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TScratch: a novel and simple software tool for automated analysis of monolayer wound healing assays

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Cell migration plays a major role in development, physiology, and disease, and is frequently evaluated in vitro by the monolayer wound healing assay. The assay analysis, however, is a time-consuming task that is often performed manually. In order to accelerate this analysis, we have developed TScratch, a new, freely available image analysis technique and associated software tool that uses the fast discrete curvelet transform to automate the measurement of the area occupied by cells in the images. This tool helps to significantly reduce the time needed for analysis and enables objective and reproducible quantification of assays. The software also offers a graphical user interface which allows easy inspection of analysis results and, if desired, manual modification of analysis parameters. The automated analysis was validated by comparing its results with manual-analysis results for a range of different cell lines. The comparisons demonstrate a close agreement for the vast majority of images that were examined and indicate that the present computational tool can reproduce statistically significant results in experiments with well-known cell migration inhibitors and enhancers.

Introduction

Cell migration has a major role in development and in physiological repair processes, and is also involved in many pathological disorders, including cancer invasion and metastasis, angiogenesis, and inflammatory reactions (1,2).

Assessment of the migrative potential of distinct cell types is important for the basic understanding of the molecular mechanisms involved, but also for screening of pharmaceutical compounds that modulate cell migration and thereby might exert beneficial effects in pathological conditions (3,4). Several assays have been developed to measure and compare cell motility in vitro, among them the Boyden chamber transmigration assay (5). The most widely used technique is the in vitro monolayer wound assay, or the "scratch assay" (5). In this assay, a confluent cell layer grown on a plastic support is artificially wounded and the coverage of open area by migrating cells is then assessed over time using bright-field imaging (6). The limitation of this method is the manual and highly subjective nature of open-area quantification (7). Overcoming this limitation will allow users to significantly accelerate analysis and translate this widely used manual assay to an automated, quantitative high-throughput system (8).

An important requirement for reliable and reproducible quantification of the open area is a robust and flexible image segmentation algorithm. So far, the performance of available algorithms has been limited by the strongly varying image quality, which depends on the acquisition mode and skills of the experimenter. Better-performing algorithms are not freely available. In this work, we applied a novel multi-purpose image analysis method based on curvelets (9, 10) in combination with an automated thresholding scheme to overcome these limitations. This combination allows customization of the quantification process and provides a consistent and efficient image analysis framework.

Here, we present TScratch, a user-friendly software tool that implements our analysis algorithm for monolayer wound healing assays, and which is freely available (www.cse-lab.ethz.ch/software.html). The software enables a reliable and reproducible quantification of open areas and an immediate readout for a broad spectrum of cell lines.

Materials and methods

Image segmentation and thresholding
We developed a novel algorithm for measuring the open image area based on a method which, for the first time in biomedical image analysis, uses the recently developed edge-detection algorithm (10) based on the discrete curvelet transform (9). The curvelet transform encodes image information for different scales, directions and positions in the form of curvelet coefficients. The transform is lossless and when all curvelet coefficients are maintained, the original image can be reconstructed. The curvelet coefficients can then in turn be processed so that the desired image properties are obtained by using different scales and orientations. For the presented application, 2 scale levels—corresponding to the levels of detail at which the cells’ boundaries appear in the image—are selected. At each position, the magnitudes of the curvelet coefficients at the different scales and directions are added together. This generates a curvelet magnitude image, with the intensities providing a measure for the amount of detail in the original image, thereby helping to separate vacant and occupied regions depending on the level of detail. In order to further enhance the separation and minimize the sensitivity to local variations, the algorithm then computes the morphological opening (an erosion followed by a dilation) of this curvelet magnitude image, using a disk.
of adjustable radius as the structural element of the opening operation.

Following this processing, a threshold is applied to separate the 2 regions by setting the threshold in a gap between 2 peaks in the histogram of the curvelet magnitude image, thus separating the low-intensity open area and the high-intensity covered area. In cases of very small, open areas, it is harder to determine the threshold automatically, and a default threshold is applied, which the user can modify if desired.

Additionally, since the quantification of contiguous open and occupied areas is desired, small, isolated open areas are marked as occupied and small isolated occupied areas are marked as open, based on a threshold parameter in the settings menu that may be adjusted by the user. An erosion step is also performed to make the edges of the marked occupied area adapt better to the cell boundaries.

Our algorithm excludes the coarsest scale of the image curvelet transform so that the method remains insensitive to moderate background intensity variations in the image. Furthermore, as the finest scale is also ignored, noise and small impurities do not appreciably affect results.

**Cell culture**

Cell lines with various morphologies were cultured and used for wound healing assays as follows: Human dermal lymphatic endothelial cells (LEC) (11), passaged 9–13 times, were cultured in endothelial basal medium (EBM; Lonza, Walkersville, MD, USA) supplemented with 20% fetal bovine serum (FBS; Gibco, Paisley, Scotland), 1% antibiotic/antimycotic solution (Gibco), 4 mM L-glutamine (Gibco), 25 μg/mL cAMP (Sigma-Aldrich, Steinheim, Germany) and 10 μg/mL hydrocortisone (Sigma-Aldrich) on plates coated with fibronectin (10 μg/mL; Chemicon, Temecula, CA, USA). The mammary carcinoma cell line MCF-7 (provided by Reuven Agami, The Netherlands Cancer Institute, Amsterdam, The Netherlands) was cultured in DMEM with 4.5 g/L glucose, 4 mM L-glutamine, 1 mM sodium pyruvate, 10% FBS and 1% antibiotic/antimycotic (all from Gibco). Primary mouse embryonic fibroblasts (PMEFs) (Institute of Laboratory Animal Science, University of Zurich, Switzerland) were cultured in DMEM GlutaMAX (Gibco) containing 4.5 g/L glucose, 15% FBS, 1% antibiotic/antimycotic, 20 mM HEPES, 2 mM sodium pyruvate (all from Gibco) and 10 μM monothioglycerol (Sigma-Aldrich). The hemangioendothelioma cell line Py-4–1 (12), stably transfected with a Prox1 overexpression vector or empty vector alone (13) was cultured in DMEM with...
4.5 g/L glucose, 4 mM L-glutamine, 20% FBS, 1% antibiotic/antimycotic (all from Gibco) and 0.8 mg/mL G418 (Sigma-Aldrich).

Monolayer wound healing assay
Cells were grown to full confluence in 24-well plates in their respective growth media and were then incubated overnight in serum-reduced medium containing 1% FBS. Cell cultures were scratched with a 200 μL sterile pipette tip and extensively washed with PBS to remove detached cells and debris. Two crosses were scratched in each well, and these were instantaneously center-imaged at 5× magnification using a Zeiss Axiovert 200 M microscope equipped with a Zeiss AxioCam MRm camera with maximum contrast (Carl Zeiss AG, Feldbach, Switzerland). Cells were then incubated in serum-reduced or full-growth medium.

For LEC experiments, 20 ng/mL recombinant human VEGF-A165 (National Cancer Institute, Bethesda, MD, USA) or 0.1 μM nocodazole (Sigma-Aldrich) were added. After 24 h, the medium was replaced with PBS and images of the same areas were acquired.

For proof-of-concept TScratch analysis of fluorescently stained wound healing assays, cells were incubated in 4 μg/mL cell body-staining calcein AM dye (Sigma-Aldrich, Buchs, Switzerland) in medium for 30 min at 37°C before being imaged by excitation at 488 nm using a Zeiss Axiovert 200 M microscope.

For manual analysis of the open, wounded areas, the cell layer margins were outlined using the polygonal selection tool of Adobe Photoshop CS3 (Adobe Systems, Inc., San Jose, CA, USA), and the area was calculated with the Photoshop analysis tool. The ratio of open area at 24 h to open area at 0 h was determined with MS Excel 2003 (Microsoft, Redmond, WA, USA) and replicates were averaged.

For automated image analysis, the same image data set was analyzed with the TScratch software using the default parameter settings, with human interaction limited to excluding the few images for which the algorithm failed in case of the Py–4–1 cell line. At least four biological replicates per experiment were used and the presented results are representative of triplicate experiments with similar outcome.

Results and discussion
The new image analysis algorithm described above was implemented in a graphical user interface in MATLAB (The Mathworks, Natick, MA, USA) (Figure 1A), and is available as a stand-alone application at www.cse-lab.ethz.ch/software.html. Using the graphical user interface, the user may automatically analyze a whole data set (consisting of any prevalent image type such as TIFF or JPEG), then easily inspect the results, modify thresholds, and directly see the results. If necessary, unsatisfactorily segmented images may be excluded from the final analysis in the case of experiments that allow a high-enough number of replicates. Alternatively, the measured open area may be manually modified by directly using the brush, eraser or polygonal selection tool on the image. As output, a tab-delimited text file is written with the computed open image area for all analyzed images, as well as quotients between later time points.
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The program also computes and outputs means and standard errors within groups of images, defined through the image file names, and presents a graphical summary. The output file may be easily imported into Excel or other spreadsheet programs for further analysis and more sophisticated visualization. Thus, TScratch allows the user to analyze wound healing assay images in an automated fashion, while also allowing for a customized analysis fitted to the experiment, available time and number of replicates.

The effects of growth factors, pharmacological compounds and genetic alterations on the migrative properties of endothelial cells are commonly tested in wound healing assays (5). We first utilized human lymphatic endothelial cells (LEC) as an in vitro lymphangiogenesis model (11). LEC were incubated with enhancers and inhibitors of LEC migration, namely VEGF-A and the β-tubulin inhibitor nocodazole, to generate wound-healing assay data for a direct comparison of manual versus automated analysis. Figure 2A shows representative images of the coverage of open area by untreated LEC before and after 24 h incubation in serum-reduced medium. Visual inspection of the manually quantified open area (outlined in red in the upper panel) and automated TScratch quantification (gray area in the lower panel) confirmed a good overlay. The quotients of 24 h to 0 h values were averaged and plotted (Figure 2B), and demonstrate a close agreement between the results of the manual and the automated analysis with low variance. For both manual and automated segmentation, the migration inhibition by nocodazole and the induction of migration by VEGF-A were statistically significant as shown by Student’s t-test analysis ($P < 0.001$). The percentage of open image area for every single replicate was plotted in Figure 2C and confirmed the close correlation between both types of image analysis. No images had to be excluded or manually adjusted due to faulty automated segmentation. Overall, the automated analysis closely matched the results of the manual analysis, recapitulating statistically significant differences in the migratory LEC response to VEGF-A and nocodazole.

We next investigated whether TScratch is capable of automating the analysis of wound healing assays performed with a range of cell lines with differing cell morphology. To this end, we analyzed MCF-7 breast cancer cells (which form densely packed monolayers and display a roundish, epithelial morphology) and primary mouse embryonic fibroblasts (which show a spindle-like, elongated morphology). For both cell lines,
the TScratch program, using default parameter settings, segmented the acquired images in an intuitive manner, which is representatively shown in Figure 3A. The results obtained by the manual and automated analysis matched equally well as in the case of the LECs (data not shown). Furthermore, we labeled LEC with calcein AM, a cell body–staining fluorescent dye, and found high agreement in the TScratch-determined open image area between images acquired in bright-field and fluorescent mode (Figure 3B). This confirms the applicability of the TScratch program for quantitative evaluation of monolayer wound healing assays performed in different cell lines with distinct morphologies and for analysis of stained samples (from, for example, historical data sets).

Finally, we studied whether TScratch can be used to analyze a recently published wound healing assay image data set (13) that was analyzed using manual open area quantification. In this study, the hemangioendothelioma cell line Py-4–1 (12) was utilized to reveal that stable overexpression of the transcription factor Prox1 enhances the migratory capacity (13). Automated analysis of the images at 0 h and 24 h was performed (Figure 3C) and the averaged results revealed a 16.9 ± 3.2% increase of cell migration for Prox1 overexpression ($P < 0.001$), which very closely resembled the results obtained by manual analysis (16.5 ± 2.2%; Figure 3D), in agreement with the previously reported results (13). An image-by-image quantification comparison showed a close agreement between manual and automated analysis, except for 7 out of 96 images that were manually excluded from the automated analysis after visual inspection (Figure 3E). The automated analysis failed for these images because of a relatively high amount of floating debris in the medium. By the use of the manual drawing tools, these images could have been rescued if replicates were limited. Here, the number of replicates was high due to a cost-efficient setup, and so images were excluded in favor of faster analysis. The results demonstrate that the present method is capable of correctly quantifying and interpreting wound healing assay images and can substitute manual analysis.

TScratch has been successfully evaluated on 2 types of normal primary cells, human lymphatic endothelial cells and primary mouse embryonic fibroblasts, and on 2 cancer cell lines (MCF7 and Py4–1), representing diverse cell types. We believe that the automated wound healing assay analysis will be applicable to a wide range of heterotypic cell populations.

Previous studies utilizing wound healing assays have been limited by missing quantification specification (14) or by time-consuming visual inspection and fluorescent staining (8,15). The time required for the quantification of a standard wound healing assay is significantly reduced by using the TScratch software. For the comparison of 6 experimental conditions in quadruplicate, with 2 crosses per well imaged at 2 time points (e.g., 0 h and 24 h), 96 images need to be analyzed. Depending on image resolution and computer performance, TScratch requires approximately 4 s per image for loading, automated segmen-

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tation, and open-area quantification including graphical output. Depending on the user and the quality of the images, visual control of all segmented images takes ~2 min, while potential threshold and manual adjustments can extend the analysis time.

For manual open-area quantification, images must be loaded, margins must be precisely drawn manually, and the area values must be extracted and transferred to a spreadsheet. For experienced users with an ideal software combination utilizing macros, the analysis time needed is ≥30 s per image. The manual approach requires constant presence and activity by the user, and constant analysis and visualization of the extracted numbers. Thus, our experiment showed that the total analysis time needed is ≥70 min for manual analysis, compared with 12 min for the TScratch program, which performs this task requiring minimal user intervention.

While TScratch is the first freely available program specifically designed for the quantification and analysis of wound healing assays, the multi-purpose image analysis tool CellProfiler (16,17), though not specialized for this kind of analysis, can discriminate between open and closed image areas. However, it does so only by differences in brightness and not by brightness-independent object variations. Additionally, it lacks an integrated statistical analysis with graphical output.

Even though the present software is capable of analyzing images of varying image quality, the results can be further improved by following some simple data acquisition guidelines: Maximizing the contrast between open and cell-covered areas is important. Usually, the best results are achieved by keeping the image slightly unfocused and using either phase contrast or bright-field with a nearly closed aperture. To reduce the number of floating particles that might disturb the image analysis and introduce artifacts, we recommend replacing the medium with PBS for imaging, as well as washing extensively after wounding to prevent reattachment of scratched cells. In some cases, images that cannot be segmented have to be excluded from the analysis, so it is advisable to evaluate at least 6–8 crosses, corresponding to 3–4 culture wells per condition within one experiment.

In summary, we have developed an easy-to-use curvelet-based image analysis algorithm and an accompanying software tool with direct graphical and statistical output named TScratch, for the reproducible and accurate automated analysis of monolayer wound healing assays. The robustness of the algorithm has been confirmed on a diverse range of cell morphologies, under conditions of pharmacological stimulation or inhibition of migration, and in genetically manipulated cell lines. By eliminating the need for staining and manual analysis, TScratch can significantly accelerate the process, suggesting its use in high-throughput screening applications.

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