More than a century ago, in 1869, the Australian pathologist Thomas Ashworth noted that some cells present in the blood of a man with metastatic cancer did not look like normal blood cells. Rather, they appeared similar to those in the patient’s solid tumors. In the article Ashworth published about his discovery in the Australian Medical Journal, he wrote the visionary words: “cells identical with those of the cancer itself being seen in the blood may tend to throw some light upon the mode of origin of multiple tumours existing in the same person”. With the advent of new technologies in the last decades, the work on these Circulating Tumor Cells (CTCs) has been revived and is growing fast. In my opinion, the field of CTCs defines one of the Grand Challenges for the microfluidics community.

Indeed, nowadays we know that Circulating Tumor Cells are released into the blood from primary and metastatic tumors and that, aside from a functional role in establishing metastatic tumors, they have high clinical diagnostic potential in oncology. The possibility to isolate and characterize CTCs with respect to their biological properties is envisioned to no less than revolutionize cancer diagnosis and therapy. Treatment of cancer, namely, is expected to change dramatically in the coming years, as an increasing number of novel targeted drugs, directed at the biological mechanism in the tumor that is directly responsible for growth of the tumor, will become available and partly replace standard chemotherapy treatment. Since this biological mechanism is not identical in all patients with the same type of tumor, it is essential to identify the biological target prior to deciding on treatment with a targeted drug in each individual patient: truly personalized medicine. An example is the antibody trastuzumab (trade name Herceptin®), which blocks the function of the membrane protein HER2 that plays a key role in cell division in some patients with breast cancer. With this development metastasized cancer can be anticipated to turn into a chronic disease where tumor growth can be kept under control for long periods of time, and people diagnosed with cancer can relatively normally continue with their lives. In this approach, it is necessary to monitor the effectiveness of the therapy at regular intervals to early detect emerging drug resistance, and this requires repeated analysis of tumor samples. Unfortunately, while the primary tumor has in general been surgically removed, most metastatic tumors are difficult and dangerous to biopsy, especially when this needs to be done repeatedly, and frequently it is even impossible because lesions are inaccessible. Isolation of CTCs from peripheral blood, as a “liquid biopsy”, is expected to be able to replace conventional tissue biopsies of metastatic tumors for therapy guidance. In fact, it may be the only way to make the new therapies possible.

The challenge is to build a system that makes it possible to perform both the isolation and the molecular characterization of CTCs, preferably at a single cell level. The biggest problem is that the CTCs, in clinically relevant concentrations, are very rare: they are found in numbers in the order of 1 to 10 CTCs per mL of whole blood in patients with metastatic disease. For comparison, one mL of blood contains a few million white blood cells and a few billion red blood cells. This poses the tremendous challenge of isolating a very small amount of CTCs from a huge amount of other cells in a relatively large total volume of blood (typically 7.5 mL or more). Another big challenge is to harvest the CTCs in such a form that they are suitable for subsequent (molecular) analysis necessary for future therapy guidance; most importantly, the captured cell population should be pure, and the cells should be viable.

Currently, there is one single FDA-approved CTC diagnostics system on the market, called “CellSearch™” of Veridex, a J&J company. The result of the analysis is a count of the number of CTCs in a blood sample. The prognostic value of CTC enumeration using this technique has been well established for several tumor types. The CTCs are captured immunomagnetically from 7.5 mL of blood by means of ferrofluidic nanoparticles conjugated to a monoclonal antibody against epithelial cell adhesion molecule (EpCAM). Subsequently, they are stained with a fluorescent nucleic acid dye and fluorescently labeled antibodies against epithelial cell specific cytokeratins 8, 18, 19, and a white blood cell specific antibody. A trained operator counts the cells manually by observing staining pattern and morphology. Prospective multicenter studies have shown that above a cut-off of 5 CTCs per 7.5 mL of blood, patients with metastatic breast, colorectal and prostate cancer have poor survival prospects.

Despite the proven clinical utility of this system, a new generation of CTC analysis that is suitable for prediction of therapy effectiveness or resistance, requires more than that. First, the method selects only EpCAM positive (epithelial) CTCs that express cytokeratins 8, 18 and/or 19 while it is known that also EpCAM negative and cytokeratin 8, 18, 19 negative carcinomas exist, which may have similar relevance for choosing the optimal targeted therapy. Perhaps more important, the majority of CTCs are not viable after capture, which interferes with cell culture and molecular diagnostics to be
For these reasons, worldwide efforts are currently directed at developing new CTC isolation methods. Many are in search of new capture and detection antibodies that would enable more specific and selective capture and detection of all subpopulations of CTCs than is currently possible with EpCAM and cytokeratin antibodies. Others are studying alternative, unlabeled, selection methods, such as the use of microfluidic devices with integrated capture features, special filtration systems, electrical approaches such as impedance spectroscopy or dielectrophoresis, or selection based on mechanical characteristics, to name a few. For some of these techniques, promising results have been published. Most of them, however, do not yet extend beyond proving technical feasibility using model samples such as cells from tumor cell lines spiked in buffers. For none, clinical validity or utility has been unequivocally shown. So we still have a long way to go.

Even a number of the proposed techniques may not be compatible with the needs and the requirements for the new generation of CTC isolation and diagnostic analysis systems.

I would like to formulate here the following Grand Challenge: developing a system, in the end suited for routine use in a general hospital setting, that can isolate viable pure CTCs (down to one copy per mL) from large volumes of full blood (≥7.5 mL), and carry out subsequent analyses ranging from single cell pathology, to protein and molecular diagnostics, to cell culture for in vitro determination of drug sensitivity. This involves the following considerations:

- **Large fluid volumes must be handled.** Since CTCs are very rare, relatively large sample volumes (5–10 mL of blood) are required for analysis. This fluid amount is not easy to handle for rare cell detection and purification strategies within a limited amount of time. It may well be that even this volume is not sufficiently large to detect CTCs in the majority of patients which would imply that we will need to search for in vivo detection technology.

- **CTCs that are viable in circulation must be kept viable after selection.** CTCs are very vulnerable cells and viability is easily compromised. Intracellular staining procedures are in general not compatible with viability. Flow-induced shear stress may easily damage the cells, which makes subsequent molecular analysis difficult, and eliminates the option for cell culture.

  - **Currently available biological markers are not sufficient.** CTCs may escape currently used isolation and detection targets because of lack of detectable antigens or technical problems with the isolation and identification of the cells amidst the blood cells. New markers, both biological and physical, must be discovered.

  - **CTCs have large morphological variability.** It is clear that there is much heterogeneity among CTCs and, evident from for example CellSearch analyses, EpCAM and cytokeratin positive cells vary significantly in size. Aside from a technical challenge to isolate the whole population, this requires sophisticated algorithms to identify the cells. Also, in assessing the potential of new isolation methods the use of cell lines should be considered with care, since cell lines may have quite different characteristics, and be more homogeneous, than CTC populations from patients.

  - **CTCs have varying molecular/functional characteristics.** Especially in the case of metastases, CTCs are likely to include cells from different metastatic and dormant sites, which are not necessarily similar. The clinical relevance of this is expected to become clear in the coming years. It represents a challenge to develop technologies to obtain a representative sample, containing all the different subtypes, from the blood.

  - **Isolated CTC subpopulations must be pure.** Other cells and cell fragments can be wrongly identified as CTCs. Apoptotic processes may interfere with easy identification, however antibodies to detect this process (e.g. against Annexin V) could be included in the protocol. Circulating fragments of cells, observed as EpCAM-expressing microparticles may potentially cause a bias in CTC counts. On the other hand they may have their own diagnostic prognostic value. Using nuclear staining helps in differentiation between CTCs and anucleate microparticles, however compromises viability.

  - **Captured CTCs must be analyzed by various diagnostic modalities.** For use in targeted therapy guidance, it is molecular characterization of CTCs, rather than “just” enumeration, which will become essential for choosing the right (combination of) targeted drugs, as well as for assessing resistance to specific targeted drugs. Basically diagnostic assays to be performed on CTCs for this purpose are expected to be similar to those performed on tissue slides obtained from the primary or metastatic tumor, e.g. immunohistochemistry, FISH, (phospho)proteomics, PCR, and DNA mutation and CNV (DNA copy number variation) analysis, as well as mRNA/miRNA profiling by sequencing. Given the extremely low concentration of CTCs in the peripheral blood, and the importance of identifying tumor heterogeneity with respect to treatment targets, it is necessary to perform such assays on single CTCs. Although methods for molecular analysis on single cells are currently being developed, they are still far from routine clinical use. In addition to pathological and molecular analysis, subsequent cell culturing will provide a way to directly confirm effectiveness of a chosen targeted drug.

  - **In the end, an integrated and automated solution is needed.** This is essential for the eventual transfer from academic centers to the routine clinic. The assay time needs to be reduced and the expert lab technician replaced by a hands-off system. The development of an integrated CTC diagnostics platform, to be ultimately also used in a routine hospital setting, requires development of several different modules, finally to be put together to obtain the complete system. A full system requires (1) sample preparation; (2) CTC recognition; (3) CTC isolation; (4) (single) CTC transport; (5) several diagnostic modalities suited for (single) CTC analysis. Especially the challenging logistics of such a system asks for smart microfluidics solutions.

Taking up this Grand Challenge requires collaboration between different fields. Microfluidics groups need to team up with molecular diagnostics groups and clinical researchers. To enable a good transition to relevant applications, diagnostic and pharmaceutical industries have to actively participate. Very importantly, clinicians (especially oncologists and pathologists) have to be involved, since the proof of clinical...
utility of any technology to be developed, should be the goal from the very beginning. At any rate, the clear needs and requirements for creating this revolution in cancer diagnostics and therapy will continue to stimulate science and research within the LOC community, as well as the formation of the multidisciplinary consortia needed to effectively tackle the Challenge.

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