Novel candidate metastasis genes as putative drug targets for breast cancer

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Chapter 2

Functional Screening with a Live Cell Imaging-based Random Cell Migration Assay

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Abstract

Cell migration, essential in cancer progression, is a complex process comprising a number of spatiotemporally regulated and well coordinated mechanisms. In order to study (random) cell migration in the context of responses to various external cues (such as growth factors) or intrinsic cell signaling, a number of different tools and approaches have been developed. In order to unravel the key pathways and players involved in the regulation of (cancer) cell migration, a systematical mapping of the players/pathways is required. For this purpose we developed a cell migration assay based on automatic high throughput microscopy screening. This approach allows for screening of hundreds of genes, e.g. those encoding various kinases and phosphatases but can also be used for screening of drugs libraries. Moreover, we have developed an automatic analysis pipeline comprising of a) automatic data acquisition (movie) and b) automatic analysis of the acquired movies of the migrating cells. Here we describe various facets of this approach. Since cell migration is essential in progression of cancer metastasis, we describe two examples of experiments performed with highly motile (metastatic) cancer cells.

Key words

Random cell migration, functional genomics, high throughput screening, quantitative image analysis, automation.

1. Introduction

Cell migration is essential for various biological processes, such as embryonic development, immune responses and tissue remodeling as well as in pathologic conditions, such as cancer progression and metastasis [1]. The complexity of the cell migration as a process can be ascribed to the involvement of many regulating genes, signal transduction pathways and numerous external stimuli such as growth factors and chemokines. Moreover, the integration and spatiotemporal coordination of the processes underlying cell migration add to its complexity [2,3,4]. Thus it is a major challenge to develop tools and approaches to study these individual processes that make up cell migration. Additionally, in the context of cancer progression and metastasis, a deeper understanding of crucial factors in the migratory behavior of cells is necessary in order to develop targeted anti-cancer therapies.

We and others have developed various approaches to unravel the key players in the mechanisms underlying cell migration [5]. One of them is the RNAi-based gene silencing high throughput screening approach [6,7,8,9]. The advantage of applying high throughput screening is that multiple genes or conditions can be tested at once, allowing a more systematic approach towards understanding mechanisms underlying cell migration. We present here a method that combines high throughput screening with a live cell imaging-based random cell migration assay. This allows for screening of hundreds of genes, e.g. those encoding various kinases and phosphatases. Moreover, we have developed an automatic analysis pipeline comprising a) automatic data acquisition (movie) and b) automatic analysis of the acquired movies of the migrating cells. This protocol can be used in combination with functional genomics as well as chemical compound screens.

2. Materials

2.1 Cell culture

1. Minimum Essential Medium (MEM) alpha medium (1x), liquid, with L-glutamine, without phenol red (GIBCO/Invitrogen) supplemented with 5% fetal bovine serum (PAA Laboratories GmbH, Pasching, Austria) (see Note 1) for the culture of MTLn3 cells [10].
2. RPMI 1640 medium (1x), liquid, with L-glutamine, without phenol red (GIBCO/Invitrogen) supplemented with 10% fetal bovine serum for the culture of H1299 cells.
3. Imaging plate CG 96 well, glass bottom, surface treated (PAA Laboratories GmbH).
4. Collagen, rat tail, type I (Bioconnect/Upstate) is diluted in PBS without Ca/Mg at 30 μg/mL (for assay with MTLn3).

5. Fibronectin from bovine plasma (Sigma Aldrich) is diluted in PBS without Ca/Mg at 10 μg/mL. Once diluted, use directly (for assay with H1299).
6. Epidermal Growth Factor (EGF, Sigma Aldrich) is dissolved at 100 μg/mL in 10 mM acetic acid containing 0.1% bovine serum albumin (BSA) and aliquots are stored at -80°C (see Note 3)
7. DharmaFECT2 transfection reagent and siGENOME smartpool targetting GFP, PTEN and RAC1 (Dharmacon, Thermo Fisher Scientific) (see Note 4).

2.2 Automated high throughput imaging

1. Nikon Eclipse TI with fluorescent lamp and 20x objective (Pan Apo, dry, NA 0.75, WD 1.0) equipped with an automated stage control and a Perfect Focus System (PFS).
2. Temperature- and CO_2- controlled imaging chamber (custom design).
3. Nis Elements AR version 3.10 SP3 build 634.
4. In Nis Elements implemented Macro: Wellplate2.mac (Nikon).

2.3 Automated quantitative image analysis

1. The conversion and analysis macros are written with Image Pro Plus software (Media Cybernetics, Inc. Bethesda, MD) and are available on request.
2. Spreadsheet software (Excel, Microsoft Corporation).

3. Methods

3.1 Preparing cells for imaging

Cell migration can be investigated in various experimental settings. Here we describe two different example experiments illustrating the type of assays that can be performed using the following protocols. In these particular examples we use the MTLn3 cell line, a rat mammary carcinoma cell line, overexpressing ErbB1 [11] and the H1299 cell line [12], a human lung carcinoma cell line; both cell-lines express ectopically the Green Fluorescent Protein (GFP). However, the protocols can be adapted to various other mammalian cell culture systems and assays.
All following steps should be performed in a sterile flow cabinet and with sterile reagents.

3.1.1 Exposure of MTLn3-ErbB1 cells to epidermal growth factor (EGF)

1. Dilute collagen type I in PBS without Ca/Mg to a concentration of 30 μg/mL. Then coat the 96-well plate by adding 50 μL of this solution to each well.

2. Incubate the plate for one hr at room temperature, then aspirate the solution and let the plate air-dry for approximately 30 min.
3. Plate 10,000 cells per well (end volume is 100 μL per well) in phenol red free medium (MEMα) containing 5% FBS (see Note 1, 2 and 5).
4. The next day, three to four hr before start of the imaging, starve the cells by replacing the medium on the cells with medium without FBS.
5. Dilute EGF in MEMα without FBS to 5 and 10 nM and expose the cells by replacing the medium covering the cells with medium containing EGF (see Note 3).
6. The cells should now be placed on the microscope as soon as possible (see Note 6).

3.1.2 RNAi mediated gene silencing in H1299-GFP

1. Dilute fibronectin in PBS without Ca/Mg to a concentration of 10 μg/mL. Then coat the 96-well plate by adding 50 μL of this solution to each well.
2. Incubate the plate for one hr at 37°C and aspirate the solution prior to plating cells. Do not dry the plate.
3. Prepare a 96-well V-bottom plate containing 50 nM siRNA per well by adding 0.25 μL/well from a 20 μM siRNA stock solution to 9.5 μL/well medium (RPMI) without serum (see Note 4).
4. In an eppendorf tube, dilute DharmaFECT 2 in serum free medium. For one well, add 0.2 μL DharmaFECT 2 to 9.8 μL medium without serum. Multiply this for the total amount of wells, and add a few spare. Incubate at room temperature for 5 min.
5. Add 10 μL of the diluted DharmaFECT2 to each well, gently pipette up and down and leave the mix at room temperature for 20 min so the siRNA complexes can form.
6. Add 3,000 cells per well in a volume of 80 μL complete medium (RPMI) containing 10% FBS to the siRNA mix in the V-bottom plate and gently pipette up and down to mix. Then transfer the total volume (100 μL) from the V-bottom plate to the coated glass bottom plate and place in the incubator.
7. After 16 hr, replace the medium covering the cells with phenol red free medium containing 10% FBS.
8. 72 hr after transfection of the cells, replace the medium one more time to get rid of floating cells and start imaging.

3.2 Automated high throughput imaging

This automated imaging protocol is developed for application on a NIKON inverted fluorescent microscope and differential interference contrast (DIC) imaging with

automated stage control, but can be adapted to similar high throughput imaging systems.

1. Prior to imaging, start heating the climate control chamber till 37°C.
2. Start the microscope including the camera, the mercury lamp (for DIC imaging) and/or fluorescent lamp (for fluorescent imaging).
3. Start the Nis Elements AR software. The 'ND acquisition control panel' will pop up.
4. Place the 96-well plate in the plate holder on the motorized stage and open the CO_2 supply.
5. In the 'ND acquisition control panel', define the exposure time and acquire one live image of your cells in a random well.
6. Open the macro WellPlate2.mac and run it. The 'WellPlate' window will pop up.
7. Position the well A1 in the centre and click on 'Alignment' (see Note 7 and Fig 1A).
8. Position the well A12 in the centre and click on 'Alignment'.
9. Position the well H1 in the center and click on 'Alignment'.

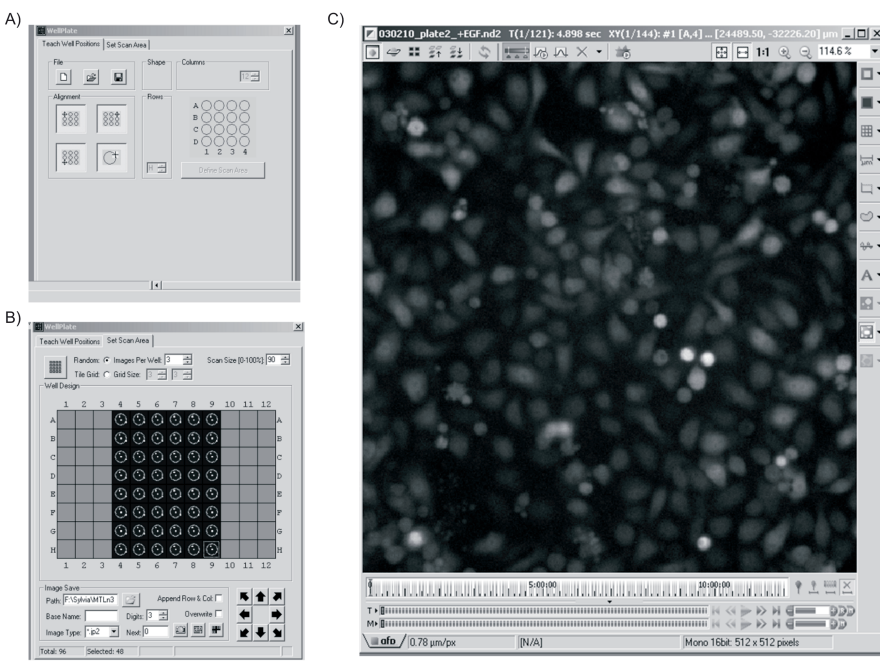

Figure 1: **Essential steps in the Wellplate2.mac macro.** A) In this control panel, define the plate, by moving the stage to well A1, A12, H1 and outside a well respectively. Click on the corresponding buttons to align the plate. B) In this control panel, define the region of wells you want to image and number of positions per well. The imaging position will be displayed as white dots in the plate scheme. C) This window shows the final .nd2 file, including images of all positions and all time frames. Movies can be viewed directly by pressing the 'play' button.

10. Position the outside of any well and click on 'Alignment'.
11. Choose for the correct number of rows (in letter, A-H) and columns (numbers, 1-12).
12. Click on the button 'Define scan area'. The tab 'Set Scan Area' will become active (see Fig 1B).
13. Select the region of wells you want to image by dragging your mouse over the wells.
14. Select at least 3 images (position) per well.
15. Select a scan size of 80% so that you do not image the extreme periphery of the well.
16. Click on the 'well design' icon and all the positions will be directly given in the 'XY position' tab of the 'ND acquisition control panel' window. Important: do not include Z (checkbox) and keep PFS ON while moving
17. In tab 'Time' and then 'Advanced' execute before Multipoint the macro 'EpiOn' and execute after Multipoint the macro 'EpiOff' by choosing then from the drag-down menu. Test if these two macros are working correctly before start imaging the entire plate by running them. To do so, go to 'Macro' in the toolbar, select 'EpiOn' or 'EpiOff' and press run. The shutter should open and close.
18. Define camera settings and keep them unchanged when imaging multiple wells and/or plates within one experiment.
19. Right click on the button with the correct imaging channel ('GFP' or 'DIC') in the upper toolbar and assign the current settings to this channel.
20. In tab 'Time', define the imaging interval (e.g. 6 min) and duration of the experiment (e.g. 12 hr). The number of loops will be calculated (see Note 8).
21. Give the experiment name and location to safe the file.
22. Click on '1 time loop' to test the imaging time of one round or click on 'run now' to start the experiment (see Note 9 and 10).
23. Finally, one .nd2 file is generated for all the wells and the entire imaging period (see Note 11 and Fig 1C).

3.3 Automated quantitative image analysis

The following protocol describes the automated quantitative image analysis that is designed to quantitatively analyze migratory tracks of all cells within each frame of a live cell movie. Firstly a number of parameters are set on a "test image/frame" followed by applying the analysis to the whole range of data (movies) recorded during an experiment.

The macros are written in Image Pro Plus Software and are available on request. The macro 'TRACKDIC' should be applied when analyzing movies recorded using differential interference contrast (DIC) microscopy, whereas the macro 'TRACKFLUOR' should be used when analyzing the movies of cells containing fluorescent probes, such as GFP. Examples of the result of both macros are depicted in Fig 2. In this particular example, MTLn3-ErbB1 cells are exposed to EGF and imaged with DIC. The result of the analysis of those movies is shown in Fig 3. In the H1299-GFP cells, the expression of two proteins (Rac1 and PTEN) is silenced using siRNA. These cells were used to acquire fluorescent movies. The result of the analysis of these movies is shown in Fig 4.

3.3.1 Conversion .nd2 to avi

1. Within NIS Elements, export the file to tagged image file (tif). You will get one folder that contains all the .tif files of the experiment and that are automatically named namet01xy01z01c01.tif where t represents time, xy the position, z the focus plane and c the channel.
2. Start ImagePro software and run the macro 'TIFfolderAVI'. A user dialog window is created and will pop up.
3. Give the number of imaged color channels (in these examples this value is 1), Z-stacks and positions. When imaging a full 96-well plate with 3 positions per well, this number is 288. Select 'auto contrast on'.
4. Select the first .tif file in your folder. The macro will automatically start to generate a time-lapse serie for each position over the entire imaging period.
5. Wait patiently until all the .tif files are converted in .avi files. These .avi files are saved in the same folder and are consequently named namet01xy01z01c01.avi for each position.

3.3.2 Analysis with TRACKDIC

1. Start ImagePro software and run the macro 'TRACKDIC'. A user dialog window is created and will pop up. In this window files or folders can be selected, minimum cell areas can be set, threshold and filter options can be set, and a status/instruction window is displayed.
2. Start Excel.
3. Select the first avi file. An avi file with the time lapse sequence of cells recorded as described in section 3.2 is opened. With this file the filters and settings can be calculated.

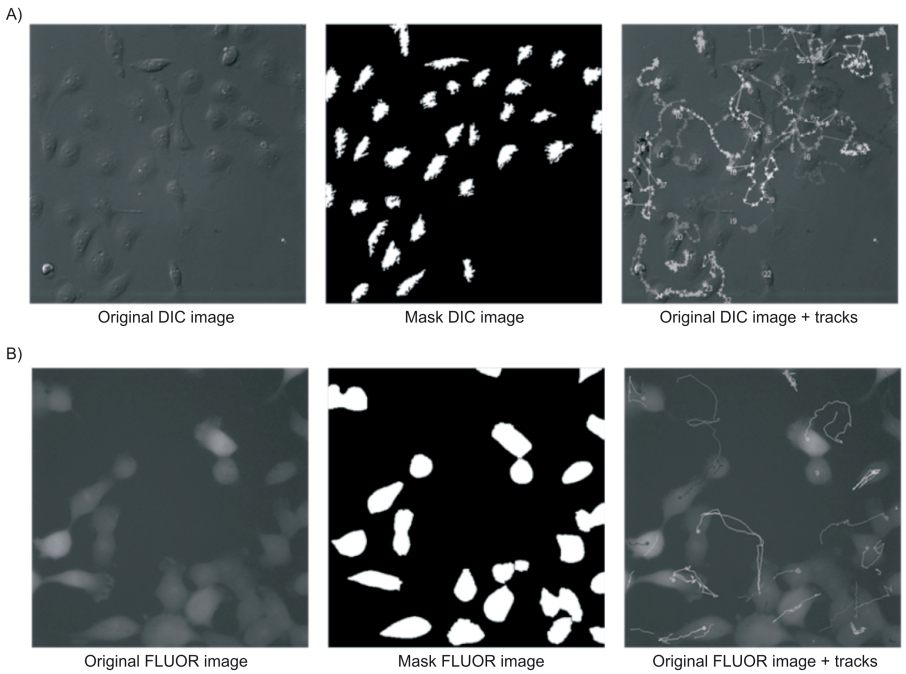

***Figure 2:* Steps in image processing.** A) An example of DIC image is shown. After applying several filters on the original image, a mask is defining the cell area and in time, the trajectory of the cells is calculated and displayed on the original image. B) As above, but now an example of FLUOR image is shown.

4. Determine manually the intensity threshold by moving the threshold ruler until the cells are red and the background is black. Determine the minimum area of 1 cell. All objects with high intensity and which are of the size equal to or larger than the set minimum area will be classified as cells. Low intensity pixels and small areas will be discarded as noise.

5. When the settings are correct, choose 'select folder' and then the first avi in that folder. All avi files in the folder will be automatically analyzed.

6. Press 'calculate' to start the analysis.

7. Calculation of the track is automatically done through the following steps:

- First 2 filters are applied to the movie. A flatten filter, which corrects for uneven background and a large edge plus filter to reduce background. The edge plus filter extracts positive edges (bright features on a dark background) from an image.
- Then a mask (binary image) sequence is made with the option to fill holes in the segmentation steps (see Fig 2A, middle panel).
- Then an open filter is applied, which will smooth object contours.
- After this, the standard Image Pro Auto track function is applied with the following

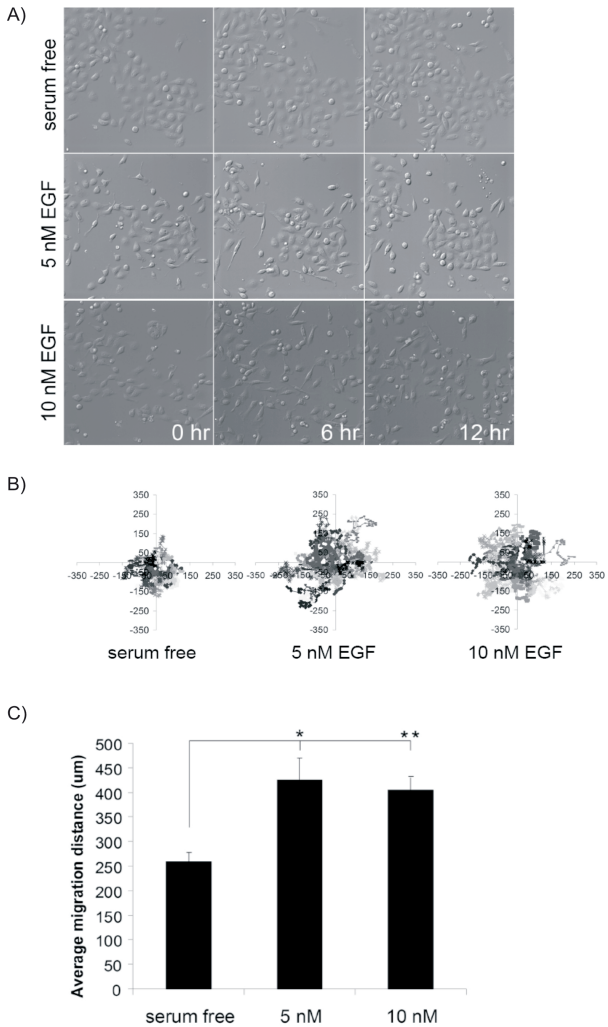

Figure 3: **Exposure of MTLn3-ErbB1 cells to EGF is triggering cell migration.** MTLn3-ErbB1 cells were starved for 3 hr and then exposed to 0, 5 and 10 nM EGF. A) Snapshots of the entire imaging period of 12 hr for different conditions. B) Representation of individual tracks within one movie. Exposure to 0, 5 or 10 nM EGF is increasing the track length. C) The calculated average migration distance of cells exposed to EGF. Exposure to EGF is significantly increasing the migration distance (*P<0.05, **P<0.01). Note that there is no additional increase when comparing 5 and 10 nM due to receptor saturation at the lower concentration.

options: velocity limit = 75 pixels, auto acceleration limit, chaotic motion type, minimum track length = 10% of total movie length, tracking prediction depth = 3 (see Note 12).

- The following parameters are automatically calculated and written to Excel: the XY positions and moved distance per cell from frame to frame. Also averages are calculated for all cells for each frame and averages for all cells in the total movie.

8. A picture of the original first image with the track annotated on it is written to Excel (see Fig 2A, right panel).

3.3.3 Analysis with TRACKFLUOR

1- 6. See steps 1-6 described in section 3.3.2, except run the macro 'TRACKFLUOR'.

7. Calculation of the track is automatically done through the following steps:

- First 6 filters are applied to the movie. A flatten filter, which corrects for uneven background, a gauss filer, which reduces high intensity noise. Contrast of the movies is optimized. The large edge plus filter is reducing background. The erode filter will separate touching cells and the open filter will smooth object contours.

- After the filters, a standard Image Pro Auto track function is applied with the following options: velocity limit = 75 pixels, auto acceleration limit, chaotic motion type, minimum track length = 10% of total movie length, tracking prediction depth = 3 (see Note 12).

- The following parameters are automatically calculated and written to Excel: The XY positions and migrated distance per cell from frame to frame. Also averages are calculated for all cells for each frame and averages for all cells in the total movie.

8. A picture of the original first image with the track annotated on it is written to Excel (see Fig 2B, right panel).

3.3.4 Processing the tracking data for representation

The TRACKDIC and TRACKFLUOR macros in ImagePro write results to Excel, but also generate .trc files within the same folder. These files can be used to generate graphs displaying all individual tracks within one well, with all tracks starting at the center of the graph.

1. Start ImagePro software and run the macro 'RelativeTracks'.

2. Open the first .avi file in the folder, which also contains the .trc files. All other .avi files will be calculated automatically.

3. Give the calibration factor for calculation of the correct distances. This is the size of 1 pixel in µm and this value is displayed in NIS Elements at the bottom left corner of the final imaging panel (see Fig 1C)

2. Start Excel and open 'makegraph.xls'. This excel file is part of the macro and available on request.

3. Go back to ImagePro and press 'ctrl' and 'G' on your keyboard. A new window will pop up.

4. Select the maximum number of tracks to be displayed in each graph, e.g. 50.

5. Enter the maximum scale in µm e.g. 200. This value needs to be enlarged for highly migratory cells and can be decreased for cells that are less migratory.

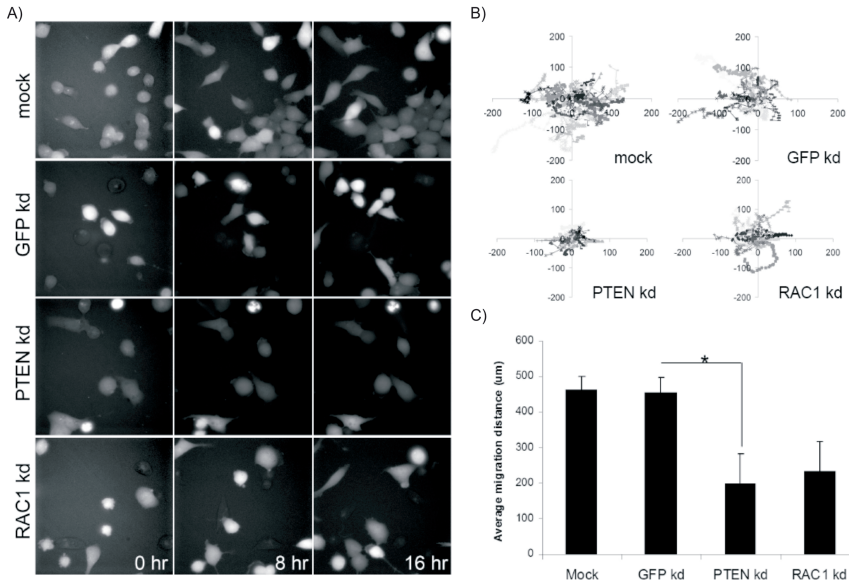

Figure 4: **Knockdown of PTEN in H1299-GFP is suppressing cell migration.** The effect of gene knockdown on cell migration was examined 72 hr after transfection. A) Snapshots of the entire imaging period of 16 hr for different genes. B) Representation of individual tracks within one movie. Knockdown of PTEN is decreasing the track length, where RAC1 knockdown partially decreases track length. C) The calculated average migration distance of cells after knockdown. Knockdown of PTEN is significantly reducing the migration distance ($P<0.05$). Knockdown of RAC1 also seems to reduce the migration distance, but this is not significant.

6. Enter the number of imaged positions, corresponding with the number of .avi files within your folder. The graphs will automatically be generated for all positions and written to the active excel file (see examples in Fig 3B and Fig 4B).

4. Notes

1. Since phenol red exhibits auto fluorescence (it is excited at 488nm and emits over a wide spectrum), it is highly recommended to use phenol red free medium while imaging in order to reduce background signal. This is in particular important when imaging GFP signal.

2. When adapting this protocol for a different cell line, the cell density should be adjusted. The required confluency at time of imaging is ~70%, so that cells have enough free space to migrate.

3. Cancer cell migration is dependent on growth factors, such as EGF. For that reason, stimulation of cells with EGF during imaging is mimicking the *in vivo* microenvironment. Exposure to other growth factors or chemokines can also be

combined with this assay. Also, exposure to growth factors can be combined with RNAi screening.

4. When performing siRNA experiments, it is essential to include the proper controls. In this case, siRNA targeting GFP is chosen as a negative control since it should not effect the cell migration. In this example, two specific genes are chosen, but this method can be applied to whole genome siRNA libraries or sub libraries. In addition, the optimal cell density and transfection reagent is cell type specific. A guideline is available at http://www.dharmacon.com/uploadedFiles/Home/Support_Center/Selection_Guides/dharmafect-cell-type-guide-dharmacon-products.pdf

5. This protocol can be further adapted for full automation by applying robotics for automated liquid handling for e.g. cell seeding and drug exposure.

6. Alternatively, first place the 96-well plate on the microscope and then carefully add the exposure medium. In this case, the concentration of EGF should be doubled, and a volume of 1:1 should be added to the medium already in the plate. Prior to placing the cells on the microscope, make sure the temperature and CO_2 levels in the camber are optimal in order to allow the natural environment of the cells and to minimize stress levels.

7. The centre of the well is easily determined by opening the shutter so that a beam of light hits the well. The beam can then be positioned at the centre of the well.

8. When adapting this protocol for a different cell line, the optimal time interval should be determined. For fast moving cells such as the MTLn3, the maximum time interval is 6 min. In general, it is advised to make the time interval between imaging frames as short as possible, to ensure a proper tracking of the cells. If the interval is too long, the cell 'jumps' between the ascending frames, making it difficult for the analysis programs to assign the track to the correct cell.

9. When imaging 96 wells and 3 positions per well, the total imaging time of one time loop is about 5 min.

10. Take note of direction of imaging (e.g. left to right or up to down) since the movies will be numbered accordingly.

11. The .nd2 file that is created at the end of the imaging period is, depending on the amount of positions imaged, ranging from 7-21 GB in size when individual images are 512x512 in size. Make sure there is enough free space on the hard disk of your computer.

12. The velocity limit (search radius) defines maximum distance in calibrated units between object positions on the neighboring frames. The parameter is used with automatic tracking to find tracks on the first two frames (on all frames with chaotic

movement type). If no object is found in the search radius, you will be prompted to click on the expected position manually. In automatic mode the track is closed at this point. Tracking prediction depth defines the number of frames ahead of the current, which are used to find most suitable track for the given object (default is 2). This parameter also defines the maximum number of consecutive occlusions on track. The occlusions number is less than the prediction depth by 1. For example, if the prediction depth is 3, the maximum number of consecutive object occlusions is 2, it means that if the tracking object in sequence disappears (or gets hidden) for 1 or 2 consecutive frames it will still be detected as a part of the track. The estimated position will be used for missed objects. The track points of missed objects do not have outlines and count/size measurement values. High prediction depth values increase computation time significantly.

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