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Analyzing the migration of cancer and stromal cells in a co-culture wound healing experiment using NYONE® imager

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Abstract

Cell Migration (i.e. the movement from one location to the other) is very important for the processes of invasion and metastasis of cancer cells. In recent years, it became clear that these processes are influenced by a complex cross-talk of malignant cells with non-neoplastic surrounding tumor stroma. Here, we co-cultured tumor and stromal cells stained with different CellTracker™ Dyes in a μ-Dish containing a Culture-insert 4 Well from ibidi. After removal of the insert, migration of the cells into the cell-free gap was imaged using NYONE® cell imager at regular intervals and quantified using image processing in YT-software®. We observed a higher migration of the co-cultured cells compared to cells in mono-culture. Unexpectedly, the stromal cells migrated towards the cancer cells and not vice versa. SYNENTEC's cell imager NYONE® proved to be a very helpful and time-saving tool in the imaging and analysis of these experiments.
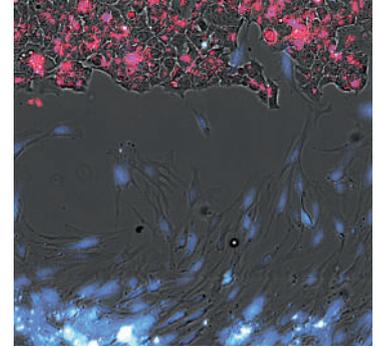
Keywords: migration, wound healing, scratch assays, microenvironment, tumor stroma, co-culture, live-cell imaging

Introduction

One of the original six hallmarks of cancer defined in a seminal paper by Hanahan and Weinberg in 2000 is 'activating invasion and metastasis' [1]. A common method to examine this hallmark is a wound healing or scratch assay. In this assay, a wound is generated in a monolayer of cells by scratching a cell-free gap. Cells start to migrate into this cell-free gap and eventually close the wound. The disadvantage of manual scratching (e.g. with a pipette tip) is the diversity in the resulting gaps making it difficult to compare different conditions and to reproducibly generate results. Therefore, commercial wound healing/ migration plates or dishes are available.

Here, we used the Culture-Insert 4 Well in a μ-Dish from ibidi. The Culture-Insert 4 Well consists of four wells, representing the four quarters of the round Culture-Insert. The wells are separated by a wall of 500 μm. When the wells are filled with adherent cells, a cell-free gap of approx. 500 μm is created between the adjacent wells after removing the Culture-insert 4 Well [2]. This format does not only allow the generation of uniform reproducible cell-free gaps, but also the co-cultivation of up to four different cell lines. This is a big advantage, as it became increasingly apparent that crosstalk between cancer cells and the tumor stroma (also

called microenvironment) is involved in the acquired capability for invasive growth and metastasis [1].
In this study, we analyzed the interaction between a cancer cell line and a stromal cell line. Therefore, we co-cultured these cell lines and compared the co-culture to mono-cultures of the two cell lines. Our hypothesis was that the cells in co-culture migrate more than the ones in mono-culture due to the cross-talk between the cells. The questions we aimed to answer were:

1) Do the cells migrate more in co-culture than in mono-culture experiments?
2) Which cell line migrates towards which?

In order to distinguish the cells in the gap, the cancer cells were stained with CellTracker™ Deep Red Dye with a previously determined, non-toxic or growth-inhibiting concentration (data not shown) while the stromal cells were stained with CellTracker™ Blue CMAC Dye. The cultures were imaged using NYONE® at regular intervals after removal of the insert.

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Material and Methods

Material

- Culture-Insert 4 Well in µ-Dish 35 mm, high (ibidi)
- Ibidi dish holder (produced by SYNENTEC)
- Cancer cell line
- Stromal cell line
- Accutase™ (Gibco/Thermo Fisher Scientific)
- DMEM medium (Gibco/Thermo Fisher Scientific)
- Fetal calf serum (PAN Biotech)

- DMEM FluoroBrite medium (Gibco/Thermo Fisher Scientific)
- CellTracker™ Deep Red dye (Molecular Probes/ Thermo Fisher Scientific)
- Cell Tracker™ Blue CMAC dye (Molecular Probes/ Thermo Fisher Scientific)
- NYONE® imager (SYNENTEC)

Methods

Cell culture and staining of the cells

Cells were cultured under routine cell culture conditions for the respective cell line. Cells were detached with Accutase™, centrifuged and resuspended in cell culture medium. Cells were counted using NYONE® cell imager and suspension cell count application to prepare a cell suspension with a defined cell number. Afterwards these suspensions were centrifuged again, resuspended in medium without fetal calf serum (FCS) containing 25 µM CellTracker™ Deep Red dye (tumor cells) or CellTracker Blue CMAC dye (stromal cells). Subsequently, they were incubated for 45 min in the incubator, centrifuged again at 540 x g for 5 min to remove the remaining CellTracker™ dye and resuspended in medium containing 10 % FCS.

Cell seeding and co-culture

35 000 cells/well were seeded in 75 µl into the ibidi dishes. Three conditions were analyzed:

1) mono-culture of tumor cells (tumor cells in all four wells)
2) mono-culture of stromal cells (stromal cells in all four wells)
3) co-culture of tumor and stromal cells (two wells with tumor cells and two wells with stromal cells).

For the mono-cultures, two of the four wells were seeded with unstained cells to observe whether the staining itself had any effect on migration. The dishes were fixed with an ibidi dish holder produced by SYNENTEC to ensure that the dishes were always imaged in the same orientation. The
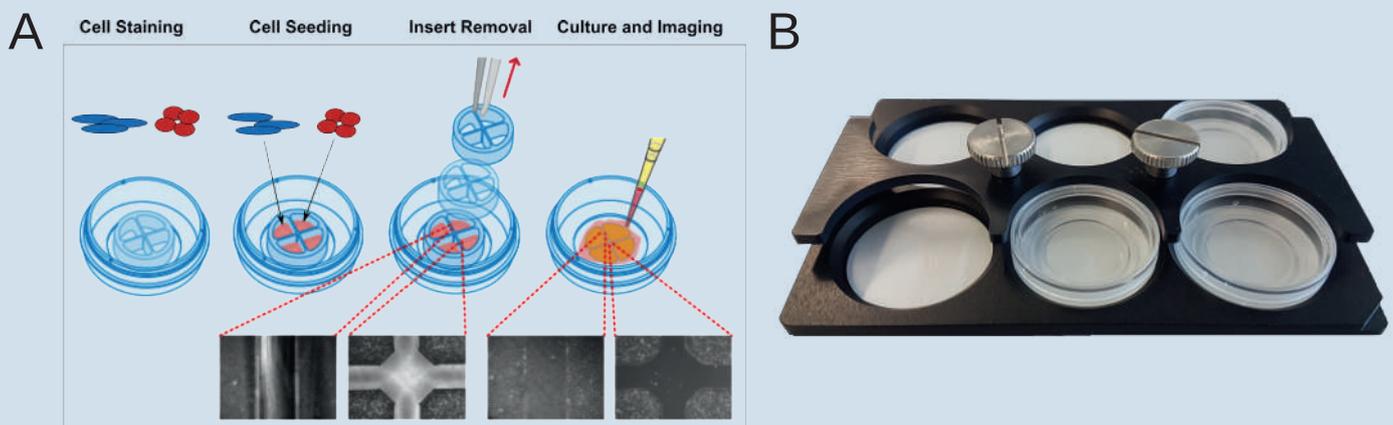
Fig. 1: Cell seeding and handling of the ibidi dishes
A) Schematic representation of the dish handling (picture modified from https://ibidi.com/removable-chambers/28-culture-insert-4-well.html) B) Picture of the ibidi dish holder produced by SYNENTEC

Tab. 1: Image acquisition parameters

Parameter	Brightfield	CellTracker™ Deep Red	Cell Tracker™ Blue CMAC
Filters (ex/em)	BF/Blue	Red/Red	UV/Blue
Intensity [%]	100	100	100
Exposure time [ms]	16	370	350
Gain [%]	16	50	60

following day, cells were washed with 100 µl DMEM FluoroBrite without FCS and the wells were filled with the same medium. This medium is a special medium for fluorescence applications and shows less autofluorescence than standard media. By removing FCS, the effect of proliferation was reduced as we were mainly interested in migration. After 4 h (and approximately 90 % confluence), the insert was removed using sterile forceps. To remove destroyed cells or debris, cells were washed twice with 2 mL DMEM FluoroBrite without FCS. Afterwards, the dishes were imaged using NYONE® imager.

Image acquisition
Cells were imaged directly after removal of the insert (0 h) and subsequently after 2, 18, 24, 44, 68 and 72 h ((image acquisition parameters shown in Tab. 1). After 18, 44 and 68 h, the medium was sterile-filtered (0.45 µm) to remove dead cells and debris in the medium. This filtration was necessary as the medium was collected at the end of the experiment to analyze it for secreted soluble

factors (cytokines, growth factors etc.). If this is not necessary, one can also just replace the medium with fresh one. However, by removing the old medium one might also remove the secreted factors and therefore disturb the co-culture system and falsify the result.

Image analysis
Images were analyzed at the end of the experiment with the Wound healing 2F (beta) operator. In this analysis, four regions of interest (ROIs) could be defined (Fig. 2A), and the confluent area in brightfield and fluorescent channels was analyzed within the ROI (Fig. 2B). However, as the CellTracker™ staining did not cover the whole cell and was very weak at the end of the experiment, we did not use the fluorescence signal for quantitative evaluation in our setting.

A **B**
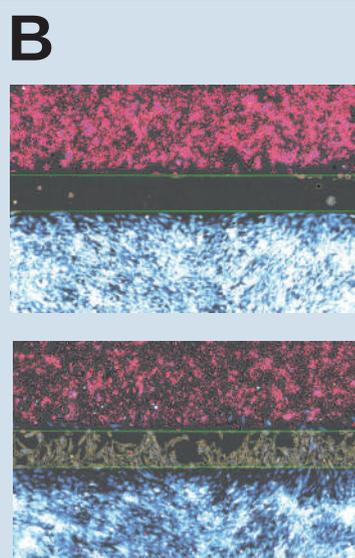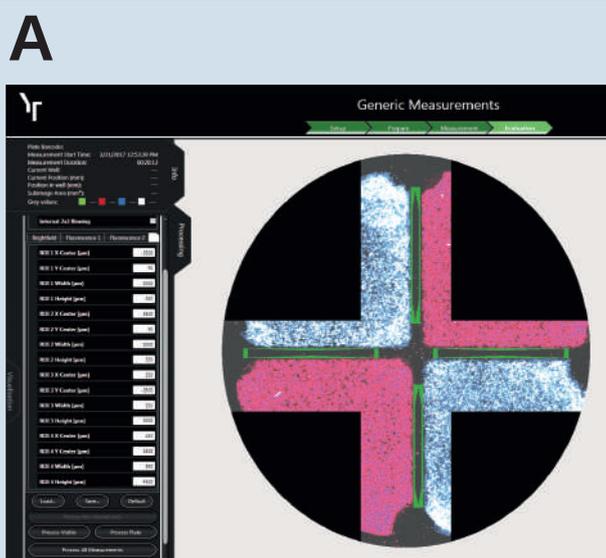
Fig. 2: Image acquisition and analysis
A) Only this areas of the ibidi µ-Dish, including the cell free gaps, were imaged. In the Wound healing 2F operator, four regions of interest (ROIs, green rectangles) were defined in the four cell-free gaps (at the first time point directly after removal of the insert). B) The operator evaluates the confluence within the ROIs for all measurements (0 h upper picture, 18 h lower picture).

Results and Discussion

Using NYONE® and the ibidi dish holder produced by SYNENTEC, it was possible to rapidly and accurately measure the ibidi dishes in an automated manner. Measurement duration for all three channels was appr. 5 min for each dish. With manual scratch assays and conventional microscopes, this process is very time-consuming as you have to relocate and image the exact same
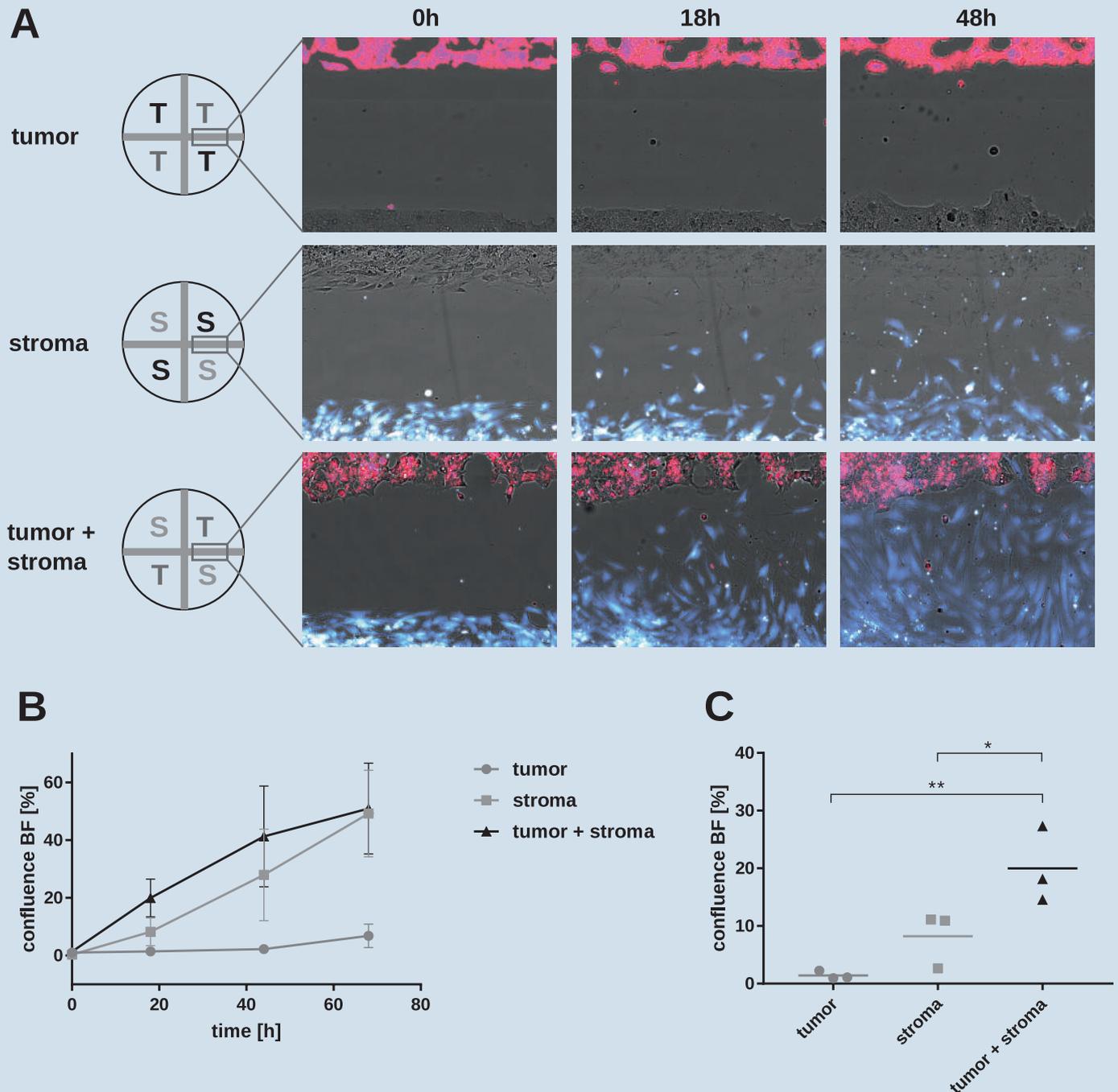
Fig. 3: Co-cultured cells showed a higher migration into the gap than mono-cultured tumor or stromal cells
A) Representative pictures of tumor (T, red or unstained), stromal (S, blue or unstained) or co-cultured (T, red; S, blue) cells at different time points. In the mono-cultured cells, only half of the cells were stained with CellTracker™ dye to analyze whether the staining had any effect on the migration. B) Analyzed cell confluence within the ROIs in the brightfield (BF) channel over time (n=3). C) Cell confluence within the ROIs in the BF image at time point 18 h.

spot at every time point. Using SYNENTEC's NYONE® imager and YT-Software® enables the user to easily acquire the images and switch between measurements in the exact same location within the experiment to visually inspect image processing and cell morphology.

We observed a higher migration of co-cultured cells than of cells in mono-culture. This was seen qualitatively and also quantified as described in Material and Methods (Fig. 3). In this setting with these specific cell lines, the stromal cells migrated more than the tumor cells as observed in the mono-cultures (Fig. 3). However, with this knowledge, one would expect a higher confluency in the stromal mono-cultures than in the co-cultures as the stromal cells migrate into the gap from both sites. However, this was not the case, but the confluency was higher in the co-cultures, indicating that the stromal cells are attracted or stimulated by the tumor cells.

An overlay of the brightfield image with the fluorescent images allowed distinguishing both cell lines. Therefore, we could not only analyze the closure of the wound but also observe, which cell line migrated into the gap. Interestingly, in this experiment, the stromal cells migrated towards the cancer cells and not vice versa as expected beforehand (Fig. 4). As the fluorescence of the stained cells was very weak and punctual at the end of the experiment, unfortunately the quantification of these signals did not represent the whole cell area and therefore, we did not evaluate it further. However, with a stronger staining intensity or other cell lines maybe expressing two or more fluorescent proteins, the Fluorescent Area 2F image processing could have been used and already has been in a different experimental setting (data not shown).

Lastly, the morphology of individual cells could be observed. Here, we saw that single stromal cells migrated into the gap. They stretched out, became very flat and changed their morphology (Fig. 4). This morphologic change is a hint that we really observed a process of migration rather than proliferation, where the whole cell front usually moves into the gap.

Conclusion

Imaging wound healing assays with automatic cell imagers is a very helpful and time-saving approach to generate reproducible results. SYNENTEC's NYONE® proved to be very useful for such experiments. The ability to measure various fluorescent colours enables users to fluorescently stain the cells in order to differentiate them in co-culture experiments. Its precise auto-focus, high-resolution optics and image analysis are great tools enabling wound healing experiments in a high-throughput manner.
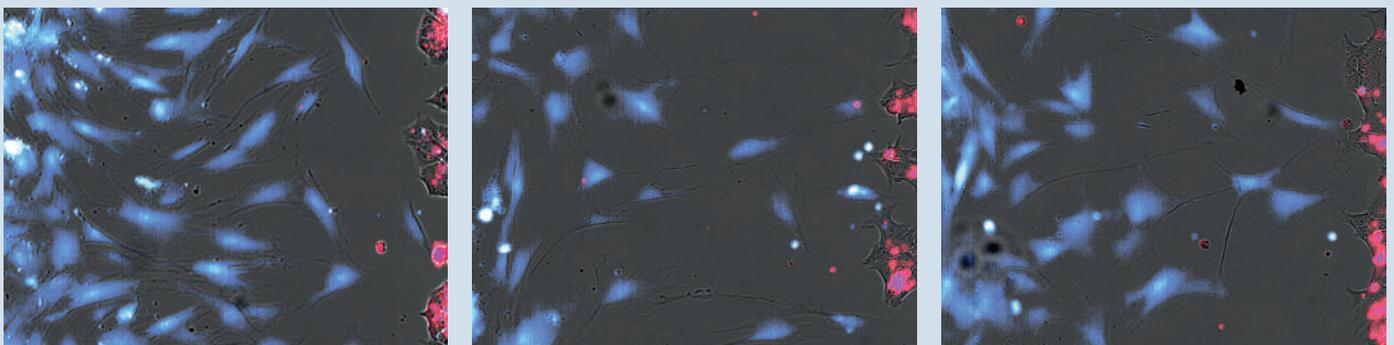
Fig. 4: Stromal cells migrated towards cancer cells in our setting
Representative pictures of co-cultured cancer (red) and stromal (blue) cells 18 h after removal of the insert The morphology of single cells could be evaluated.

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Comments

As this Application note contains confidential/unpublished data, no further details about the used cell lines etc. can be released at the moment by SYNENTEC.

References

[1] D. Hanahan and R. A. Weinberg, 'Hallmarks of cancer: the next generation.', Cell, vol. 144, no. 5, pp. 646–674, 2011.

[2] Ibidi, 'Instructions Culture-Insert 4 Well'. 26-Sep-2018.

All applications are compatible with all models of NYONE® and CELLAVISTA® and are readily available in YT-software®.

Cellavista® 4.0   NYONE®

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