Bioscience Solutions

Simple and Easy Monitoring of Tube Formation and Migration Assays with the CytoSMART™ Live Cell Imaging System

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Introduction

Movement of cells plays a critical role in the development of cancer. Analyzing the motility of cells *in vitro* is therefore important for many cancer researchers. This White Paper describes the set-up and analysis of three different cancer-relevant assays – the endothelial cell tube formation assay, a 2D cancer cell migration assay and a 3D cancer cell invasion assay. In all cases, the CytoSMART™ Lux 10X Cell Monitoring System was used to follow cell movement in real-time and label-free. Label-free live cell imaging, thus live cell imaging without the addition of any dyes, markers or reporter genes, allows capturing dynamic processes in cell culture without risking potential side-effects of the used markers or dyes.

Endothelial Cell Tube Formation Assay

Angiogenesis, the formation of new blood vessels, is required to ensure sufficient nutrient and oxygen supply and to allow solid tumors to grow beyond a certain size. It also plays a critical role in cancer metastasis. Inhibition of angiogenesis is thus a key target in a number of cancers including breast, prostate, ovary, lung, colon, rectum, and brain (glioma).[1–6]

Angiogenesis is a multi-step process that results in the creation of new blood vessels from pre-existing vasculature and is mediated primarily by endothelial cells. It involves multiple steps: basement membrane disruption, endothelial cell migration, invasion, proliferation and differentiation into capillaries. One of the key steps of this process is the assembly of endothelial cells into tubes – this is known as tube formation. This process can be modeled *in vitro* by plating endothelial cells onto Basement Membrane Extract (BME) and examining the branching structures within 12 to 24 hours of cell plating.[7,8]

In this White Paper, we analyze the dynamics of tube formation of Human Umbilical Vein Endothelial Cells (HUVEC) and the impact of Suramin on tube formation. Suramin is a specific and competitive inhibitor of G-protein-coupled receptor (GPCR) activity and impacts multiple outputs of tubule formation, i.e. number of junctions and the total tubule length.[9]

2D Cell Migration Assay

In cell biology research, the term migration generally refers to the movement of cells on two-dimensional (2D) surfaces that lack an obstructive fiber network (such as plastic or glass, or thin coatings of extracellular matrix proteins like collagen I or basement membrane extract). However, the term migration also includes the movement of cells through three-dimensional (3D) structures, without destruction of the 3D barrier. This process is for example observed when passenger leukocytes migrate through 3D tissues and do not require proteolytic action and tissue remodeling.[10]

A basic test to measure the non-destructive migration potential of cells in 2D is to monitor the closure of a so-called wound or scratch in a confluent monolayer of cancer cells.[11] Scratches can be introduced into cellular monolayers by simply scraping of an area of cells with a plastic pipet tip. Scratches of more defined size can be created using specialized replacement devices or silicone inserts. Another advantage of the use of silicone inserts for scratch formation is the homogeneity of the biochemical composition of the scratch. Silicone inserts create a scratch surface that is largely free of extracellular matrix (ECM) and cell debris. Pipet tip induced scratches can contain undefined remains of ECM and cell debris in various quantities. Therefore tools like silicone inserts for scratch formation can lead to less result variability.

In this White Paper, we analyze the migration of the human colorectal carcinoma cell line HCT 116 with a specialized silicone insert system (ibidi). The impact of DL-Sulforaphane, a phytochemical with migration inhibitory properties and anticarcinogenic activity,[12] was quantified.

3D Cell Invasion Assay

In contrast to the non-destructive migration of cells, invasion is defined as the movement of cells through a 3D matrix and typically includes attachment to, as well as degradation and restructuring of matrix components. One typical *in vitro* invasion assay is the so-called Boyden chamber assays. The porous filter of transwell inserts is overlaid by a layer of extracellular matrix (ECM) and the invasion of cells from the upper side of the porous filter to the lower side is observed.[11]

Cancer cells *in vivo* often show invasion-type movement through ECM. This process can be mimicked in an assay that is an alternative to Boyden chamber assays. Cancer cell aggregates – so called spheroids – are embedded into a 3D matrix and their invasion into the 3D matrix is monitored microscopically. Non-invasive cancer cell lines remain as compact spheroids with a distinct border to the surrounding 3D matrix, whereas invasive cell lines start to invade into the surrounding matrix and display astral outgrowth from the spheroid.[11]

In this White Paper, we analyze the invasion properties of two invasive cell lines – the human, presumably glioblastoma cell line U-87 MG and the human fibrosarcoma cell line HT-1080 – from spheroid structures into a 3D matrix. The impact of DL-Sulforaphane on cell invasion was quantified. For both cell types DL-Sulforaphane reduced the "sprouting activity" in a concentration dependent manner. Interestingly, while the shape of the invading U-87 structures was unaffected by DL-Sulforaphane, medium DL-Sulforaphane concentrations impacted the HT-1080 "sprouting structures".

Materials and Methods

The CytoSMART™ Lux 10X System

The CytoSMART™ Lux 10X System (Lonza) is an easy-to-use and affordable live cell monitoring system. The small footprint is ideal for placing into a standard cell culture incubator. It has been designed for applications, which require a larger field of view, such as cell culture documentation and migration assays. The field of view is 2.4 × 1.5 mm and the magnification is similar to that typically achieved with a 10x objective using a conventional microscope. The images and videos can be monitored anytime and anywhere, via smart phone, tablet or computer with the integrated cloud functionality.

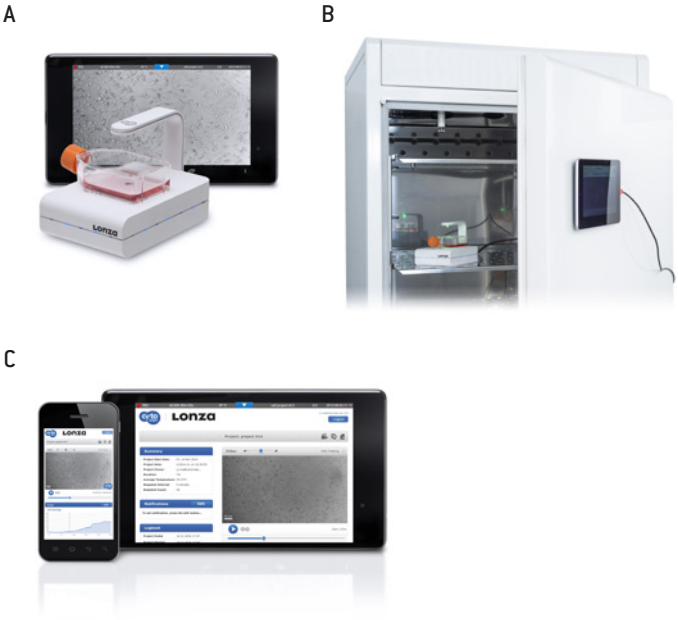

Figure 1
A) The CytoSMART™ Lux 10X System **B)** The CytoSMART™ Lux 10X System installed in a cell culture incubator **C)** Results can be monitored anytime and anywhere, via smart phone, tablet or computer

Endothelial Cell Tube Formation Assay

Human Umbilical Vein Endothelial Cells (HUVEC, Lonza Cat. No.: C2519A) were cultured in EGM™-2 Medium (Lonza Cat. No.: CC-3162) according to the supplier's instructions. Engelbreth-Holm Swarm Sarcoma-derived Basement Membrane Extract (BME, Matrigel™, Corning Cat. No.: 356237) was thawed overnight on ice at 4°C. 150 µL of BME were transferred using ice-cooled pipet tips into ice-cooled standard 48-well cell culture plates (Corning™ Costar™ Cat. No.: 3548). If air bubbles were observed in the BME, plates were centrifuged at 300 g for 10 minutes at 4°C. Subsequently, plates were incubated for 10 minutes at room temperature, followed by 30 minutes incubation at 37°C / 5% CO_2. 125 µL of EGM™-2 Medium was added to each well and incubated for 30 minutes at 37°C. HUVEC were harvested according to the instructions given by the supplier. 50,000 HUVEC in 125 µL of EGM™-2 Medium were added to the BME-coated cell culture wells. The plate was positioned onto the CytoSMART™ Lux 10X Device inside a standard 37°C / 5% CO_2 cell culture incubator (Heraeus, HERAcell™ 240). Formation of endothelial tubes was monitored live over a period of 16 – 24 hours in 5-minute intervals in selected wells.

In order to evaluate the effect of Suramin (Sigma Cat. No.: S2671) on tube formation, Suramin was diluted at various concentrations in EGM™-2 Medium. 125 µL of Suramin-containing EGM™-2 Medium was added to each well of the BME-coated cell culture plate and incubated for 30 minutes at 37°C. 50,000 HUVEC in 125 µL of EGM™-2 Medium were added and selected wells were monitored as described above. For quantitative analysis images were captured 18 hours after seeding in three different positions of each well by using the CytoSMART™ Lux 10X Device like a standard cell culture microscope.

In order to evaluate the viability of HUVEC in BME-coated cell culture plates, 2.5 µL of Calcein AM (Life Technologies Cat. No.: C3100MP) and 5 µL of Propidium Iodide (PI, Sigma Cat. No.: P4170) were added to each well resulting in a final concentration of 0.4 µM Calcein AM and 10 µg/mL PI. Images were captured after 15 – 30 minutes with Zeiss AxioObserver Z.1 microscope equipped with a Zeiss N-Achroplan 5x objective, appropriate fluorescence filters and an AxioCam MRm camera.

2D Cell Migration Assay

The human colorectal carcinoma cell line HCT 116 (ATCC Cat. No.: CCL-247) was cultured in McCoy's 5A medium (Lonza Cat. No.: BE-12-688F) supplemented with 10% FBS, 100 U/mL Penicillin and 100 U/mL Streptomycin (Lonza Cat. No.: DE17-602E) according to the instructions given by the supplier. 40,000 cells were resuspended in 70 µL of cell culture medium and seeded into each chamber of the culture-insert (ibidi Cat. No.: 81176). This corresponds to a seeding density of 1,800 cells/cm^2. The culture-insert was placed into 35 mm micro-dishes. HCT 116 cells adhered and reached confluence after approximately 24 hours in a standard 37°C / 5% CO_2 cell culture incubator. Subsequently, the culture insert was gently removed using sterile tweezers. A defined scratch with a gap width of 500 µm is formed. The 35 mm micro-dish was washed carefully with 1 mL of cell culture medium to remove any non-adherent cells that could otherwise settle into the gap. 2 mL of medium were added to each micro-dish,

containing various concentrations of DL-Sulforaphane (Sigma Cat. No.: S4441-5mg). The micro-dishes were positioned onto the CytoSMART™ Lux 10X Device inside a standard 37°C / 5% CO_2 cell culture incubator. In order to facilitate image analysis, it is critical that the scratch is positioned either horizontal or vertical on the CytoSMART™ Device. Gap closure was monitored live over a period of 40 hours in 5 – 15-minute intervals. In our experience optimal results for scratch assays are obtained when monitored with an imaging frequency of 15 minutes.

3D Cell Invasion Assay

The human, presumably glioblastoma cell line U-87 MG (ATCC Cat. No.: HTB-14) and the human fibrosarcoma cell line HT-1080 (ATCC Cat. No.: CCL-121) were cultured in EMEM medium (Lonza Cat. No.: BE-12-662F) supplemented with 10% FBS, 100 U/mL Penicillin, 100 U/mL Streptomycin (Lonza Cat. No.: DE17-602E) and 2 mM UltraGlutamine I (Lonza Cat. No.: BE17-605E/U1) according to the instructions given by the supplier. 3,000 cells were plated in 50 µL 1x Spheroid Formation ECM (Trevigen Cat. No.: 3500-096-K) in culture medium into round-well ultra-low attachment plates (Corning Cat. No.: 7007). Plates were centrifuged for 3 minutes at room temperature and 200 g and subsequently cultured for 3 – 4 days in a standard 37°C / 5% CO_2 cell culture incubator. Once spheroids have formed, they were overlaid with BME cell invasion matrix (Trevigen Cat. No.: 3500-096-K). To that end, the cell invasion matrix was thawed overnight on ice at 4°C. If air bubbles were observed, the matrix was centrifuged at 300 g for 5 minutes at 4°C. 50 µL of invasion matrix were transferred using ice-cooled pipet tips onto the spheroids. Plates were centrifuged at 300 g for 10 minutes at 4°C. Subsequently, plates were incubated for 1 hour at 37°C / 5% CO_2. 100 µL of cell culture medium containing various concentrations of DL-Sulforaphane (Sigma Cat. No.: S4441-5mg) was added to each well. The culture plates were positioned onto the CytoSMART™ Lux 10X Device inside a standard 37°C / 5% CO_2 cell culture incubator. Invasion of cancer cells into the surrounding matrix was monitored live for up to 6 days in 60-minute intervals.

Results

Endothelial Cell Tube Formation Assay

Live-cell monitoring showed that HUVEC seeded into BME-coated micro-well plates started to aggregate into tube like structures immediately after seeding (Figure 2). Defined tube-like structures became visible after 4 – 6 hours and were stable for up to 24 hours. Subsequently the structures started to disintegrate (data not shown).

In a second set of experiments, the impact of Suramin on tube formation was quantitatively analyzed. Using the live cell monitoring data, it was determined that 18 hours after seeding is the optimal time-point for quantitative analysis.

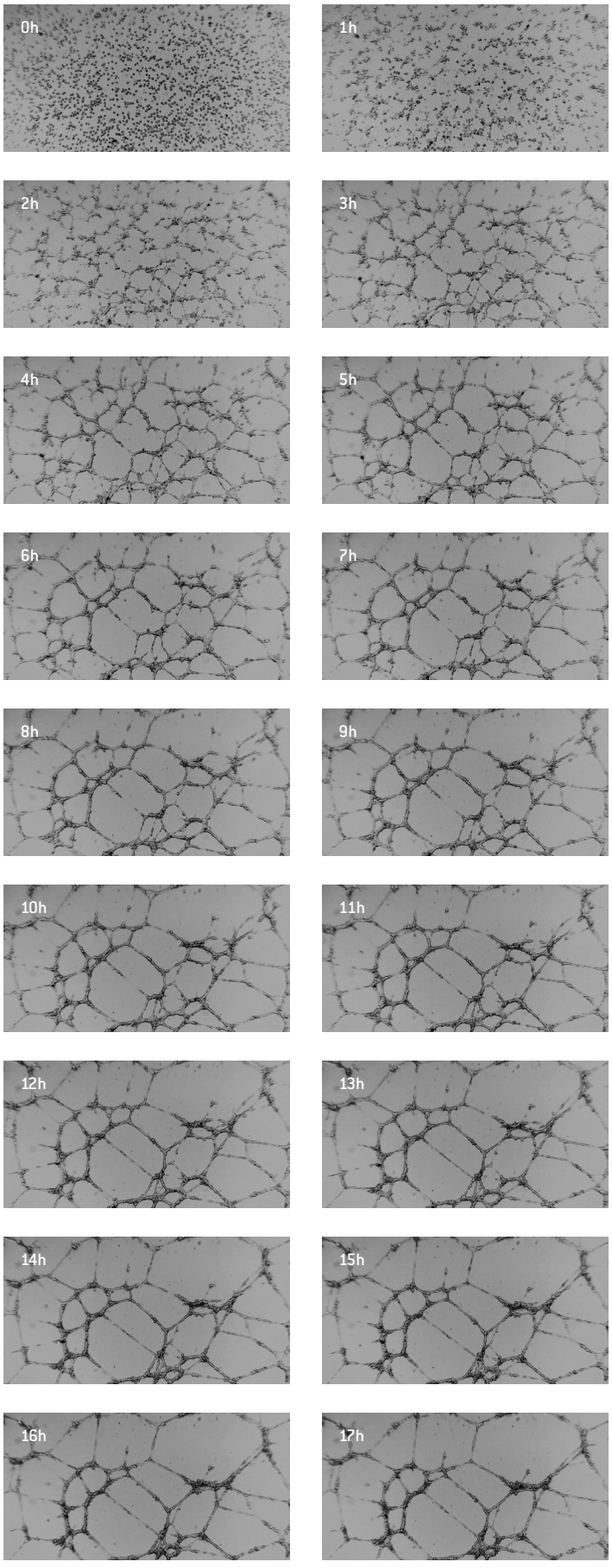

Figure 2
Tube Formation of HUVEC on BME 0 – 17 hours (h) after seeding.

It was observed, that Suramin inhibited the formation of tubular structures in a concentration dependent manner (Figure 3). While Suramin concentration of 5 µM and 7.5 µM impaired tube formation, almost no tube formation was visible at Suramin concentration of 10 µM and above.

Using Calcein AM and PI staining, the viability of the HUVEC exposed to different Suramin concentrations for 19 hours was analyzed. Independent of the Suramin concentration almost all HUVEC were Calcein AM positive and therefore viable, with only a small percentage of PI positive dead cells (Figure 4). This indicates that Suramin has an impact on tube formation, however not on cell viability.

In order to get quantitative information about the impact of Suramin on tube formation, the Angiogenesis Analyzer Module of ImageJ (https://imagej.nih.gov/ij/) was used to determine the number of master junctions and the total segment length per image. For both parameters Suramin inhibited the formation of tubular structures in a concentration dependent manner up to a Suramin concentration of 7.5 µM (Figure 5). In the presence of 10 µM Suramin or higher concentrations, no clear distinction between actual tubular structures and debris could be made by the software.

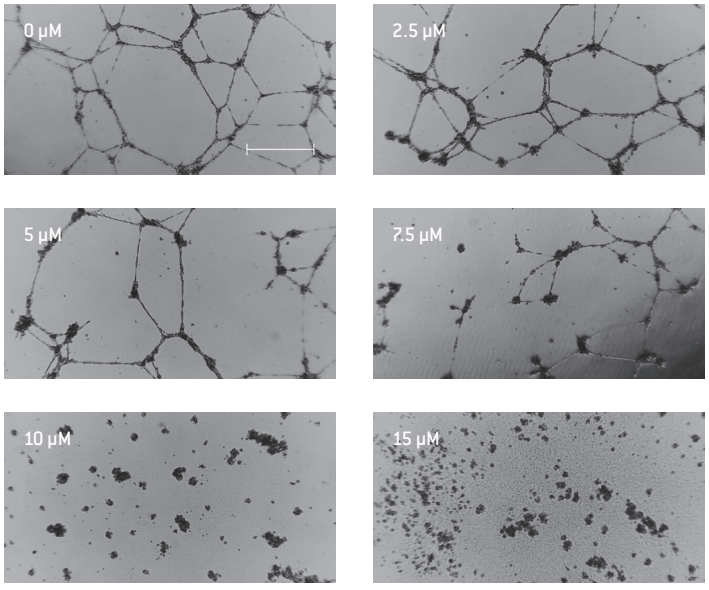

Figure 3
Tube Formation of HUVEC is inhibited by Suramin 18 hours after seeding. Final Suramin concentration is indicated below each image. Scale bar: 500 µm

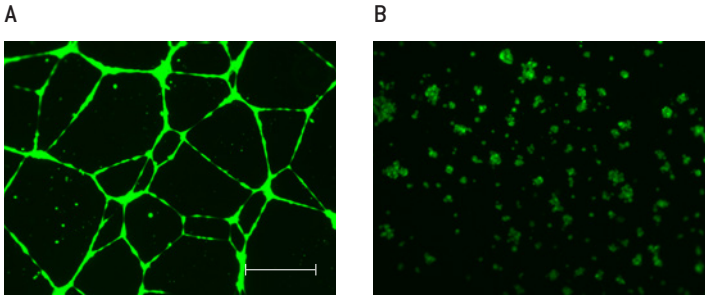

Figure 4
Suramin has an impact on tube formation, however not on cell viability. HUVEC on BME were exposed for 19 hours either to **A)** 0 µM Suramin or **B)** 30 µM Suramin. Subsequently, cells were stained with Calcein AM and PI. Independent of the Suramin concentration almost all HUVEC were Calcein AM positive (green) and therefore viable, with only a small percentage of PI positive dead cells (red). Scale bar: 400 µm

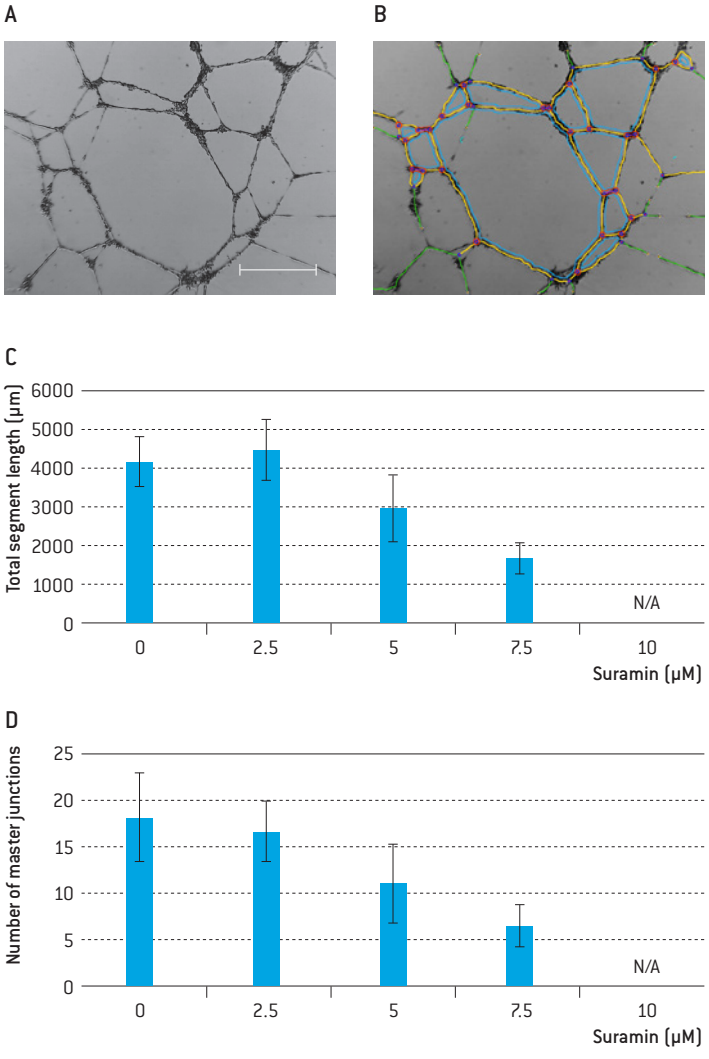

Figure 5
A) Tube Formation of HUVEC on BME 18 hours after seeding. **B)** Image Analysis with the Angiogenesis Analyzer Module of ImageJ. Tube segments are colored in yellow, green and dark blue and master junctions in pink. Impact of Suramin on **C)** the total segment length and **D)** the number of master junctions. (n=6) Scale bar: 500 µm

2D Cell Migration Assay

The migration properties of the human colorectal carcinoma cell line HCT 116 was analyzed in a so-called scratch assay. A gap of defined size was created in a confluent monolayer of HCT 116 cells using a silicone insert system (ibidi). In untreated samples wound closure was observed after 35 – 40 hours (Figure 6).

Gap closure time was analyzed in the presence of DL-Sulphoraphane. Various concentrations of DL-Sulphoraphane were added to the cell culture medium. In samples treated with low amounts of Sulphoraphane (2.5 µM), gap closure was observed after 35 hours, which is comparable to untreated samples (Figure 7). In samples treated with 5µM Sulphoraphane gap closure is delayed. In the presence of 10 µM Sulphoraphane almost no reduction of wound size was observed.

In order to get a more quantitative understanding of the gap closure kinetics, the images were analyzed with the Image J MRI Wound Healing Tool or with the intrinsic cell coverage measurement tool of the CytoSMART™ Lux 10X System. Both tools turned out to be suitable to quantify gap closure (Figure 7).

One benefit of combining "wound healing" assays with live cell imaging tools is that the speed of gap closures varies among different cell types (data not shown). While some cell types close gaps within hours others take days to close a gap of similar size. Live cell imaging allows to capture the kinetics of wound closure and to identify the optimal time point for quantitative image analysis. A practical example for the relevance of "wound healing" assays is the analysis of potential molecular targets that play a role in cancer cell migration. If a certain molecule that is relevant for cancer cell migration is mutated, knocked-out or inhibited by potential active pharmaceutical ingredients the capability and speed of gap closure will be reduced in those samples.

One general caveat in the analysis of cell migration assays is to distinguish between cell proliferation and actual cell migration. In our proof-of-principle study, no differentiation between cell migration and cell proliferation has been made. If it is required to distinguish both processes, it is recommended to reduce the amount of serum in the assay medium to e.g. 0.1% or to add specific cell proliferation inhibitors like arabinofuranoside, actinomycin-C, mitomycin C or lovastatin. The amount of cell proliferation can be analyzed using specific markers like Ki-67. Please refer to corresponding literature for details.

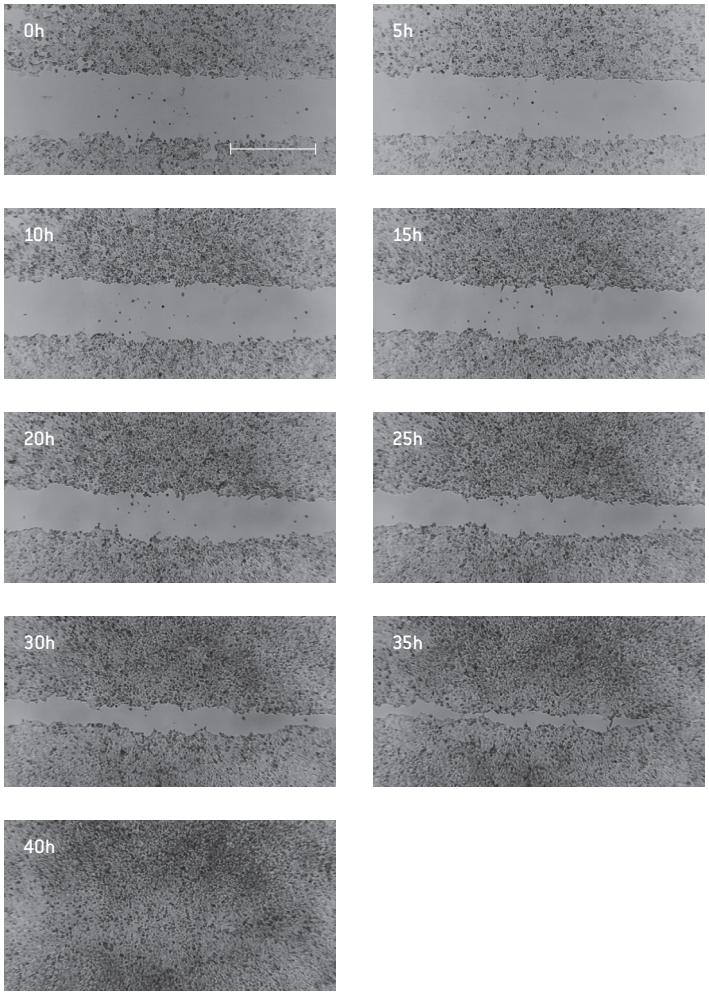

Figure 6
Gap closure by HCT 116 cells after the indicated number of hours (h) of "wound creation"
Scale bar: 500 µm

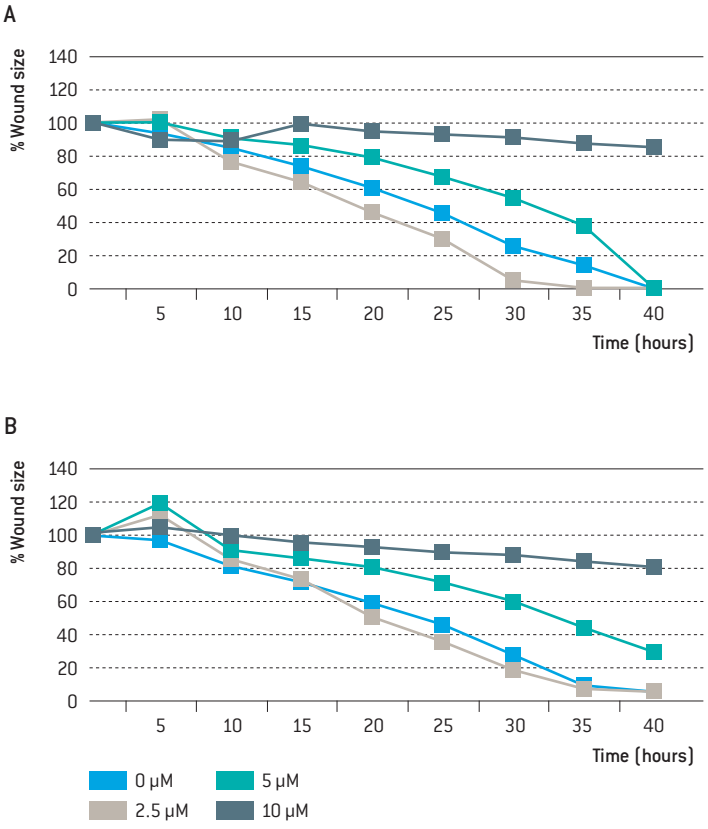

Figure 7
Kinetics of "wound closure" of HCT 116 cells exposed to different concentrations of Sulphoraphane with the A) ImageJ MRI Wound Healing Tool or the **B)** cell coverage measurement tool of the CytoSMART™ Lux 10X System. (n=1)

3D Invasion Assay

The invasion properties of the human, presumably glioblastoma cell line U-87 MG and the human fibrosarcoma cell line HT-1080 were analyzed in a so-called 3D invasion assay. Spheroids of the respective cell types were formed in 96-well U-bottom ultra-low attachment plates. These spheroids were overlaid with a BME cell invasion matrix. Invasion into the surrounding matrix could be observed within a few days using the CytoSMART™ Lux 10X System (Figure 8).

The increase of spheroid area due to sprouting was quantified using Image J (data not shown). In addition, the effect of DL-Sulphoraphane on the invasion properties of both U-87 and HT-1080 cells was determined. As shown in Figure 9, DL-Sulphoraphane blocked the sprouting of U-87 cells in a concentration dependent manner without influencing the morphology of the cells that invade into the surrounding matrix (Figure 10). At a concentration of 30 µM DL-Sulphoraphane almost no sprouting of U-87 cells was observed any more.

Using Calcein AM and PI staining after 3 days of the invasion assay, it was confirmed that even the highest DL-Sulphoraphane concentration of 30 µM has no visible impact on cell viability for both U-87 MG and HT-1080 cells (data not shown).

For HT-1080 cells, DL-Sulphoraphane not only blocked the invasion properties of the cells at high compound concentrations, but also changed the morphology of the invading cell structures (Figure 10) at Sulphoraphane concentrations between 5 µM and 20 µM.

The CytoSMART™ Lux 10X System was a useful tool to monitor the invasion of cancer cells from spheroid structures. Invasion could be monitored under constant temperature and CO_2 levels and without agitating the cultures. There was no risk of disturbing the sometimes fragile cell protrusions by carrying the culture plate fourth-and-back between the incubator and the microscope. In addition, the migration properties of individual cells could be observed in real-time. This allows understanding cell-type specific cell invasion properties in detail – both qualitatively and quantitatively when combined with the appropriate image analysis software.

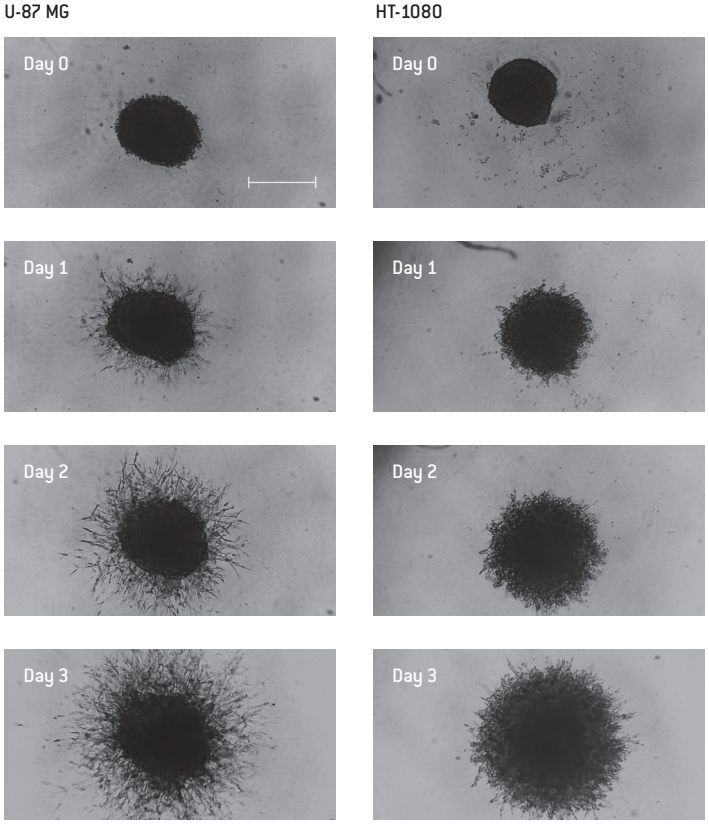

Figure 8
Sprouting of U-87 MG and HT-1080 from a spheroid structure into a 3D matrix. Time of image capture after invasion matrix overlay is indicated in the images. Scale bar: 500 µm

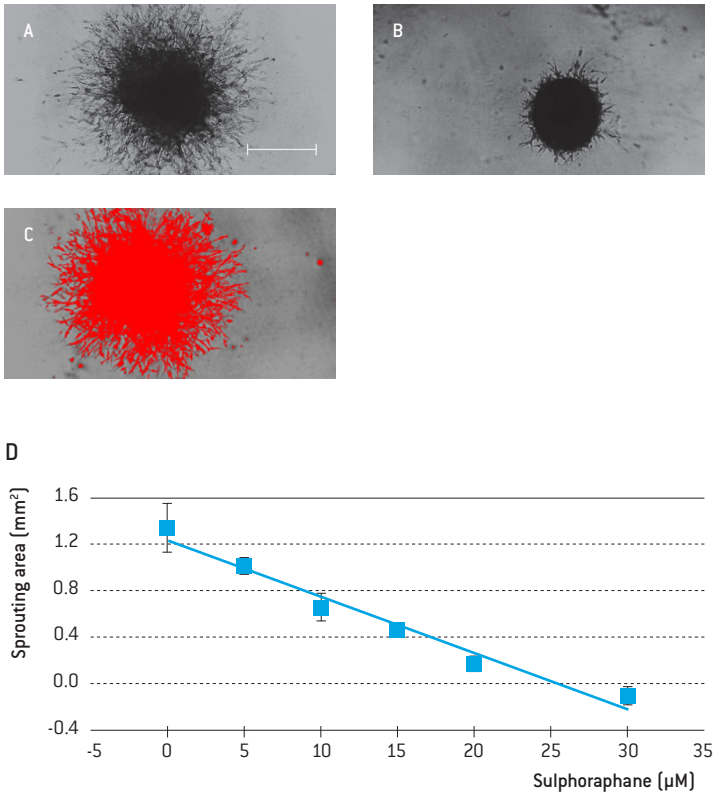

Figure 9
Sprouting of U-87 MG from a spheroid structure into a 3D matrix after 3 days of culture in the A) absence or **B)** presence of 30 µM Sulphoraphane. **C)** Quantification of sprouting with Image J. **D)** Impact of Sulphoraphane on sprouting (normalized to the size of spheroids on Day 3 cultured in the absence of Sulphoraphane and invasion matrix; n=2).
Scale bar: 500 µm

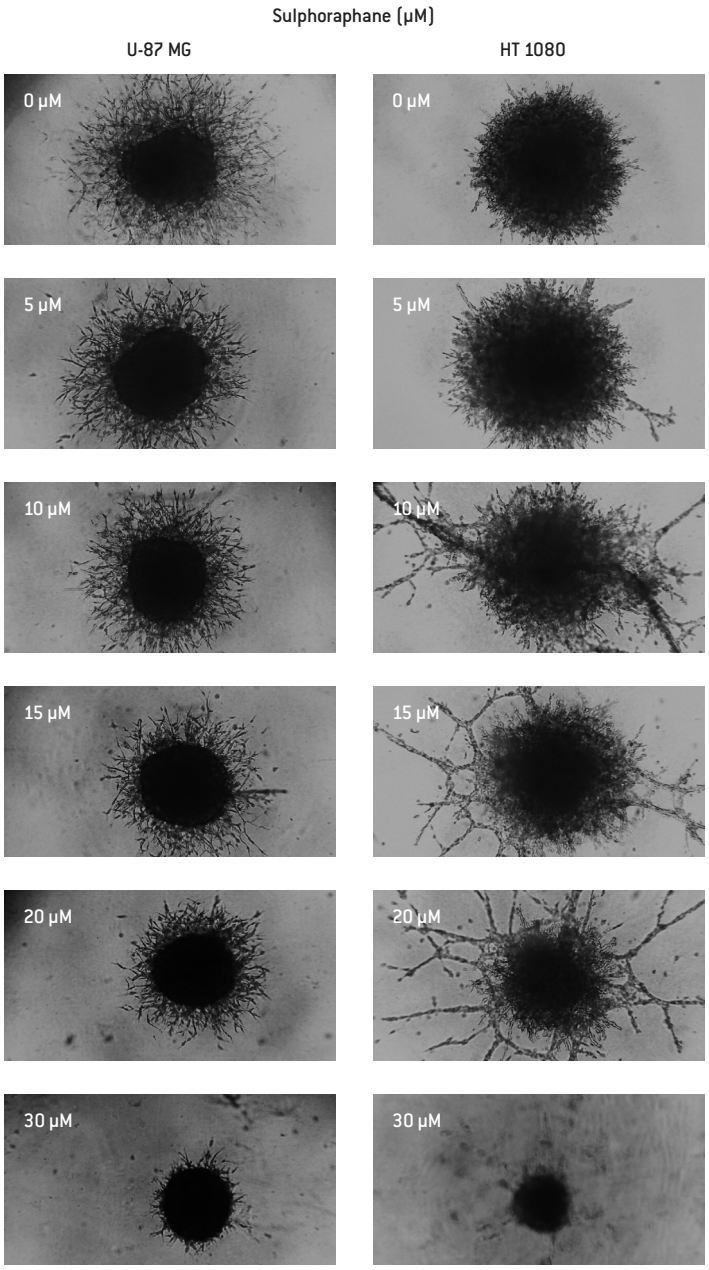

Figure 10
The sprouting morphology of HT-1080 cells from a spheroid structure is dependent on the Sulphoraphane concentration in the cell culture medium. Interestingly, this effect is not observed for U-87 MG cells. Images were taken 3 days after invasion matrix overlay.

Summary and Conclusions

The CytoSMART™ System is an easy-to-use, small and affordable live cell imaging system suitable for analysis of different cancer-relevant assays. Individual cells can be recognized in the resulting images. Therefore they can be easily quantified using software tools like ImageJ or the CytoSMART™ Analysis Software.

References

1. Carmeliet, P. and Jain, R. K. (2000) Angiogenesis in cancer and other diseases. *Nature* 407 (6801): 249–257.
2. Rolfo, C. et al. (2013) BIBF 1120/nintedanib: a new triple angiokinase inhibitor-directed therapy in patients with non-small cell lung cancer. *Expert Opin. Investig. Drugs.* Jun 22.
3. Mackey, J. R. et al. (2012) Controlling angiogenesis in breast cancer: a systematic review of anti-angiogenic trials. *Cancer Treat. Rev.* 38 (6): 673–688.
4. Wei, X. W. et al. (2013) Anti-angiogenic drugs currently in Phase II clinical trials for gynecological cancer treatment. *Expert Opin. Investig. Drugs.* Jun 20.
5. Kast, R. E. et al. (2013) A conceptually new treatment approach for relapsed glioblastoma: coordinated undermining of survival paths with nine repurposed drugs (CUSP9) by the International Initiative for Accelerated Improvement of Glioblastoma Care. *Oncotarget* 4 (4): 502–530.
6. Tímár, J. et al. (2001) Angiogenesis-dependent diseases and angiogenesis therapy. *Pathol. Oncol. Res.* 7 (2): 85–94.
7. Goodwin, A. M. (2007) In vitro assays of angiogenesis for assessment of angiogenic and anti-angiogenic agents. *Microvasc. Res.* 74: 172–83.
8. Kubota, Y. et al. (1988) Role of laminin and basement membrane in the morphological differentiation of human endothelial cells into capillary-like structures. *J. Cell Biol.* 107: 1589–1598.
9. Khoo, C. P. et al. (2011) Comparison of methods for quantifying angiogenesis in the matrigel assay *in vitro*. *Tissue Eng. Part C* 17 (9): 895–906.
10. Fackler, O.T. and Grosse, R., Cell motility through plasma membrane blebbing, *J. Cell Biol.* 181 (2008) 879–884.
11. Kramer, N. et al. (2013) *In vitro* cell migration and invasion assays. *Mutation Research* 752: 10–24.
12. Jee, H.G. et al. (2011) Sulforaphane inhibits oral carcinoma cell migration and invasion *in vitro*. *Phytother. Res.* 25 (11): 1623–8.

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