide a suitable biological readout of cell migration for our pipeline to quantify. In other words, because AGR2 is known to promote cell migration, it is ideal for testing a computational platform's ability to detect changes in cell migration.

In this work, we describe an experimental and computational pipeline to quantitate cell migration. We demonstrate this pipeline by quantifying migration of MDA-MB-231 cells, a breast cancer cell line known to migrate aggressively⁶. We show that blocking the extracellular AGR2 (eAGR2) with a neutralizing antibody that binds specifically to AGR2 (referred to as AGR2-Ab) in cell medium prevents the migration of the MDA-MB-231 cells³. Our pipeline aids in the verification of well-established hypotheses and it can be used to test new hypotheses, thus aiding in and accelerating the drug discovery process.

Methods

Cell culture

MDA-MB-231 cell line was obtained from the American Type Culture Collection (ATCC, No. HTB-26) and tested for mycoplasma contamination before usage. Cells were maintained in DMEM media (Thermo Fisher, No. 21063045), supplemented with 10% fetal bovine serum (FBS), they were kept at 37°C in a humidified incubator with 5% CO₂. Cells were used within 6 months of purchase.

Cell migration assay

The Oris™ migration assay (No. CMA1.101, Platypus Technologies, Madison, WI, USA) uses a physical barrier “stopper” to create a defined circular region that is intended to prevent cell adhesion at the start of the assay. This central cell-free detection zone is in the center of each well of a 96-well plate. As the cells migrate to the cell-free zone over 24–48 hours, real-time assessment of migratory cells allows acquisition of richer data sets. Since there are no artificial membranes or inserts in the light path through which cells must pass, this assay is amenable to quantification with microscopy. We used the Oris cell migration assay from Platypus Technologies to create migration regions by inserting stoppers in each of the 96 wells on a plate. Shortly after inserting the stoppers, we seeded MDA-MB-231 cells and waited until they reached 80% confluent (approximately 24 hours). We then fed cells with either treated or untreated media. Treated media included Taxol (5 nM), mouse anti-AGR2 antibody (1:50 of 2 µg/ml) (Santa Cruz Biotechnology, No. sc-101211), and mouse-IgG (Santa

Cruz Biotechnology, No. sc-2005), while the untreated media was Dimethyl sulfoxide (DMSO) (VWR, No. 97061-250). Next we removed the stoppers and allowed the cells to move into the migration region. 48 hours after removal of the stoppers, we imaged each well using a fluorescence microscope (Zeiss Observer.Z1 microscope at 2.5x objective with AxioCam MRm camera and Axio Vision 4.8 software).

Implementation

This tool reads microscopy images which are placed in the same folder as the main code. First, the script first trains using the negative control (i.e., the image of a well where the stopper was not removed) to find the optimal disk which represents the migration region. Next, the script finds all the images present in the same folder as the code (by default it looks for TIF files) and applies the migration quantification metric to each of them, recording its output in a text file called `output_c3.txt` for easy access and plotting (a Python 3 script which creates a publication-quality plot based on this output is provided as well for any user who wishes to use it).

Operation

Typically a user only needs to make one modification the main script called `code.m`, make one minor modification to the `img_name` variable (on the third line of the script), and run the script. The change to the `img_name` variable needs to reflect the name of the image which contains the negative control.

If the user desires change the format of the images the script uses for the quantification, the line `file_list= dir('* .tif')`; needs to change to reflect the desired format. The images need to be in the same directory as the script.

This tool has been tested in multiple laptops running Matlab spanning releases 2015a–2018a.

Automatic selection of migration region

We first created a mask corresponding to the area covered by cells using standard deviation filtering and applying a series of morphological operations in Matlab R2016a (MathWorks) as shown in Figure 1.

Note that these images are gray scale (green is used throughout to highlight software outputs as is shown in the right panel of Figure 1), hence every pixel's value belongs to the interval [0,1] where a completely black pixel has value 0 and a completely white pixel has value 1. Also note that a mask is a binary matrix that indicate which pixels belong to the mask (with value of 1, these pixels are referred to as “cell pixels”) and which pixels do not belong to the mask (with value 0).

We then used a genetic algorithm to determine the coordinates of the center and the radius of a circle according to Equation (1). This optimal circle determines the migration region.

$$\left[c_x^*, c_y^*, r^* \right] = \underset{c_i, c_j, r}{\operatorname{argmax}} \sum_{m_{i,j} \in M} m_{i,j} - p \left(\#M - \sum_{m_{i,j} \in M} m_{i,j} \right), \quad (1)$$

Figure 1. Sample images immediately after the stopper was removed. In order to identify the migration region, we took images of each well (left), then we select a mask that covers the area utilized by cells, highlighted in green (right).

where c_x^* , c_y^* , and r^* are the optimal parameters of the migration region, M is the Matlab mask we are evaluating (i.e., a circle with center at coordinates (c_x, c_y) and radius r), so $\sum_{m_{i,j} \in M} m_{i,j}$ is the sum of all the pixel intensities ($m_{i,j}$) which belong to the mask M , $\#M$ is the cardinality of M (i.e., the number of pixels which belong to M), and p is a penalty parameter. If $p = 1$, we have:

$$\left[c_x^*, c_y^*, r^* \right] = \underset{M}{\operatorname{argmin}} \#M - 2 \sum_{m_{i,j} \in M} m_{i,j} \quad (2)$$

Hence, the maximization problem from Equation (1) is equivalent to the minimization represented in Equation (2) (when $p = 1$). From Equation (2), we can interpret the optimization performed by the genetic algorithm as finding “the largest circle which contains the least number of cell pixels.” Figure 2 shows the optimal circular region selected by the genetic algorithm when the input is the image from Figure 1.

Percent of migration region covered by cells: We defined a metric to quantify the migration of MDA-MB-231 cells. This metric is Q , the percentage of migration pixels inside the migration region. We define a migration pixel as any pixel whose intensity value is greater than or equal to a threshold T . We chose T equal to 1.25 times the median pixel intensity of the migration region immediately after the stopper was removed (i.e., the green region in Figure 2). This is:

$$Q = \left(\sum_{i \in \{M^* \geq T\}} i \right) / \#M^*, \quad (3)$$

Where M^* is the optimal circle defined by the three parameters $\left[c_x^*, c_y^*, r^* \right]$ from Equation (1) and Equation (2) and the set $\{M^* \geq T\}$ includes all of the pixels inside M^* with intensities higher than T .

Preprint

A previous version of this manuscript is available from bioRxiv: <https://doi.org/10.1101/130526>

Results and discussion

To test the hypothesis that MDA-MB-231 cells' migration is reduced in the absence of AGR2, we designed an experiment (utilizing the cell migration assay described in the Methods section) with 5 experimental conditions: a positive control (untreated cells), a negative control (wells where the stopper was not removed), cells treated with 10nM of Taxol (a non-cytotoxic dose level which prevents cell migration but does not promote cell death⁸), cells treated with a 1:50 dilution of AGR2-ab to inactivate eAGR2, and with a 1:50 dilution of IgG, a control antibody (Ctrl-Ab) which does not affect cell migration. Representative images from these conditions (i.e., replicate 1) are shown in Figure 3 (top).

For the untreated case and the control peptide we observe increased migration, with 46 ± 2 (mean $\pm$ standard error of the mean) percent of the migration region covered in the untreated case and 42 ± 1.7 percent of the migration region covered in the control antibody case. We fail to reject the null hypothesis that these two are the sample means from the same distribution (p value of 0.084). In the Taxol case, 21 ± 1.9 percent of the migration region is covered. We reject the null hypothesis that the mean of the Taxol population and the mean of the untreated case are sample means from the same distribution (p value of 8.16×10^{-5}). Similarly, for the AGR2-Ab case, 13 ± 3.2 percent of the migration region is covered. We reject the null hypothesis that the mean of the AGR2-Ab population and the mean of the untreated case are sample means from the same distribution (p value of 2.19×10^{-5}). Not only do we confirm the hypothesis that MDA-MB-231 cells' migration is reduced in the absence of AGR2, but our method allows for reproducible quantification of these qualitative observations. Furthermore, the algorithms used to compute Q requires a single input from the user (a string with the names of the control experiments) and it can run on a desktop machine with Matlab (R2015a-2018a and above) installed.

Conclusions and future work

We have designed and implemented a pipeline for quantifying cell migration *in vitro*. It is worth noting that this metric

Figure 2. Automatically-selected migration region. The genetic algorithm selects the largest circle which contains the least number of masked pixels from the cell area mask. This optimal circle is the migration region.

Figure 3. Quantifying cell migration. Representative images (replicate 1) of each condition are shown (top). 10nM of Taxol and the 10µg/mL of the H10 peptide show similar levels of migration inhibition compared to the positive and negative controls. Our metric (bottom) allows us to quantify the qualitative results (top).

may not discern between cell motility and proliferation, hence in order to use it to estimate parameters for a mechanistic model, a parameter estimator such as CellPD⁹ should be used to estimate parameters of processes which decouple proliferation and motility^{10,11}. However this metric is robust to image noise,

open source, replicable, replicable and user friendly. In particular, we show that H10 aids in the reduction of migration of MDA-MB-231 cells by blocking sAGR2. This pipeline can be expanded to various cancer cell lines and model systems.

Data availability

Raw, unedited microscope images from this analysis are available from the project GitHub repository: <https://github.com/edjuaro/cell-migration-quantification>

Software availability

The source code used throughout this manuscript can be accessed in the public GitHub repository: <https://github.com/edjuaro/cell-migration-quantification>.

Archived source code at time of publication can be found here: <http://doi.org/10.5281/zenodo.1323923>¹²

This code is released under the permissive [MIT license](#).

Competing interests

No competing interests were disclosed.

Grant information

This work was supported by the USC Center for Applied Molecular Medicine, the National Institutes of Health (Physical Sciences Oncology Center [5U54CA143907] for Multi-scale Complex Systems Transdisciplinary Analysis of Response to Therapy (MCSTART), and [1R01CA180149]), the Breast Cancer Research Foundation, the USC James H. Zumberge Research and Innovation Fund, and a USC Provost's PhD fellowship.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Open Peer Review

Current Peer Review Status: ? ?

Version 1

Reviewer Report 18 September 2018

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Assaf Zaritsky

Department of Software and Information Systems Engineering, Ben-Gurion University of the Negev, Be'er Sheva, Israel

In their Software Tool Article "Quantification of cancer cell migration with an integrated experimental-computational pipeline", Juarez et al present a software to quantify circular monolayer migration assay. The assay itself is quite basic and widely used, although mostly not with circular geometry, which is used as an assumption by the segmentation algorithm. Multiple algorithms and tools exist for this task and the authors should compare their methods to alternatives. Quite a few details are missing making the algorithm very hard to read and interpret.

Specific major concerns / revisions:

- The authors missed to cite a significant body of work methods for quantification of monolayer cell migration. Also, the experimental assay should be put into context – there are many alternatives assays to perform monolayer migration, Oris is just one example.
- As a "Software Tool" article, I am expecting the authors to benchmark using a ground truth annotated dataset and compare its performance to at least one of the existing alternatives. Here is one easy-to-use optional method, Gebäck et al¹ (that does not use the information about the circular geometry).
- The authors missed to discuss the limitations of their method. Specifically, the assumption that the pattern is circular does not fit most experimental settings.
- Missing technical details:
 - The description of the algorithm is not clear to me. Is there a first stage of segmentation (thresholding?). Second stage of using the binary mask as an input to optimize a fit to a circle as the initial geometry (is it the negative control?) using a genetic algorithm (GA). Third stage where segmentation is performed by simple thresholding and Q is calculated in relation to the initial circle as a measure to migration? This is very hard to interpret from the text. A major revision is essential.
 - No details are provided for the GA. Many (most?) of the readers will not even know what is a GA. Moreover, there are practically no implementation details provided in the text.
- I am not certain from the text whether m_{ij} values are binary (0/1) or the actual pixel

intensity. In the latter case, the assumptions used to define the function to optimize with the GA do not necessarily hold as they are heavily dependent on the values and variability of the pixel's intensity in the background and foreground. The sentence "we can interpret the optimization performed by the genetic algorithm as finding "the largest circle which contains the least number of cell pixels." is not necessarily true when using the raw pixels intensities and has to at least be discussed - how was this equation derived? what were the assumptions? how to set the value of the parameter p ? I would like to see the complete algorithm and how the equation was derived in any case.

- What statistical tests were performed? Why do we see only 4 replication per experimental condition when the experiments were performed in a 96-well plate.

Other revisions and suggestions:

- "A key aspect of metastasis is cell migration¹" - this association of cell migration and metastasis is not established, definitely not through a 2D monolayer migration assay. Also, bone cancer (ref #1) is very different than the model used for this study (breast cancer cell line). Monolayer cell migration assays are quite prevalent and a more relevant argument as motivation can be articulated. Overall, I suggest to tune-down the relevance to cancer throughout this article.
- I would suggest to switch the order of the 2nd and 3rd paragraphs in the introduction. The focus of this article is the software tool. The experimental perturbation (AGR2) can come later (or better, not at all in the introduction).
- In the same paragraph, why mention solely the AGR2 perturbation and ignore the Taxol-perturbation. The authors should also mention somewhere that Taxol is a drug that inhibit microtubules.
- "Our pipeline aids in the verification of well-established hypotheses" – it is the other way around: the known perturbation that is known to impair migration can be used to validate the method.
- Typos / grammar:
 - "ZeoissObserver.Z1"
 - "First, the script first trains"
 - "If the user desires change the format of the images"
- Missing detail:
 - "and applying a series of morphological operations"
 - "We then used a genetic algorithm to determine the coordinates of the center and the radius of a circle"

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[PubMed Abstract](#) | [Publisher Full Text](#)

Is the rationale for developing the new software tool clearly explained?

Partly

Is the description of the software tool technically sound?

Partly

Are sufficient details of the code, methods and analysis (if applicable) provided to allow replication of the software development and its use by others?

Partly

Is sufficient information provided to allow interpretation of the expected output datasets and any results generated using the tool?

Yes

Are the conclusions about the tool and its performance adequately supported by the findings presented in the article?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Computational cell dynamics Quantitative cell biology Cell migration High throughput phenotyping Computer vision application

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 29 August 2018

<https://doi.org/10.5256/f1000research.17017.r37224>

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Mohammed El-Kebir

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The authors introduce a tool for identifying the migration region in microscopy images from an in vitro experiment for metastasis in cell lines. Mathematically, the problem is to find the largest circle that contains the fewest cells. There are a couple of issues/inconsistencies in this paper that I will outline below.

1. Why do you need to train the tool on a negative control?

Please specify which parameters are being learned? Are you learning the value of parameter p ? Please be specific.

2. More motivation is needed

Is the Oris cell migration assay the only assay where a circular stopper is used? How often is

this assay used? Please describe this so that the reader can understand if your tool is widely applicable.

3. Description of related work is missing

In computational geometry, there is a problem that is called minimum enclosing circle (MEC). Please describe how and if your problem is different. (I'm not convinced it is - as there is a weighted version of MEC.)

4. Tool needs a name

Right now the tool is called code.m. Please give it a name.

5. Notation

Please don't use $\#M$ to denote the cardinality of M -- rather use $|M|$.

Equation (1): don't use c_i, c_j this clashes with $m_{\{i,j\}}$. Use c_x and c_y .

Equation (2): don't use M in $\arg \min$. For consistency with (1) use c_x, c_y, r .

What is the relevance of $p=1$? Is this what you used in your experiments? If so, no need for this parameter in (1).

Is the rationale for developing the new software tool clearly explained?

Yes

Is the description of the software tool technically sound?

Partly

Are sufficient details of the code, methods and analysis (if applicable) provided to allow replication of the software development and its use by others?

No

Is sufficient information provided to allow interpretation of the expected output datasets and any results generated using the tool?

Yes

Are the conclusions about the tool and its performance adequately supported by the findings presented in the article?

Partly

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

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