High-throughput monitoring of cancer cell/fibroblast co-cultures, and their interaction with TNF-α

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Introduction

Live-cell imaging enables researchers to determine not only whether, but also when and how certain cellular events occur in culture. When compared to brightfield-only imaging, fluorescence live-cell imaging multiplies the number of read-outs– and consequently the obtained information from one experiment – with the number of fluorescence channels. In cellular co-cultures, fluorescence imaging facilitates distinction of the cell types, when cell types are assigned specific fluorescent labels. Quantitative and qualitative read-outs per cell type are then based on the respective fluorescent labels [1].

Co-culture setups are commonly applied in cancer research, since tumors and their environment involve many different cell types: e.g., immune cells aim for elimination of the cancer cells [2], endothelial cells are stimulated to vascularize a tumor [3], and fibroblasts can either promote or inhibit tumor growth [4-6]. Two types of fibroblasts have been found in tumor cell microenvironments. On the one hand, cancer-associated fibroblasts (CAFs) have tumor-promoting and immunosuppressive activity. These fibroblasts get activated by tumor necrosis factor-α (TNF-α), which is secreted by cancer cells [6-9]. On the other hand, normal-associated fibroblasts (NAFs) secrete TNF-α themselves, thereby inhibiting tumor growth [6, 7]. The fact that TNF-α can perform both conflicting functions relates to the TNF receptors expressed by tumor cells, TNFR1 and TNFR2. These receptors influence each other's downstream effects, and thereby together orchestrate TNF-α functionality in a tumor microenvironment-dependent manner [7].

The confluence per cell type can provide insight into interactions between cancer cells and different fibroblast types: CAFs supporting cancer cell proliferation or NAFs competing with and eliminating tumor cells [6]. Fundamental knowledge involving these cellular interactions can be relevant in cancer research, where potential clinical therapies could be based on insight in conditions in which cancer cells are eliminated by surrounding fibroblasts. To study interaction effects of cancer cell/fibroblast co-cultures and TNF-α, all possible combinations of conditions per experimental variable have to be covered. However, this two-factor experiment design requires an imaging system that enables high-throughput fluorescence live-cell imaging of the co-cultures over time. Currently, fluorescence live-cell imaging is predominantly performed using a fluorescence microscope with a stage-top incubation box. However, regulation of the culture conditions in the incubation box – usually 37°C and 5% CO_2 – is more sensitive to variations compared to a dedicated incubator [10, 11]. Besides that, motorized movement of the sample stage is necessary to image all wells in a culture plate used for high-throughput experiments. This increases not only the total cost of the setup, but medium flow resulting from sample movement can also alter cell behavior and consequently the experiment results [12].

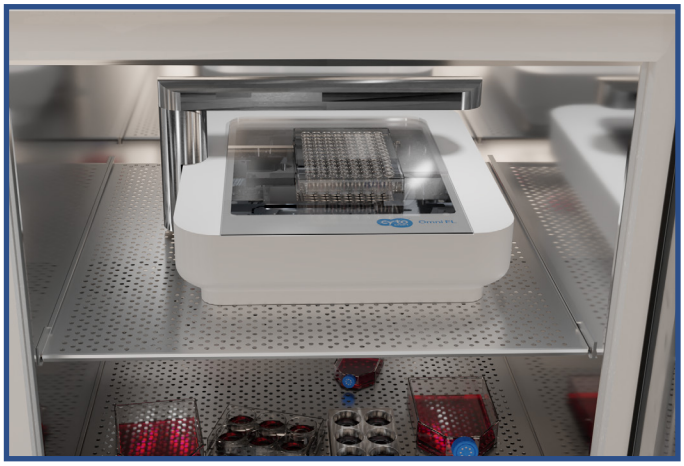

Fig. 1. The CytoSMART® Omni FL

The CytoSMART® Omni FL (Fig. 1) is a high-throughput fluorescence live-cell imager that could overcome these issues. The device can be placed on a single shelf of a regular incubator, and therefore enables two-channel fluorescence live-cell imaging (green, red) of a well-plate in a constant and optimal culture environment. Since the fluorescence module and camera are moving while the plate remains stationary, all wells in a culture plate can be imaged without medium flow resulting from sample movement disturbing the cells.

In this proof-of-concept study, we determined the effect of both the seeding ratio and TNF-α concentration on proliferation of a cancer cell line and fibroblasts in co-culture. The CytoSMART® Omni FL and corresponding Cloud-based image analysis algorithm for brightfield and fluorescent channel confluence were applied. With these tools, the kinetic confluence profiles of fluorescently labeled HeLa cells (cervical carcinoma cell line) and 3T3 cells (fibroblasts) in co-cultures with six different seeding ratios and four different TNF-α concentrations were determined. This high-throughput experiment provided fundamental insight into TNF-α-dependent interactions between the cell types, which may ultimately be related to tumor growth.

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Materials and methods

tGFP-labeled HeLa cells (Innoprot P20107; green fluorescent) and non-fluorescent 3T3 fibroblasts (ATCC® CL-173™) were separately cultured to sub-confluency in DMEM (Gibco) supplemented with 10% FBS (Gibco) and 1% pen-strep (Gibco), under standard culture conditions (37°C; 5% CO_2). Co-cultures of HeLa cells and 3T3 cells were seeded in a 24-well plate at a total density of 50,000 cells per well, at ratios of 1:0, 10:1, 2:1, 1:2, 1:10, and 0:1 (HeLa : 3T3).

Co-cultures at each of the cell ratios were exposed to various concentrations of TNF-α (Merck H8916): 0 ng/ml, 0.2 ng/ml, 2 ng/ml, and 20 ng/ml [13, 14]. All conditions were monitored for 2 days using the CytoSMART® Omni FL (37°C; 5% CO_2), at 3 locations per well, and a 1 h imaging interval. At each location, the brightfield and green fluorescence channel were imaged. Confluence per cell type was determined using the integrated algorithm for brightfield and fluorescence confluence in the CytoSMART® Cloud.

Results

End-point images per well are displayed in Fig. 2, and the quantified confluence graphs per cell type over time are shown in Fig. 3.

The final ratio HeLa : 3T3 cells was dependent on the seeding ratio, where the abundant cell type in seeding inhibited growth of the other cell type in the 10:1 and 1:10 seeding ratios. TNF-α shifted the final cell ratio, with the 3T3 cells thriving in the co-cultures with increasing TNF-α concentration. Remarkably, with higher TNF-α concentrations, confluence of HeLa cells in mono-culture increased, whereas it decreased for 3T3 cells in mono-culture.

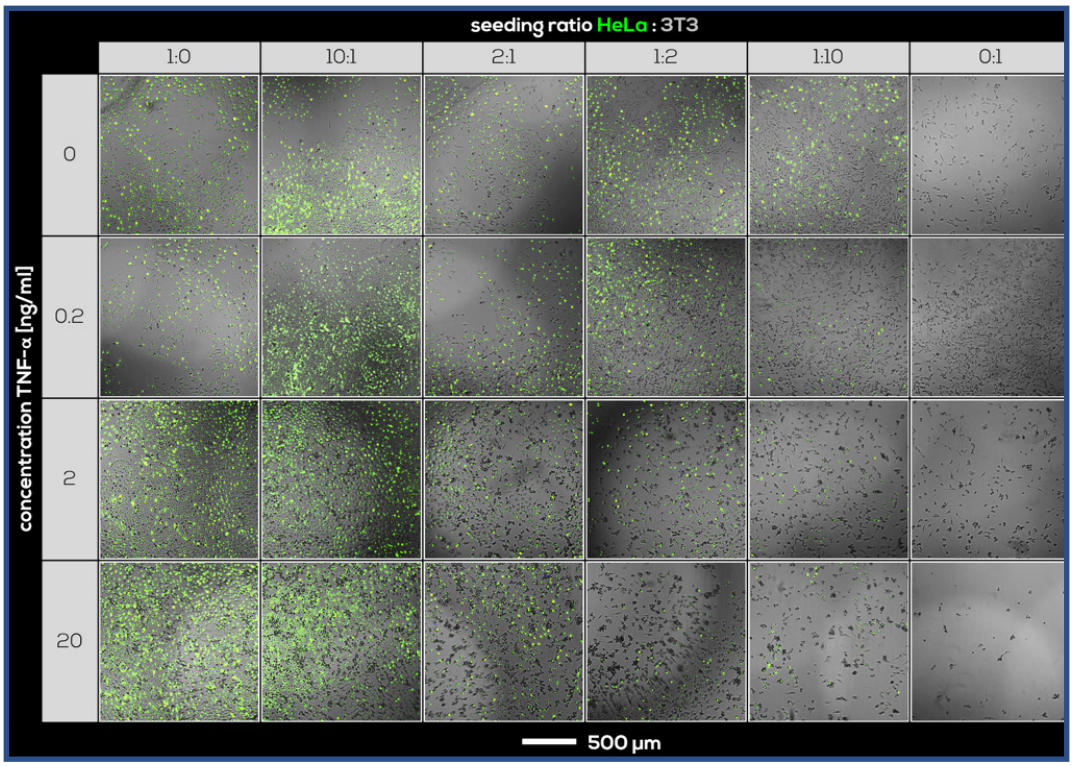

Fig. 2. Representative end-point images of HeLa : 3T3 co-cultures, corresponding to the various seeding ratios and TNF-α concentrations. Green fluorescent HeLa cells and non-fluorescent 3T3 fibroblasts were seeded at 1:0, 10:1, 2:1, 1:2, 1:10 or 0:1 ratios, and exposed to 0-20 ng/ml TNF-α for 48 h. 3 images per well were made at each time point.

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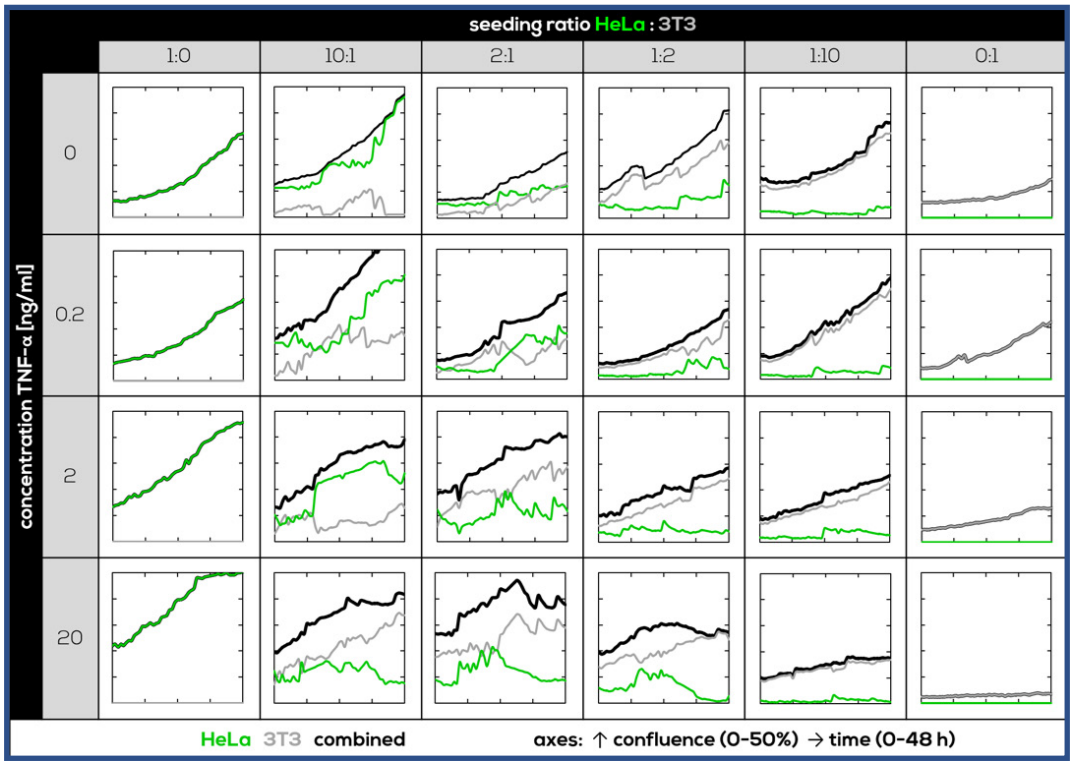

Fig. 3. Confluence quantifications per cell type over time for HeLa : 3T3 co-cultures, corresponding to the various seeding ratios and TNF-α concentrations. All conditions were monitored with the CytoSMART® Omni FL over 48 h with a 1 h imaging interval and 3 images per well. The confluence per channel was assessed in the CytoSMART® Cloud, and averaged over the 3 images per well. All graphs have a vertical axis range of 0-50% confluence, and a horizontal axis range of 0-48 h.

Discussion

Fluorescence live-cell imaging enables distinction of cell types in co-cultures, by assigning a fluorescent label to a cell type. This can provide fundamental knowledge regarding cell types either supporting each other's proliferation or competing and eliminating each other, which can serve as base for potential therapies in e.g., cancer research. Literature reports the existence of CAFs supporting and accelerating tumor growth on one hand, and NAFs with tumor-inhibiting properties on the other hand. A key role in orchestrating the interaction between cancer cells and fibroblasts is ascribed to TNF-α. Therefore, we aimed to demonstrate the use of the CytoSMART® Omni FL for long-term high-throughput experiments with fluorescence live-cell imaging, and investigate the interaction effect of cancer cell/fibroblast ratio and TNF-α concentration on cell proliferation in HeLa : 3T3 co-cultures.

Application of fluorescence live-cell imaging in co-culture experiments is limited due to various (practical) issues: the culture environment inside a top-stage incubation box on a high-end fluorescence microscope is sub-optimally regulated. Besides that, a moving sample stage increases the costs of the setup and resulting medium flow can disturb the cells. These issues were overcome in our experimental setup with the CytoSMART® Omni FL. The compact system enabled the cells to be cultured in the optimal and consistent environment of a regular incubator. Besides that, the well plate could remain stationary because of the Omni FL's moving camera, avoiding unnecessary medium flow.

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The confluence quantifications over time indicated competition between the HeLa cells and the 3T3 cells. However, which of the cell types was eliminated was dependent on the initial ratio. In previous research, it was found that NAFs can prevent tumor growth [6]. We found this is only possible with a minimal initial percentage of fibroblasts. NAFs can suppress tumor growth via TNF-α excretion [6]. However, we observed that tumor-suppressing properties of NAFs became stronger with higher environmental TNF-α concentrations. The Omni FL enabled monitoring of this multi-factor experimental setup, thereby revealing the interaction between the seeding ratio and the TNF-α concentration in establishing the dominant cell type.

Conclusion

In this study, we successfully showed the application of high-throughput fluorescence live-cell imaging in cell confluence monitoring in a co-culture experiment with a two-factor experimental design: cell seeding ratio and TNF-α concentration. The integrated image analysis of the CytoSMART® Omni FL enabled automated confluence measurements for both channels – brightfield as well as green fluorescence – and at all conditions. This revealed the competition between HeLa cells and 3T3 fibroblasts in co-cultures, where the initially most abundant cell type seemed to eliminate the other cell type. Besides that, higher concentrations of TNF-α suppressed cancer cell survival and promoted fibroblast growth in co-cultures, whereas this effect was opposite in mono-cultures.

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