Monitoring the confluence ratio per cell type in co-cultures with varying seeding ratios

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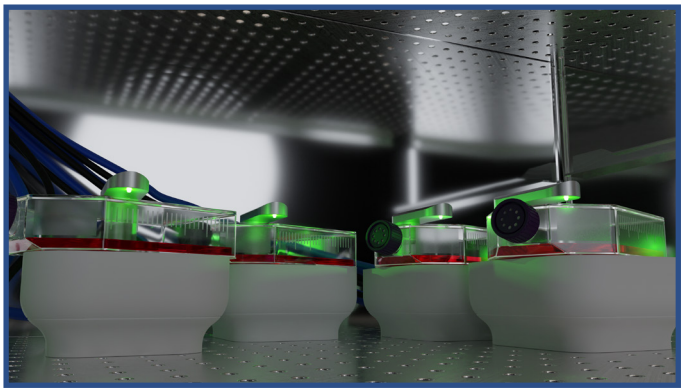
Introduction

Live-cell imaging enables researchers to determine not only whether, but also when and how certain cellular events occur in culture. By including one or multiple fluorescent probe(s) and corresponding channel(s) – besides a brightfield channel – the number of read-outs and consequently the obtained information from one experiment increases. This strategy is for example applied in co-cultures. Each included cell type is then assigned a particular fluorescent label, which facilitates distinction of the cell types based on their label and (quantified) read-outs per cell type [1]. Co-cultures are widely used in cancer research, since many different cell types play a role in or around tumors: immune cells aim for elimination of the cancer cells [2], endothelial cells are stimulated to vascularize a tumor [3], fibroblasts can either promote or inhibit tumor growth [4-6], and many more. A relevant determined read-out in this context is the confluence per cell type in the co-culture, which provides insight into the cell types either supporting each other during proliferation or competing and eliminating each other [1]. These interactions relate to the number of cells of the various cell types, where the cell type with the largest relative presence is generally dominant, but this depends on the specific interaction [7]. Potential clinical cancer therapies could be based on fundamental knowledge regarding the interactions between cancer cells and other cell types. However, a proper imaging system for the fluorescence live-cell imaging of the co-cultures is a prerequisite to gain sufficient insight in the interactions.

Currently, fluorescence live-cell imaging is predominantly performed using a fluorescence microscope with a stage top incubation box. However, the regulation of the culture conditions in the incubation box – usually 37°C and 5% CO_2, with the latter regulating the culture medium pH – is more sensitive to variations compared to a dedicated incubator [8, 9]. If the cells in a co-culture have different sensitivities to temperature and pH [10, 11], the variability in these factors may disturb the cultures and distort results.

There are also practical issues when using a regular microscope. Although images are only captured at certain time intervals, the microscope is unavailable for other users during the entire live imaging experiment. It is practically infeasible to optimize microscope use by performing continuous imaging



experiments during the waiting intervals of the time-lapse experiment. Besides that, only one culture vessel can be imaged per run. If multiple conditions in separate vessels need to be covered, repeated runs of the same experiment may be required, or multiple culture vessels may need to be placed under the microscope alternately to be imaged manually. Both strategies are labor-intensive, time-consuming, and increase variability in culture conditions and consequently in results.

A system that could overcome these issues is the CytoSMART® Multi Lux3 FL. This is a dedicated system for long-term time-lapse imaging, enabling optimal use of imaging systems within a laboratory. It fits in a regular incubator, and therefore enables undisturbed two-channel fluorescence live-cell imaging (green, red) in a constant and optimal culture environment. By connecting four devices within the same incubator to a single laptop, multiple conditions can be monitored simultaneously without variation in temperature and CO_2 level. The automated cloud-based image analysis enables confluence analysis per cell type over time. Consequently, co-culture comparison studies can be performed with minimal variability in culture- and imaging conditions, and directly available confluence data per imaging channel – and consequently per cell type.

In this proof-of-concept study, we demonstrate the applicability of simultaneous fluorescence live-cell imaging to determine the effect of various seeding ratios on proliferation of a cancer cell line and fibroblasts in co-culture. The CytoSMART® Multi Lux3 FL and corresponding cloud-based image analysis algorithm for confluence were used to determine confluence over time of fluorescently labeled HeLa (cervical cancer cell line) and 3T3 cells (fibroblasts) in co-cultures with various seeding ratios. This provided fundamental insight into 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 fibroblasts were seeded in 24-well plates at a total density of 50,000 cells per well, at ratios of 1:1, 3:1, 10:1 and 20:1 (HeLa:3T3).

The co-cultures were monitored for 90 h using the CytoSMART® Multi Lux3 FL (37°C; 5% CO_2), making a snapshot every 1 h, with the brightfield and green fluorescent channel as well as confluence analysis for both channels activated. Confluence per cell type was determined in the CytoSMART® Cloud, using the integrated algorithm.

Results

Figure 1 displays images from the time-lapses of the four seeding ratios, as well as the determined confluence per cell type over time. In all seeding ratios, the HeLa cells almost completely eliminated the 3T3 fibroblasts. At the more equal ratios, the 3T3 cells initially proliferated, increased in confluence until total confluence reached ±50%, and were eliminated afterwards. However, in the more skewed ratios, the 3T3 cells barely proliferated. No major difference in total proliferation rate between the conditions could be observed.

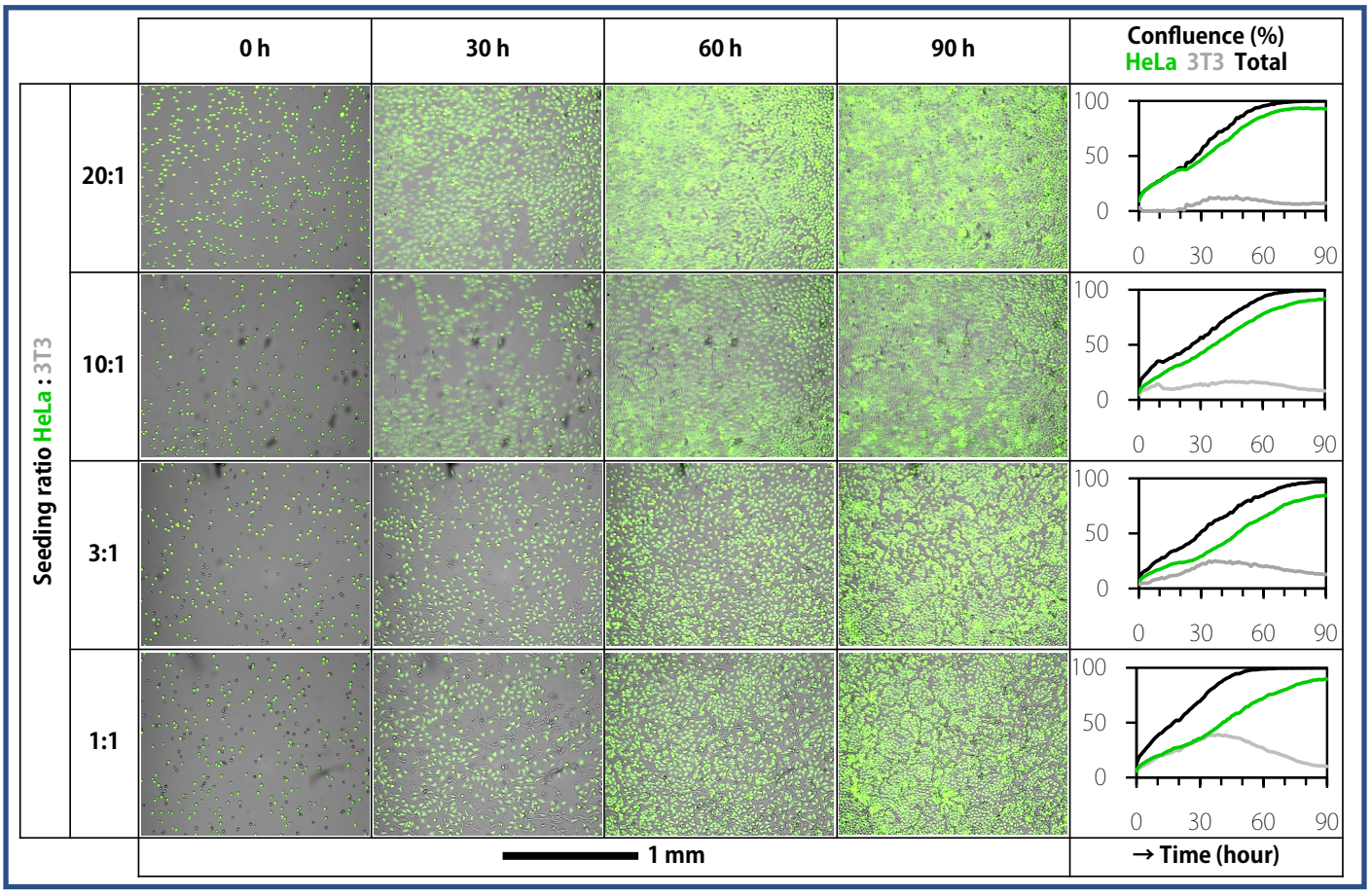

Fig. 1. Simultaneous comparison of confluence per cell type in HeLa-3T3 co-cultures. Green fluorescent HeLa cells and non-fluorescent 3T3 fibroblasts were seeded at ratios of 1:1, 3:1, 10:1 and 20:1, and monitored by making an image every 1 h during 90 h. Confluence per cell type was determined from confluence per imaging channel.

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Discussion

In co-cultures, fluorescence live-cell imaging can facilitate distinction of the cell types, when each included cell type is then assigned a particular fluorescent label. In cancer research, potential therapies could be based on fundamental knowledge regarding cell types either supporting each other's proliferation or competing and eliminating each other. However, various (practical) issues impede the extensive use of fluorescence live-cell imaging in such fundamental co-culture experiments: the culture environment in a stage-top incubation box on a high-end fluorescence microscope is generally regulated sub-optimally, the high-end microscope is occupied during the entire experiment whereas images are only captured at certain intervals, and comparison of multiple experimental conditions is difficult and labor-intensive. Therefore, we aimed to demonstrate the use of the CytoSMART® Multi Lux3 FL for long-term comparison experiments with fluorescence live-cell imaging, and investigate the interactions in cell proliferation in co-cultures of HeLa cells with 3T3 fibroblasts.

In our experimental setup with the CytoSMART® Multi Lux3 FL, the existing (practical) issues of fluorescence live-cell imaging were overcome. The cells could be cultured in the optimal environment of a dedicated laboratory incubator. Since the incubator remained undisturbed during the entire experiment, temporal and inter-sample variability in culture environment was minimized. This made comparison of conditions straightforward, and no manual intervention was required. The dedicated system for long-term monitoring was used, rather than a high-end microscope that could also be used for other applications.

The confluence quantifications over time indicated that the HeLa cells eliminated the 3T3 cells at any seeding ratio, although the initial ratio affected the time before this elimination was initiated. This effect was clearly visible with live-cell imaging, whereas end-point imaging would only have shown the similar final ratios.

In a previous co-culture study involving HeLa cells and fibroblasts [4], it was found that in high seeding densities, the HeLa cells overgrew the fibroblasts at any ratio of the cell types. However, at low seeding densities, both cells could proliferate. This seems to match the profile over time found in our study, with the 3T3 cells proliferating until the total confluence was around 50%, and the HeLa cells eliminating the fibroblasts when densities became higher.

When comparing the seeding ratios, no clear trend in total proliferation rate was observed: these were similar for all conditions. In previous research [5], it was found that metabolic rates of tumor cells were lower in co-culture than in mono-cellular systems, indicating no stimulating role for the investigated fibroblasts in tumor growth. However, the fibroblasts also did not appear to play an inhibitory role in our experiments.

This role of regular fibroblasts preventing tumor growth was described before [6]. Consequently, the existence of specific cancer-associated fibroblasts was introduced, which are more active than regular fibroblasts, and create an optimal environment for tumor cells to grow. Therefore, tumor growth is supported and accelerated by the presence of these cancer-associated fibroblasts. In our research, the culture niche for the 3T3 fibroblasts was apparently insufficient to act as regular fibroblast and prevent HeLa proliferation. However, these cells also did not possess the properties of cancer-associated fibroblasts, since they did not accelerate cancer cell growth.

Conclusion

In this study, we provided a successful proof-of-concept for the use of fluorescence live-cell imaging in simultaneous monitoring of cell confluence throughout a co-culture experiment with various seeding ratios. Confluence measurements for both channels (brightfield, green) and all conditions could be performed simultaneously and fully automatically with the integrated image analysis of the CytoSMART® Multi Lux3 FL. This revealed elimination of 3T3 fibroblasts by HeLa cells in a co-culture, where the initial seeding ratio determined how long the 3T3 cells could proliferate before elimination.

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