MetaSystems White Paper

Metafer CTC Application

Automated identification and analysis of CTCs enriched and isolated by SE-i•FISH® technology

Introduction

Circulating Tumor Cells (CTCs) are cancer cells shed from primary or metastatic solid tumors into peripheral blood, but these rare cells can also be found in other forms of biofluid such as pleural effusion, ascites, urine, bone marrow and cerebrospinal fluid, where they are commonly referred to as disseminated tumor cells (DTCs) [Lin PP, 2015]. Liquid biopsy is an umbrella term that includes not only CTCs, but also other tumor-derived material such as circulating tumor DNA (ctDNA), tumor-derived exosomes and circulating miRNAs [O'Flaherty et al., 2017]. Enumeration and characterization of these rare cells holds promise in the early detection, screening, monitoring, and treatment of cancers.

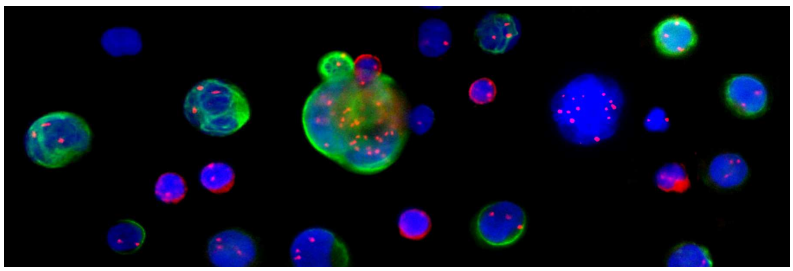

Figure 1: Examples of normal (diploid) white blood cells (WBCs), as well as aneuploid marker-positive and marker-negative CTCs (image provided by Cytelligen)

CTC Enrichment and Identification

As CTCs occur at very low concentrations in the range of one tumor cell per millions of blood cells, the first challenge is to enrich the sample from a typical starting point of 5-10 ml blood. To this end, numerous approaches have been utilized, from physical properties such as size and deformability, to biological properties, which include surface marker expression and invasive potential. Another route is to not enrich the sample, in which case the whole population of tens of millions of cells must be analyzed; naturally this poses other challenges.

The next step is identification of CTCs from the enriched sample. Classically, CTCs are negative for the blood marker CD45, and positive for an epithelial marker such as CK or EpCAM, though the dynamic expression and even absence of these epithelial markers means that this is often not the real situation [Lin PP, 2015]. Diagnostic information relating to CTCs includes enumeration, as well as characterization of morphological / marker-based subtypes. Individual CTCs can even be isolated and subject to other assays such as genomic profiling.

The general process can be summarised as follows:

- Sample collection (e.g. 5-10 ml peripheral blood)
- Sample enrichment (e.g. by surface marker expression)
- Fixation and staining (e.g. surface marker expression as CTC)
- CTC identification, classification (e.g. imaging to visualise stained cells and reveal subtypes)
- Further assays or Reporting (e.g. NGS)

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Cytelligen SE-i•FISH® Technology

The Cytelligen SE-i•FISH® kit uses an unbiased CTC enrichment approach called subtraction enrichment (SE), which combines non-haemolytic removal of red blood cells and immuno-magnetic removal of white blood cells. In contrast to most other methodologies, this is independent of cell size and independent of molecular markers, such as the commonly used EpCAM and Cytokeratins (CKs), and can therefore find cells that are overlooked by these other approaches (superiority to e.g. CellSearch is documented [Li et al., 2014]). The SE-enriched samples are stained by immunofluorescence for CD45 and other markers of interest, as well as centromeric FISH for chromosome 8 as an indicator of aneuploidy [Lin et al., 2017; Li et al., 2016].

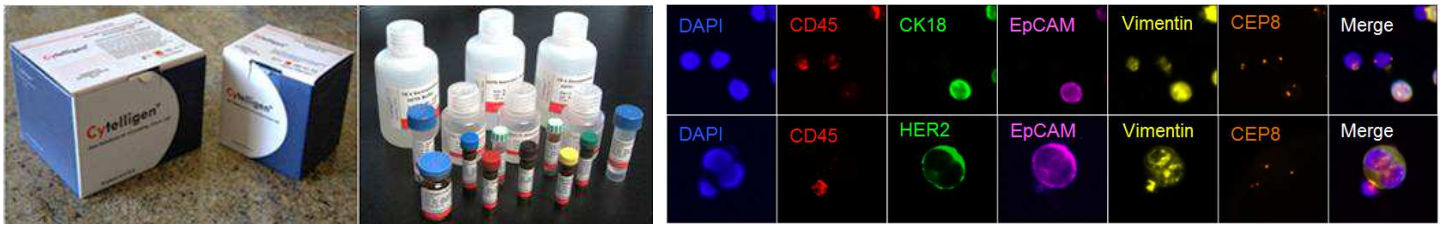

Figure 2: Cytelligen technology. CTCs are negative for the WBC marker CD45, and can be positive for epithelial and/or mesenchymal markers, such as CK, EpCam and Vimentin, as well as tumor related markers such as Her2 (images provided by Cytelligen)

Multiplexing using unique fluorescent dyes allows simultaneous analysis of the standard CD45 marker and up to 3 markers of interest, as well as centromeric FISH for chromosome 8. Aneuploidy of chromosome 8 examined by CEP8-FISH has been reported on neoplastic cells from tissues of several types of tumors including lung, bladder, gastric, esophageal, pancreatic, colon, and hepatocellular carcinomas. [Ge et al., 2015]. In CTCs, aneuploidy of Chromosome 8 has been shown to correlate with prognosis in patients with advanced gastric cancer [Li et al., 2016].

Metafer CTC Application

Manual analysis of SE-iFISH samples, as with CTC samples in general and other many other microscope-based analyses, is a time consuming and strenuous process that can take several hours, even for experienced operators. This limits its utility as a routine clinical application. The Metafer CTC Application was developed specifically to address this bottleneck, detecting CTCs processed by SE-i•FISH® and enabling analysis within a clinically relevant timeframe. It enjoys all of the benefits of the Metafer automated scanning platform, such as uninterrupted, around-the-clock imaging at a scale that suits the needs of the laboratory and grows with its requirements (8-880 slides). Cytelligen's proprietary CTC microscope slides have a sample/iFISH staining area of 18 x 18 mm, which can be imaged automatically (DAPI + CD45 + CEP8 + Single Marker) at 10x magnification in under 45 minutes. Additional markers can be added according to specific requirements (up to a Triple Marker maximum).

CTC identification is complex, particularly when multiple markers are introduced simultaneously. The Metafer platform is ideally suited to on-screen review of candidate cells, their morphological properties and individual marker expression levels, allowing clear, reliable confirmation of all CTC types. A combination of automatic and interactive measurements classifies these cells by size, marker status and ploidy. These often complex results can be automatically reported in customizable formats to eliminate errors in the clinical setting. For parties interested in extending the downstream capabilities for single cell studies, Metafer is compatible with laser microdissection technologies like the Zeiss PALM MicroBeam, and provides coordinate transformation for integration with other coordinate-based cell excision equipment such as micromanipulators.

Figure 3: The Metafer CTC platform with full SlideFeederX robotic module, which increases capacity in modular 80-slide units. Example gallery of circulating endothelial cells (CECs) and both marker negative and positive CTCs identified by the Metafer CTC Application. CECs can be identified by CD31 staining (see Lin et al., 2017 for a comprehensive guide).

Applications of CTC Technology

CTCs are currently being used to monitor response to therapy as well as relapse. It is hoped that they will also be predictive at early stages of cancer that currently cannot be detected by other tests. Some of the applications of CTCs are highlighted below:

- *Efficacy of treatment regime* – by monitoring CTC levels before and after treatment, we can decide how effective the treatment is
- *Monitoring relapse (MRD)* – changes in CTC levels could be a sensitive way of detecting relapse earlier and less invasively than it would be seen by other traditional tests
- *Molecular characterization of the cancer* – expression of particular markers relates to aggressiveness of the disease, response to certain therapies etc. Therefore sub-classification of CTCs by marker and ploidy status can directly inform clinical decisions
- *Research* – Basic research as well as R&D for drug development

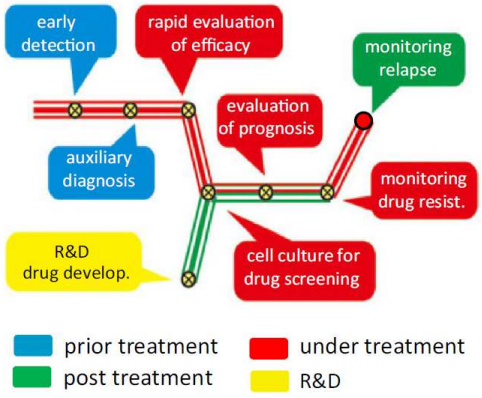

Figure 4: Proposed clinical and scientific significance of CTCs [taken from Lin PP, 2015]

Technology Comparison

The entire integrated solution spans three technology areas: isolation of CTCs by SE, identification of CTCs by iFISH, and automated imaging/analysis of iFISH positive cells by the Metafer CTC scanning platform.

Most of the recognized methodologies for CTC isolation fall into three major categories – enrichment, antibody capture and cell filtration [Lin PP, 2015]. The major benefit of enrichment over the other two strategies is no loss of CTCs due to the inherent

fundamental flaws of the methodology – marker-negative CTCs in the case of antibody capture (most commonly CK and/or EpCAM), and smaller CTCs in the case of cell filtration. A notable example of the importance of size was demonstrated in hepatocellular carcinoma (HCC) patients, where the majority (almost 70%) of detected CTCs was smaller than WBCs (≤5um) both pre- and post-surgery [Wang et al., 2018].

The preferred form of enrichment in the context of CTCs is negative enrichment, which applies both hypotonic lysis to remove RBCs and anti-CD45 antibody to deplete WBCs at a range of 2-3 logs [Liu et al., 2011]. Subtraction Enrichment (SE), in contrast to conventional negative enrichment, has a number of improvements including minimal hypotonic injury and higher fold removal of WBCs [Lin PP, 2015]. Subtraction Enrichment is contrasted with antibody captures and cell filtration in *Table 1*.

Isolation Process	Subtraction Enrichment	Antibody Capture	Cell Filtration
Type of enrichment	Negative depletion	Marker positive selection	Size selection
Enrichment-related CTC loss	**No**	Yes	Yes
Hypotonic injury	**Minimal***	NA	NA
Antibody perturbation	**No**	Yes	No
Loss of smaller CTCs	**No**	No	Yes
Loss of marker low/negative CTCs	**No**	Yes	No
Retention of CTCs of all sizes and marker expression levels	**Yes**	No	No

*Table 1: Comparison of Cytelligen's SE technology with other enrichment methods. *Hypotonic injury is significant in conventional enrichment protocols*

Epithelial markers are used not only in antibody capture isolation methods, but also for the most common CTC identification strategy – immunostaining. Not all CTCs, however, have strong expression of CK / EpCAM (which is precisely the limitation of isolation by antibody capture). EpCAM is expressed in many but not all tumors, while CK is heterogeneously expressed, and both EpCAM and CK have been shown to be downregulated during the progression of the epithelial-to-mesenchymal transition (EMT) as part of an oncogenic pathway towards invasiveness and metastasis [Mikolajczyk, 2011]. It is therefore clear that expression of the common surface markers cannot be used as the sole method of CTC identification. We solve this problem via iFISH, which allows CK / EpCam low or negative cells to be distinguished by their aneuploid status. This offers a unique additional layer of diagnostic information that cannot be revealed by immunostaining. In addition to determination of aneuploidy, iFISH is also uniquely capable of detecting cytogenetic gene rearrangement, such as ALK in non-small cell lung cancer (NSCLC) [Lin PP, 2018].

The third and critical technology area that maximizes the advantages of the negative enrichment approach, is an effective imaging and analysis platform. The power and flexibility of Metafer allows us to compound the negative enrichment with a negative image-based identification approach by only selecting cells that lack expression of the CD45 blood cell marker. To our knowledge, this is unique among other imaging systems. Checking the CEP8 status of these CD45-negative cells is a rapid CTC confirmation strategy that can be overlaid with the additional marker data.

SE-i•FISH® and Metafer CTC Application Scientific Results

The SE-i•FISH® method has been validated extensively [Li et al., 2014; Lin PP, 2015], and has been shown to be superior to CellSearch, with a positive detection rate of 90.5% vs 54.8% for CellSearch in advanced gastric cancer patients [Li et al., 2014]. The Metafer CTC Application has been used in several studies (described as 'Metafer-iFISH') to analyze samples processed by SE-i•FISH® [Lin et al., 2017; Lin PP, 2018; Wang et al., 2018] and has already been adopted by a number of institutes for both research and clinical purposes. Furthermore, the CE marked Metafer platform has been established for over 30 years, and is successfully used by clinicians and researchers worldwide [*].

References

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