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White Paper

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Exploring the Hallmarks of Cancer with Real-Time Live-Cell Analysis

Our understanding of cancer cell biology has progressed enormously since Hanahan and Weinberg published their landmark paper describing six hallmarks of cancer in 2000[1]. Since this time, breakthroughs in areas such as immune modulation and the tumor microenvironment have led to these hallmarks being revised and updated—not once, but twice[2,3]—with the most recent review identifying seven:

1. Selective proliferative advantage
2. Altered stress response
3. Vascularization
4. Invasion and metastasis
5. Metabolic rewiring
6. Immune modulation
7. An abetting microenvironment

This constantly evolving picture of tumor biology is helping to shed new light on the behavior of many cancers, as well as providing new pharmacological targets and treatment strategies. Much of this progress has been driven by technological advances, enabling previously unachievable studies to be performed. The last decade in particular has seen huge strides made in the field of *in vitro* translational models and live-cell assays, using both traditional 2D and advanced 3D cell culture systems. The advent of purpose-built live-cell analysis instruments—such as the Incucyte® system (Essen BioScience)—has revolutionized the field, offering the ability to observe and quantify cancer cell biology over time, in a completely non-perturbing way. This white paper takes a look at some of the current research trends across the seven hallmarks of cancer, and highlights the contribution live-cell analysis is making to elucidating the biological mechanisms underpinning these behaviors.

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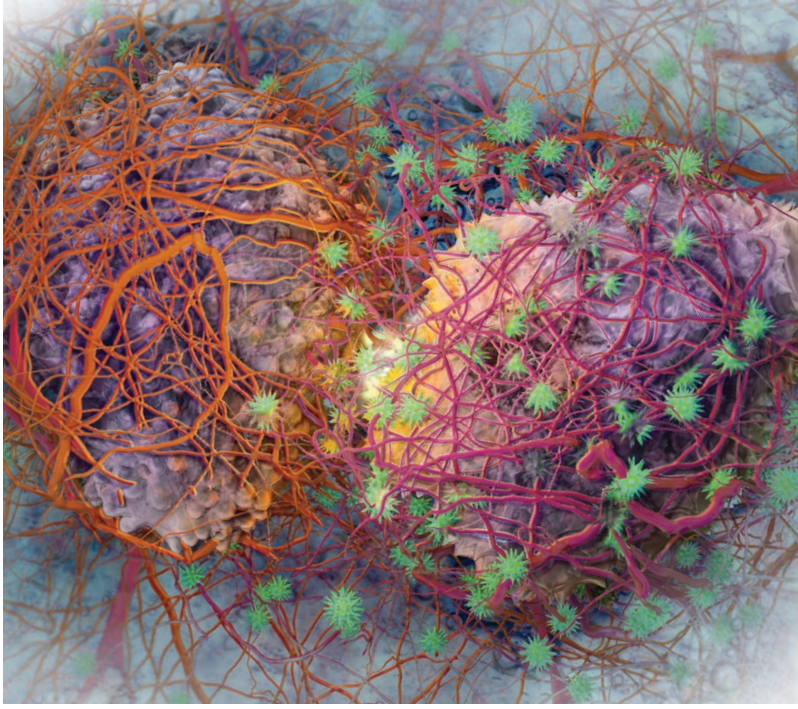
1. Selective Proliferative Advantage

Continual unregulated proliferation is a fundamental abnormality to the development of cancers. As such, almost every study of cancer cell biology includes measures of cell growth and proliferation in one form or another. Live-cell analysis, both in 2D and 3D formats, has become the "gold standard" method for these measurements—due to the unique combination of morphological insights, full time-course data and non-perturbing workflows that the approach provides—and there are now over 700 research publications referring to this application of the technology.

Cancer stem cells (CSCs) are responsible for tumor development and relapse, and result from one or more significant changes to cell signaling pathways that affect growth ligands, their corresponding receptors or cytosolic signaling molecules. Novel fluorescent reporters targeting stem cell transcription factors can be combined with live cell analysis to assess the spatial distribution of CSCs in cell models that retain tumor microenvironmental and structural cues, and this same approach can also be used to monitor CSC plasticity and response to therapeutics in real time[4]. Another critical property of CSCs is self-renewal, and the signaling pathways involved in this process have been extensively characterized using the Incucyte system[5].

One of the current hot topics in this area is chemoresistance, as this is a major hurdle in the treatment and ongoing management of many cancer patients. Several cancers—particularly glioblastoma and epithelial ovarian cancer—currently have a poor prognosis due to the rapid recurrence of aggressive, chemoresistant tumors. A number of interesting studies using live-cell analysis have been published looking at how this resistance may be conferred by a subpopulation of CSCs which comprise only a small proportion of the primary tumor, as well as the potential of combination therapies to overcome this chemoresistance[6,7].



2. Altered Stress Response

Cancer cells face a wide range of stresses—including excessive signaling, DNA damage, hypoxia, nutrient starvation and anticancer therapies—and have developed or adapted a number of stress responses to ensure their survival and propagation. Live-cell imaging is ideally suited to investigating these altered stress responses, as well as looking at how these changes can be exploited in cancer therapies[8,9].

All tumors feature persistent mutations which are not eliminated by the cells' various DNA repair mechanisms and, logically, many cancers have characteristic defects in DNA repair pathways. But some malignancies—such as leukemia, breast and pancreatic cancers—over-express DNA repair proteins. Several recent studies have used real-time analysis to look at the potential of over-expression patterns as therapeutic targets[10]. This approach also naturally lends itself to drug validation studies, providing an effective and convenient method to look at, for example, synthetic lethality mechanisms[11].

The Incucyte platform is widely recognized as a versatile tool for real-time multiplexed measurements of proliferation, cell health and apoptosis[12,13], and can be combined with numerous validated assay reagents (e.g. caspase 3/7 substrates, annexin V labels or cytotoxicity probes) to establish how cancer cells avoid the effects of apoptotic stimuli[14]. Similarly, it can be used to investigate the complex interplay between oncogenes and tumor suppressors which lead to senescence[15,16].

3. Vascularization

Despite their self-renewal capabilities and differing stress responses, tumors are not immune to the effects of hypoxia, and so require a blood supply to grow beyond a few millimeters in size. Angiogenesis—the sprouting of new vasculature from existing vessels—is therefore key to tumor growth and development.

Live-cell analysis is ideally suited to following the proliferation, clustering and differentiation of endothelial cells that occur during vascularization—as well as the critical functions of fibroblast and pericyte stromal cells[17,18]. Using advanced cell models, it is possible to dissect these processes, yielding critical morphological information and insights into the sequence of events. A range of chemical and physical stimuli has been characterized using this approach, including classical growth factors (e.g. VEGF, FGF), established anti-angiogenic therapies (e.g. bevacizumab) and novel angiogenic regulators—such as HSP70-1A[19], piezo1[20] and melflufen[21]. Scratch wound studies on human vascular endothelial cells (HUVECs) are also commonly used to probe the signaling pathways involved in cell migration.

Approaching this same topic from the opposite direction, a number of studies have used Incucyte to study the effects of hypoxia in both tumors and healthy cells. One particularly interesting analysis looked at the uptake and efficacy of colloidal gold nanoparticles—which are increasingly being used as drug delivery vehicles and radiation dose enhancers—and identified that hypoxia significantly reduced the toxicity of the nanoparticles compared to normoxic conditions in HeLa and MCF-7 cells[22]. Another looked at the accumulation of intracellular H_2S in clear cell renal cell carcinoma (ccRCC), combining live-cell imaging, ATP assays and flow cytometry studies to suggest that inhibition of H_2S production could be an attractive new therapeutic target for ccRCC[23].

4. Invasion and Metastasis

The ability to invade surrounding tissues and seed secondary sites is a defining feature of malignancy. Split into distinct phases—invasion of the extracellular matrix, escape into the vasculature, survival in circulation, seeding of secondary sites and adaptation of the new microenvironment—invasion and metastasis is a broad and complex subject area. There are a number of Incucyte assays that can be used to dissect the sequential steps of metastasis, including 'scratch wound' migration and invasion assays[24], chemotaxis experiments[25], and growth and colonization studies[26]. Combining these live-cell assays with 3D cell culture models has also provided valuable information which might otherwise be overlooked[27].

Elucidating the various signaling pathways involved in each step of invasion and metastasis is essential to understanding these processes[28]. For example, a panel of Incucyte migration assays—combining over-expression, shRNA knockdown and pharmacological inhibition studies—has been used to characterize a novel signaling pathway regulating cell migration and metastasis in liver, lung, colon and breast cancer cell lines[29]. This technology can also be used to investigate how the invasion and metastasis process is affected by chemotherapy drugs[30], and to screen for potential therapeutics to reduce the risk of secondary tumor formation or relapse[31]. Colonization of the secondary tumor site can also be followed using real-time imaging of co-culture invasion assays to identify the various mechanisms by which invading cancer cells remodel the extracellular matrix to enable rapid proliferation[32].

5. Metabolic Rewiring

There are various ways that the metabolism of cancer cells can be 'rewired' to provide selective advantages, such as increased uptake of glucose, amino acids or nitrogen, opportunistic nutrient acquisition, use of glycolysis and TCA cycle intermediates, or altered gene regulation. Characterizing these aberrant metabolic activities can provide greater insight into tumor progression, as well as potentially offering new therapeutic targets.

Investigating how this reprogramming of the metabolic pathways occurs is a priority for researchers looking to target these activities, and long-term, live-cell studies are proving vital. The rewiring of both metastatic breast cancer and malignant melanoma have been looked at using this approach[33], with a further study also looking at how alterations to metabolic activity affect characteristics such as invasion[34] and chemoresistance[35].

Abnormal nutrient uptake and usage is another area currently garnering a lot of interest, due to the obvious potential to 'starve' tumors. In one recent example, real-time apoptosis assays were used to demonstrate that endothelial cells infected with Kaposi's sarcoma-associated herpesvirus become glutamine 'addicted'—similar to many cancer cells—and that glutamine starvation leads to apoptosis[36]. Live-cell imaging has also been used to monitor and quantify the effects of NAD depletion—a proposed therapeutic strategy—on proliferation, motility and cell death[37].

6. Immune Modulation

The immune system's constant surveillance for developing cancer cells clearly plays a role in tumor suppression and eradication, but this also places selective pressures on these cell populations. This can lead to so-called 'cancer immunoediting', providing a selective advantage to variants which can 'escape' the innate and adaptive immune responses. This has become an increasingly hot topic over the last five years, particularly the interactions between cancer cells and natural killer or cytotoxic T cells.

However, it has become clear that this is an over-simplification of the tumor-host immunological interactions, and many cancer cells may evade immune destruction by actively disabling immune system components. The Incucyte system is proving a valuable tool for investigating the behaviors of immune cells—such as T cell activation, clustering, and chemotaxis—both independently and in co-culture with tumor cells, using 2D and 3D immune cell killing and phagocytosis studies. The system's unique design, where the optical path moves around the static cell plate, allows non-perturbing imaging studies on a wide range of cell types, including non-adherent cells (e.g. blood).

A number of new reagents—including nuclear-targeted fluorescent probes (e.g. Nuclight™) for labeling tumor cells and pH-sensitive dyes for tracking internalization—are now being widely used in combination with the Incucyte to create time-lapse movies and analysis. This strategy has been particularly helpful in elucidating the physical interaction between immune and cancer cells, as well as the resulting morphological changes. This frontline technology is also now proven for developing new immunotherapies, such as CAR-T[38], checkpoint inhibitors[39], T cell activators[40], cancer vaccines[41] and oncolytic viruses[42].

7. An Abetting Microenvironment

Tumors do not grow in isolation, and there is continuous communication between cancerous and stromal cells throughout all stages of carcinogenesis. There is still much confusion—and several conflicting theories—around how this microenvironment influences tumor growth, but over the past decade, tumors have increasingly been seen as complex organs. When viewed from this perspective, the biology of a tumor can only be understood by studying the individual specialized cell types within and around it.

Live-cell imaging has been used to analyze the function of a wide range of biomatrices and important tumor-associated cells—including stroma[43,44], fibroblasts[45], endothelia[46,47] and pericytes[48]. This technology is also allowing researchers to observe spatial and temporal cellular interactions of co-culture tumor models in real time, providing unprecedented insights into dynamic changes in the tumor microenvironment, as well as highlighting previously overlooked therapeutic targets. For example, Incucyte co-culture invasion assays have helped to elucidate the role of cancer-associated fibroblasts in cancer cell invasion driven by extracellular matrix remodeling[49]. In another co-culture study, the system was used to demonstrate the transfer of microRNAs between cells in *in vitro* models of osteosarcoma and ovarian cancer[50].

Summary

Our understanding of the hallmarks of cancer has changed significantly over the last decade, and will probably progress just as much in the next 10 years. There are several hot topics—such as metabolic rewiring and tumor microenvironments—which are currently seeing rapid progress, and other hallmarks are likely to undergo similar revisions soon. One of the key factors dictating the rate of this progress is the availability of suitable investigative tools, from biochemical assay reagents and sequencing chemistries to patient-derived primary tissue samples, 3D cell culturing methods, and novel imaging systems. As this review demonstrates, live-cell imaging and analysis using the Incucyte system is playing a central role in the elucidation of cancer biology, as well as providing more effective and relevant ways of evaluating potential therapeutics. Based on the evidence so far, this real-time imaging capability will become increasingly important to furthering our understanding of the complex behavior of tumors.

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