Research Article

Standard Operating Procedure for Wound Healing Cell Migration Assay within the Nanostructurome Pipeline

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Abstract

Standardization of scientific procedures is a key issue in making research reproducible, cost-effective, shareable and easily accessible for technology transfer and data reuse. Here we present a standard operating procedure (SOP) for setting-up, running and analyzing a cellular wound healing assay. Wound healing assays allow the ability of different substances, such as conditioned medium, natural extracts, natural and synthetic compounds, drugs, extracellular vesicles, and synthetic nanoparticles to influence the ability of cells to migrate to be evaluated. Different cell types can be tested, including both normal and tumor cell lines. The output of the assay is to determine the effect of the tested sample on the migratory ability of specific cells, compared to untreated cells, by measuring the percentage of wound closure over time. These data are relevant in all the biological contexts in which cell migration occurs, including morphogenetic events during embryonic development, tissue repair and regeneration, tumor infiltration and invasiveness, and metastasis.

The SOP provides a step-by-step description of the procedure, lists the materials and equipment required, and identifies safety measures, instrument cautions and critical process parameters that must be controlled to ensure the quality of the output. It is therefore a valuable tool for staff training, competence transfer, quality assurance and management of the process, as well as facilitating technology transfer.

Keywords: Cell migration; Wound healing assay; Wound closure rate; Tumor invasion and metastasis, Tissue repair and regeneration; Standard operating procedure

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1. Definitions

EVs: extracellular vesicles
FBS: fetal bovine serum
N: number
NT: non treated
PBS: phosphate-buffered saline
PF : paraformaldehyde
qPMO: quality and project management OpenLab
SOP: standard operating procedure
T (T_0,T_1,T_2): time
T: treated
V_f: final volume
WA: wound area
WH: wound healing

2. Background

Cell migration is a key biological process for embryo development and adult life as well as an integral mechanism of many pathological processes, including tissue repair, tumor cell infiltration, invasiveness and metastasis. Cell migration depends on complex and integrated relations with the surrounding cells as well as the extracellular space. External biochemical cues that can induce cell migratory behaviour include soluble factors, chemoattractants and extracellular vesicles released by surrounding cells and present in the extracellular milieu. These stimuli have to be internalized in order to induce the cytoskeletal remodelling and adhesion rearrangements capable of conferring cell polarization and migratory behaviour (Merino-Casallo et al., 2022; Liguori and Kralj-Iglič, 2023)

Among the experimental assays available to assess cell migration is the wound healing assay, which is based on the formation of a wound on a confluent layer of cells and the subsequent monitoring of the healing process as cells migrate into the wound to close it. This assay has been used in several studies to determine the effect of different treatments or samples, including growth factors, drugs, natural products and extracellular vesicles, on the ability of different types of cells to migrate (Tong and Wang, 2017; Mantile et al., 2022; Alqarni et al., 2025). This assay is capable of assessing the ability of different samples/substances to induce or inhibit cell migration. Substances capable of inducing cell migration may be of interest in tissue and wound repair, whereas substances capable of inhibiting cell migration may be relevant in the context of tumour therapy to reduce the invasiveness and spread of cancer cells.
Several factors can influence the performance of the assay and the reproducibility of the results, including cell culture density, wounding method, the use or not of antiproliferative drugs, and the amount of sample to be tested. Therefore, a Standard Operating Procedure (SOP) describing the method and the critical steps to be controlled is useful for setting up, running and analysing a cellular wound healing assay.

3. Purpose, Scope and Applicability

The **purpose** of this SOP is to describe a procedure to determine the effect of a specific sample or treatment on the cell migratory behaviour over time.
The **scope** of the SOP is to describe how to perform and analyse a wound healing assay, mainly using silicone cell culture inserts with a defined cell-free gap (Ibidi) to increase the reproducibility of the wound and the reliability of the results.
The SOP is **applicable** to all types of adherent cells, especially those involved in tissue repair or tumour dissemination, and to all types of extracellular substances (natural compounds, synthetic peptides and drugs, chemokines, growth factors, extracellular vesicles, synthetic nanoparticles), purified or not, that can be diluted in the cell-conditioned medium.

4. Health & Safety Warning

All recommended measures for working with cell cultures should be followed. All experimental procedures should be carried out under a vertical laminar flow hood, wearing gloves and coat. All surfaces should be cleaned with 70% alcohol before and after use. Cell culture material should be discarded with biological waste soon after experiments. For further details, please refer to the guidelines for working in cell culture laboratories (Lacerra et al., 2013) identified by the quality and project management OpenLab (qPMO) network of the National Research Council of Italy (Bongiovanni et al., 2015), according to a quality-based model for life science research guideline (Digilio et al., 2016).

5. Cautions

All measures recommended for working with cell cultures must be followed to avoid cell culture contamination, as previously described (Lacerra et al., 2013). For imaging at different times, if the microscope used is equipped with a top incubator, it is possible to leave the cell samples in the top incubator throughout the experiment. If not, the cell samples are normally grown in the cell incubator and then transferred to the microscope at fixed times in clean, closed containers for image acquisition. In the latter case, the image acquisition step must be as fast as possible so as not to disturb the cell cultures. The image coordinates must be determined very carefully in order to monitor the same field of view over time and thus ensure the reliability of the results. All surfaces must be cleaned with 70% alcohol before and after use.

6. Personnel Qualifications / Responsibilities

The personnel responsible for the assay must be trained to work in both the cell culture and microscopy facilities. Personnel must know the basic rules for working with cells under a laminar flow hood, how to use an inverted microscope, perform live image acquisition, and analyse images and data using specific software (e.g. ImageJ, Excel, Prism, Power Points, Adobe Photoshop).
The cell culture and microscopy facility managers, together with the technical staff, ensure the performance of the facilities and equipment involved and are responsible for identifying and resolving any malfunctions, taking also advantage of external specialized technical support.
The Principal Investigator in charge of the project supervises the design, execution and analysis of experiments.
The other users of the facilities are responsible for respecting the operating rules and their reservation times so as not to interfere with the other experiments.

7. Materials, Equipment and Supplies

The following facilities and equipment are required to carry out the wound healing process:
- a cell culture facility equipped with a laminar flow hood that allows work to be carried out under sterile conditions; an incubator that maintains constant temperature, O_2 and CO_2 parameters; a bench-top centrifuge to collect cells from the cultures; an inverted phase contrast microscope to observe and count the cells;
- a microscopy facility with an inverted light microscope equipped with a motorized stage (e.g. DMI6000 microscope); automated image acquisition software that allows specific positions to be registered for time-lapse analysis, preferably with a top incubator to ensure that optimal growth conditions and sterility are maintained during the experiments.

The experiments will also require the following supplies:
1. sterile cell culture plates and/or flasks, strippettes and pipette tips;
2. pipettes and electronic pipettor;
3. a Burker chamber for cell counting;
4. 24-well cell culture plates;
5. removable 2-well silicone culture inserts to create a cell-free gap for migration assays (Ibidi, cat.no. 8209);
6. sterile tweezers.

and the following reagents:
1. Growth medium specific for the cell line used, complete with antibiotics, and specific supplements;
2. PBS1X for cell washing;
3. Mitomycin C to block cell proliferation;
4. Trypan Blue 0.4% for live cell counting.

8. Computer Hardware & Software

The microscope has to be equipped with a PC, a camera and a software for automated image acquisition. Specifically, we used the Leica DMI6000 inverted light microscope and Leica LAS AF software. Different software is required for image analysis, panel construction and data analysis (Image J, Adobe Photoshop, Power Point, Microsoft Excel and GraphPad Prism).

9. Step by Step Procedure

Scheme of the wound healing procedure is shown in **Figure 1.**

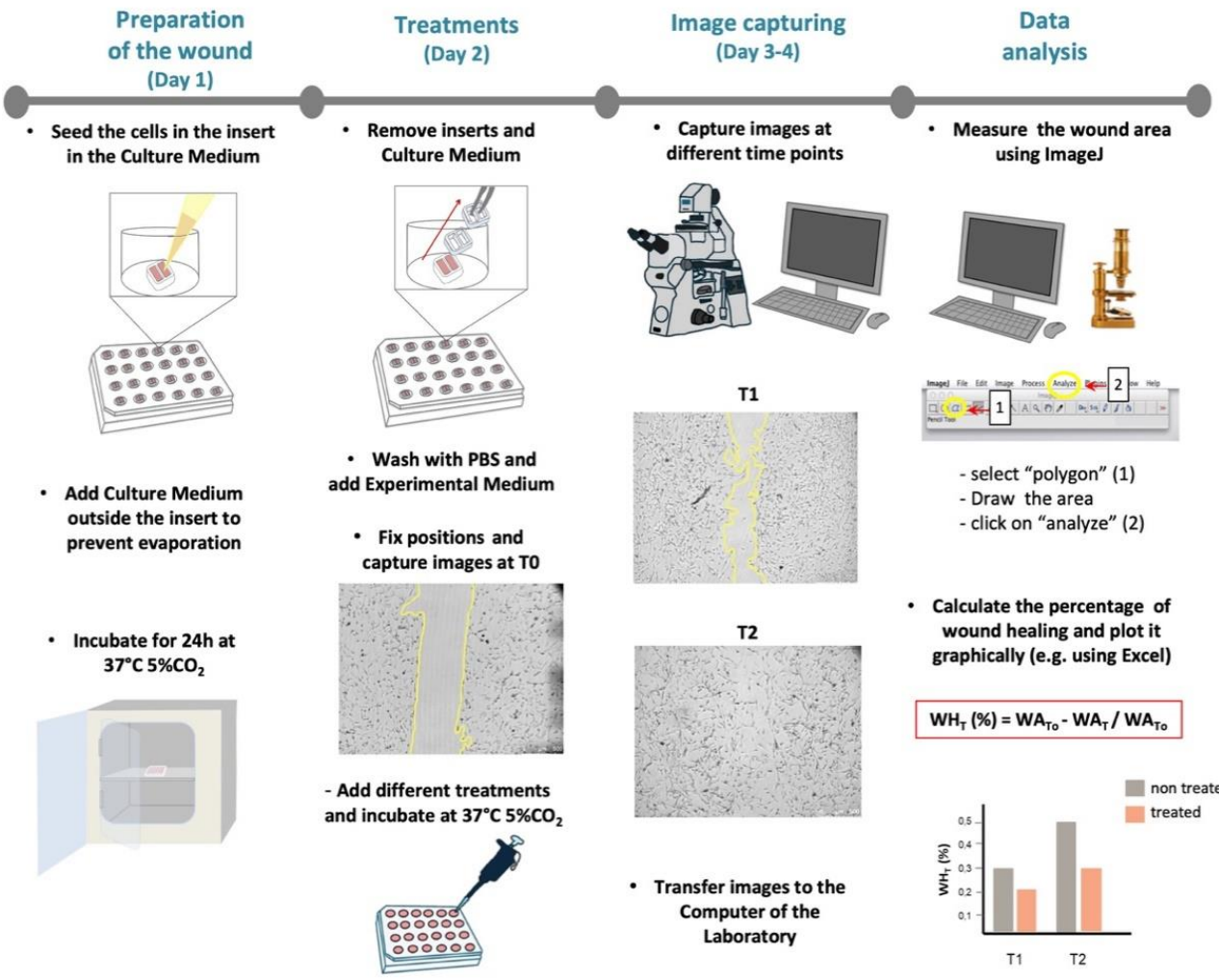

Figure 1. Scheme of the key step in the wound healing procedure.

9.1 Preparation of the cells and samples to test

9.1.1 Cell cultures

Cells are grown under their specific growth conditions (culture medium, supplement(s), T, CO_2 and/or O_2 atmosphere), which may vary from one cell line to another. It is recommended to use cell cultures at low passages, which have not been grown in culture for a long time and which have been tested for the absence of mycoplasma, whose contamination could alter the metabolism and behaviour of the cells and affect the reproducibility of the results. It is recommended that each assay is repeated three times using different batches of cells but at comparable passages.

9.1.2 Samples to test

The sample to be tested should be carefully prepared to reduce the risk of cross contamination, accurately quantified, aliquoted and stored at the optimum temperature to maintain its characteristics and to avoid repeated freezing and thawing which may affect the integrity and performance of the samples. It would be advisable to repeat each experiment three times using different batches of samples and testing different sample concentrations to measure a dose-response curve.

EVs are emerging as key particles capable of influencing cell migration (Sung et al., 2021) during both tumorigenesis (Mantile et al., 2020; Liguori and Kralj-Iglič, 2023) and wound repair (Narauskaitė et al., 2021; Li et al., 2025). In our case study, we determined the ability of EVs isolated from teratocarcinoma cells to affect cell migration of glioblastoma cells (Mantile et al., 2022).

9.2 Execution of the assay

Detailed description of the assay is given in **Table 1.**

Table 1. Detailed description of the wound healing assay procedure.

Day 1_Preparation of the Wound	
Step	Description
1	Calculate the number of cells required for the wound healing experiment: TOT N cells = N cells/well x N treatments x N replicates for each treatment The **Number (N) of cells** to be seeded in each well must be sufficent to form a confluent or subconfluent cell monolayer 24 hours after seeding. Therefore, it should be defined separately for each cell type. For U87 glioblastoma cells we used 15000 cells per well.
2	Detach cells with Trypsin 0.25% (incubation time and temperature depend on the specific cell line) or other methods if specifically recommended. Wash with PBS1X, and collect cells in Culture Medium in a 15ml Falcon tube. A single 100mm cell culture dish at 80% confluence should be sufficient.
2	Centrifuge at 1200 rpm for 5-8 minutes, preferably with gentle acceleration/deceleration, to collect cells. Discard the supernatant and resuspend the cells in 0.5-1 ml of Culture Medium.
3	Count the cells using a Burker chamber, making a cell dilution of 1:5 or 1:10 in a vital dye such as Tripan Blue 0.4%.
4	Transfer the amount of cells required for the entire experiment to another 15 ml Falcon tube.
Optional	Dilute in Culture Medium, resuspend well with Stripettes and count cells again with Burker chamber (1:5 dilution) to ensure that the desired number of cells are present.
5	Add Culture Medium to obtain the required V_f **V_f = 70ul (V for half insert) x 2 x N wells**
6	Under a laminar flow hood, open the box of inserts and, using sterile tweezers, place them in the centre of the well in a 24-well plate. Ensure that the Ibidi insert is firmly attached to the plate and does not move.
7	Always resuspend the cells just before aliquoting them and add 70 ul of cell culture to each side of the insert (140 ul for each well.)
8	Incubate at 37°C and 5%CO_2 approximately 1 hour to allow the cells to adhere to the multiwell plate.
9	Add 500 ul of Culture Medium to each well, outside the insert, to prevent evaporation. Incubate at 37°C 5%CO_2 for at least 24 hours to reach the required confluence.

Day 2_Treatments	
Step	Description
1	Check the cell density under the microscope to ensure that a monolayer is visible, then remove the medium outside the insert.
2	Using sterile tweezers, remove inserts from the wells by gently pulling on one corner. Check under the microscope that the monolayer is still attached and that the wound is homogenous.
3	Wash the wells with PBS 1X and add 550 ul of Experimental Medium containing a reduced concentration of FBS and an antiproliferative agent (e.g. mitomycin C) to block cell proliferation. The concentration of Mitomycin C and FBS to be used in the Experimental Medium should be determined separately for each cell type. For U87 cells we used 2% FBS instead of 10% in the Culture Medium and 2 ug/ml of Mitomycin C to block cell proliferation.
4	Place the multiwell under the inverted light motorized microscope (e.g. DM6000, Leica). Fix the coordinates of the positions in the culture cell wound and take the images for the T0 point (2 images for each well, at 5X magnification).

5	Under the hood, add 50 ul of the appropriate treatments (NB. For non-treated samples, add 50 ul of the Experimental Medium). It is recommended to test different concentrations and each concentration at least in duplicates, preferably in triplicates. For our test with extracellular vesicles we use a range of concentration between 2 and 20 ug/ml (2-5-10-20 ug/ml).
6	Incubate at 37°C and 5%CO_2 in the top stage incubator (if the microscope is equipped with one) or return the well to the cell culture incubator.
7	At fixed times (e.g. T1=8h, T2=24h, T3=40h), take new images at the same positions as previously fixed.
Optional	At the end of the experiment collect the medium and store it at -20°C, while the cells are fixed in PFA 4%, then stored at -20°C in PBS/Glycerol 1:1 for further analysis.
8	Transfer images from the microscope PC to your own laboratory computer for futher storage and data analysis (e.g. using Image J for wound area quantification, Adobe Photoshop and Microsoft Power Point for image processing, Microsoft Excel and/or GraphPad Prism for data analysis and graphing) .

9.3 Data acquisition and calculation

Using ImageJ software, the area of the wound can be measured in the images at different times. Two main parameters can then be calculated and plotted:

1) the Relative Wound Healing at different time shows the percentage of healing compared to initial wound area (**Figure 2**). It is defined as

$$WH_T\ (\%) = (WA_{T0} - WA_T)/\ WA_{T0}\ , \qquad (1)$$

where WH_T (%) is the percentage of wound healing at a given time t, WA_{T0} is the area of the wound at time t0; WA_T is the area of the wound at a given time t.

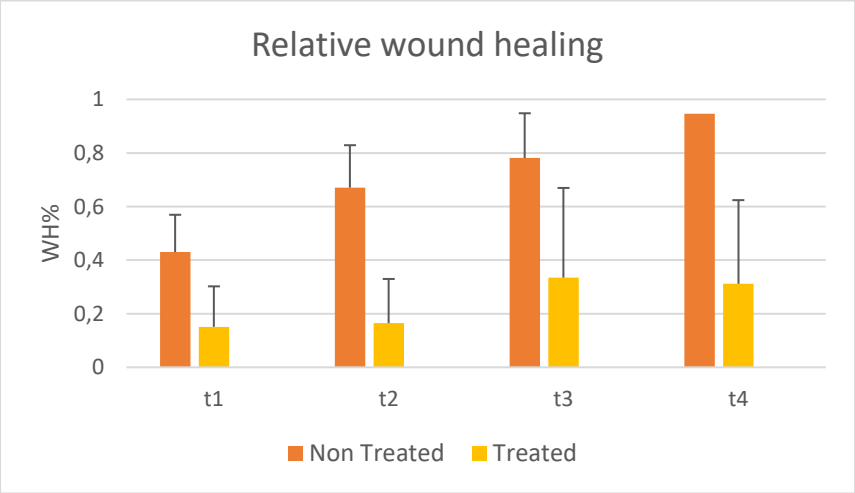

Figure 2. Example of the graphical output of the relative wound healing in dependence on time.

2) the Absolute Rate of Gap Closure (in um² or pixels over time) as the slope of the line obtained by reporting the area of the wound covered by cells (ordinate) at different times (t1, t2, t3, t4) (abscise) (**Figure 3**), before reaching the saturation phase.

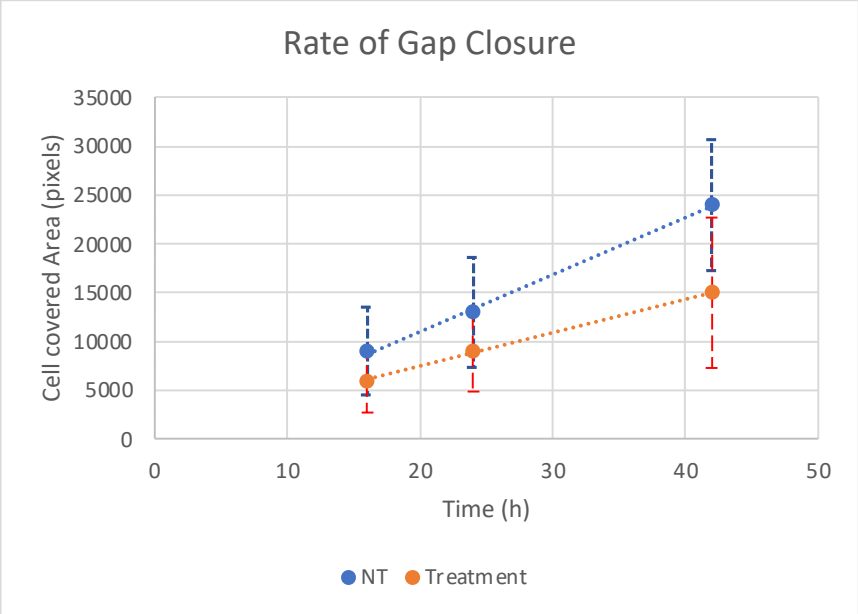

Figure 3. Example of the graphical output of the Cell covered area in dependence on time.

9.4 Troubleshooting

Envisaged problems and possible solutions are given in **Table 2.**

Table 2. Envisaged problems and possible solutions

Step	Problem	Possible reason	Solution
Step1, Day2	Cells density too low or too high	• Inaccurate cell counting • Poor cell resuspension	• Count cells twice • Pipette cells again just before plating cells into the inserts
Step1, Day2	Evaporation of the medium	Multi-well plate not closed properly	Check that the well plate is closed
Steps2 and 3, Day2	Cells detach from the plate	• Rough removal of culture inserts • Strong PBS1X washes	Be gentle and check the integrity of the cell monolayer and of the wound after insert removal
Steps6 and 7, Day2	Cells continue to proliferate	• Suboptimal concentration of FBS and/or Mitomycin C • Mitomycin C has lost its activity	• Test different concentrations • Check expiry date and/or change batch
Step7, Day2	The image field of the wound at different times does not match	Problem with position fixing at the microscope	Check that the microscope and software are working properly before starting the experiment
Step7, Day2	The technical replicates are very heterogeneous	Heterogeneity in plating cells, in aliquoting treatments, and/or in removing inserts	Prepare more technical replicates, to exclude those with technical problems
Step9, Day2	Treatment has no effect or is toxic	Insufficient concentration of treatment or presence of contaminants	• Increase or reduce the amount of treatment. A dose-response curve would be helpful. • Try different batches

10. Data and Records Management

All output files are temporarily stored on the PC connected to the microscope and then transferred to the server and to the laboratory computer(s) for further analysis. At the end of the project, the data are stored on external hard drives.
The protocols used are stored in electronic and paper registers. Any stable modification to the protocol is adequately documentd and included in the update version of the SOP.
The experimental plan and the results are recorded in the Laboratory Notebook and in the electronic files of the experimenter and are presented periodically to the supervisor, which also keeps a copy of the files on his/her computer.

11. Waste Management

At the end of the experiment, all materials used will be disposed of as liquid or solid biological waste.

12. Related Protocols or SOPS

For more informations on the use of culture inserts in wound-healing assays please visit https://ibidi.com/img/cms/downloads/ag/FL_AG_033_Wound_Healing_150dpi.pdf

13. Quality Control and Quality Assurance

13.1 Instrument Maintenance

Laminar flow hoods, incubators and microscopes are regularly inspected by internal technical services. In the event of a malfunction, users inform the facility managers, who investigate the problem and, if necessary, contact external technical support.

13.2 Critical Process Parameters and Checkpoints

A list of critical processes parameters and checkpoints is given in **Table 3**.

Table 3. A list of critical process parameters and checkpoints.

Critical Process Parameter	Checkpoint
Cell density	Check under the microscope that the cells have reached the adequate confluence before starting the experiment. If necessary, leave the cells in the incubator for a longer period of time
Wound area	Check that all the wounds at T0 are regular and measure approximately 500um, as foreseen for using Ibidi culture inserts. It is recommended to prepare more wells than necessary, so to choose the best ones for the experiment
Cell proliferation	Check that the cell density does not increase during the experiment, indicating that cell proliferation is occurring and contributing to the wound healing, thus confounding the results. In this case, repeat the experiment and check that the conditions used (serum and/or antimitotic drug concentration) are adequate. Check also the expiry date of the drug
Amount of Sample	Test different amounts of sample to obtain a dose-response curve
Reproducibility of technical replicates	Check the standard deviation and/or mean error among technical replicates. If there are visible problems in some replicates (e.g. cell density too low or too high; heterogeneity of initial wounds), consider discarding the corresponding data. In any case, the experiment must be repeated. Consider checking the quantity and quality of the cell and treatment batches used and/or using different batches
Reproducibility of biological replicates	Check the standard deviation or mean error between biological replicates. Check the quality of the cell culture batches (presence of mycoplasma, vitality, cell metabolism) and treatment batches (quantity and quality of preparation) and discard batches with problems. Repeat the experiment

14. Data on procedures and samples

Data on procedures and samples are given in **Table 4**.

Table 4. Data on procedures and samples

Description of the outcome	Relative wound healing and Absolute rate of gap closure as a measure of the effect of the treatment on cell migration
Time required to obtain the results	1 week
Amount of the sample needed	pg-ng for purified molecules ug for heterogenous samples
Estimated cost without manpower	10 EUR for each single test
Contact person	Giovanna L. Liguori IGB, CNR, Naples giovanna.liguori@igb.cnr.it

15. Conclusions

Optimization and standardization of the procedures and multivariable cellular assays are fundamental to increase the performace of the experiments, the reliability and reproducibility of the results, and to minimize the time and resources required, as the scientific community is increasingly aware and committed (Mancinelli et al., 2015, 2021; Hollmann et al., 2020, 2022). Based on our personal experimental practice, the current literature and the manufacturers' guidelines, we present a SOP describing the fundamental steps to perform a wound healing assay using silicone cell culture inserts with a defined cell-free gap. This method is more reproducible than the scratch method using the yellow tip to create the wound, and, unlike the scratch, does not risk to cause cell damage. The SOP focuses on the materials and equipment required, the critical parameters to control in order to obtain reliable and reproducible results, as well as the possible causes of failures and relative solutions. The SOP also includes safety measures, cautions and the required competence of personnel. It is worth noting that the wound healing assay can be used as a preclinical in vitro assay to test potential cell migration agonists or antagonists as possible candidates for use in wound repair or anti-tumor therapy, respectively. The SOP, therefore, is an essential tool for training staff, transferring competence, managing processes, ensuring the quality of the output, and ultimately facilitating technology transfer.

The assay can be used with purified compounds as well as more complex and heterogeneous samples such as cell conditioned medium, EVs or synthetic nanoparticles. The nature of the sample to be tested and the method used to prepare and/or purify and quantify it can also affect the reproducibility of the results. Therefore, all input variables and upstream processes must be also carefully checked in order to analyse the data correctly. This is particularly true in the context of EV research due to the high heterogeneity of both the methods used and the different batches and preparations obtained, which requires a large number of chemico-physical, biochemical and functional parameters to be checked, as well as the implementation of tools for quality management and control (Reiner et al., 2017; Ayers et al., 2019; Nieuwland et al., 2020; Liguori and Kisslinger, 2021, 2022; Loria et al., 2023; Shekari et al., 2023; Welsh et al., 2023).

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