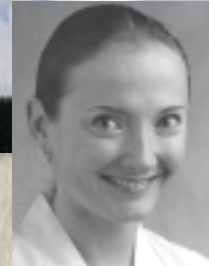


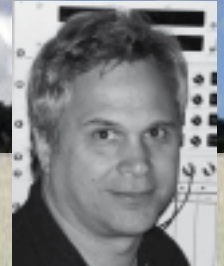
Finding a Needle in a Haystack

Applications of Fast Rare-event Analysis in Flow Cytometry

Detecting rare events is akin to finding a needle in a haystack. Until recently, using flow cytometry-based assays for oncological applications, such as detecting tumourigenic stem and progenitor cells, was even harder because of their limited event acquisition speed. Although the newest cytometers can rapidly capture data from millions of cells, analysing the large files they generate caused bottlenecks. This is now also changing – here we examine the possibilities that are opening up.



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Old Ways of Searching Hay

Rare-event analysis (REA) is the art of finding a needle in a haystack, confirming that it is a needle, and making measurements to determine what kind of needle it is. A rare event is typically one occurring at a frequency of below 1 in 1,000 (0.1%) [1, 2]. Numerous measurements are needed to detect rare events, such as tumour cells in peripheral blood, and it was historically a long-winded process as conventional-speed flow analysers had a maximum acquisition speed of 1,000–3,000 events per second. Modern flow cytometers can detect up to 10,000 events per second [3], leading to flow cytometry-based assays increasingly being used for oncological REA. Results from up to 10 million cells [3] can now be collected but, often, the bottleneck shifted to data analysis since most current flow cytometry software has problems analysing large data files.

This problem is worsened as the event detection method's signal-to-noise ratio (SNR) depends on the number of parameters collected. SNR maximisation is critical to distinguish the rarest events from background noise, especially when analysing tumours, which often contain dying cells and other noisy debris. The flow cytometer uses laser beams to excite

fluorescent labels attached to particles suspended in a narrow fluid stream. Scattered and fluorescent light is detected when the fluid passes the lasers. Histograms can be plotted showing brightness fluctuations at each detector, and used to determine the physical and chemical characteristics of cell sub-populations. With solid-state lasers now available in most colours, multiple laser beams can capture up to fourteen fluorescence parameters for each event, increasing the SNR, but also expanding the file sizes analysis software must deal with.

Pain-free Ways to Find Needles

A new generation of flow cytometry software that can work ten-times faster than traditional systems is now making REA routine. This uses parallel processing and can access large blocks of random access memory directly. The VenturiOne software, by Applied Cytometry, runs on 64 or 32 bit MS Windows systems and, for any dataset, automatically plots previews of all possible one- and two-parameter histograms on-screen. Interesting histograms can be dragged into an on-screen analysis box where other tools can be applied.

These software developments will allow more oncological applications of REA

to be performed in clinical laboratories using flow cytometry. Early detection, relapse and minimal residual disease were early applications of REA, and can be performed using cytometry. In these studies, numerous human peripheral blood or bone marrow cells are assayed for markers present on tumour cells, but absent in normal peripheral blood or bone marrow. These include tissue-specific differentiation markers such as cytokeratins [4] and the absence of leukocyte-specific markers CD45 [5, 2]. Additionally, immune regulators, such as the identification and listing of dendritic cell subsets and their precursors, are useful to tumour immunology studies, induction of tolerance and studies of immune responses to novel antigens [6].

An Example of a Haystack

An important recent application of rapid oncological REA is testing the cancer stem cell hypothesis (CSCH). CSCH explains cancer initiation and recurrence as being due to mutated tissue stem/progenitor cells, which retain or re-gain properties common to normal adult tissue stem cells. Normal stem cells are confined to anatomic niches where they are protected from toxins by, for example, interactions with other niche cells

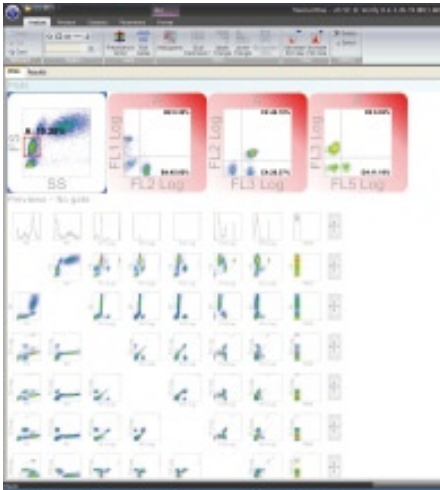


Fig. 1: Example screenshot from VenturiOne showing preview histograms

and detoxifying enzymes. They proliferate into a progenitor daughter, which migrates from the niche and produces mature cells with tissue-specific characteristics. CSCH claims that a small number of mutated stem cells retain their self-protective and self-renewal properties, and that some mutated progenitor cells can also dedifferentiate into tumorigenic stem cells. This protects them from cancer therapy meaning they can subsequently reactivate and redifferentiate into non-tumorigenic cells, which form the therapy-sensitive bulk of a tumour [3]. CSCH's main contribution is recognising that traits retained from normal tissue stem cells, or acquired due to mutation, guarantee that some tumour cells will self-renew and self-protect [7].

CSCH studies are now possible that were impractical with older flow cytometry

systems and software. Rapidly analysing numerous parameters is important, because the event frequency may only be 0.01% for bone marrow micrometastases [3] and cannot be boosted without physically pre-processing the sample. The SNR for a noisy sample with limited parameters may be too low to distinguish very low frequency events. Furthermore, rapid processing is required to filter out noise and unwanted data. Detecting cancer stem cells in disaggregated solid tumour samples is complicated by spurious signals generated by, for example, dead cells, debris, cell clusters and autofluorescent cells [3]. Autofluorescence can be reduced by using a laser wavelength longer than that which excites it. Dead cells and debris appear in even freshly collected samples. However, they have broad emissions spectra and bright DNA staining, meaning they are distinguishable from live cells with overlapping emissions spectra and can be eliminated from rare-event data by, for example, detecting decreases in forward scattering properties, such as pulse height and width [2]. Comparing forward scatter pulse height and width can also remove malignant cell clusters, which remain in disaggregated samples. Finally, cells expressing certain markers, such as the pan-hematopoietic marker CD45, must also be removed.

Sweeping Away the Chaff

New flow cytometry software is revolutionising REA. Analysing millions of

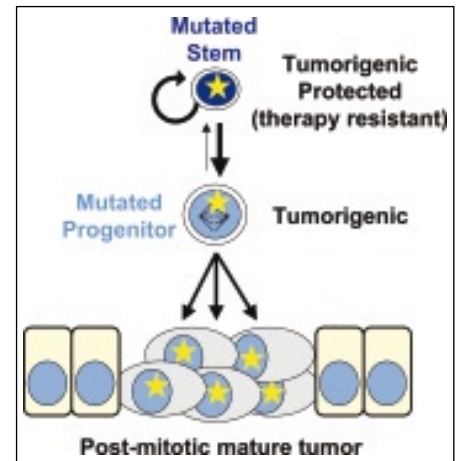


Fig. 2: Graphical representation of CSCH [2]. Mutated (star) stem cells are rare in epithelial tumours. The cancer stem cell can self-replicate (circular arrow). The two-way arrow between the stem and progenitor cells shows progenitor cells can revert to being stem-like.

events now takes minutes. Visually interpreting cell properties in 14-dimensions is simple now that numerous histograms can be viewed simultaneously on-screen. The next step is using software like VenturiOne to aid multi-parameter functional assay development. New CSCH studies use flow cytometry to study stem cell differentiation and proliferation over time using rare event data including up to 14 parameters.

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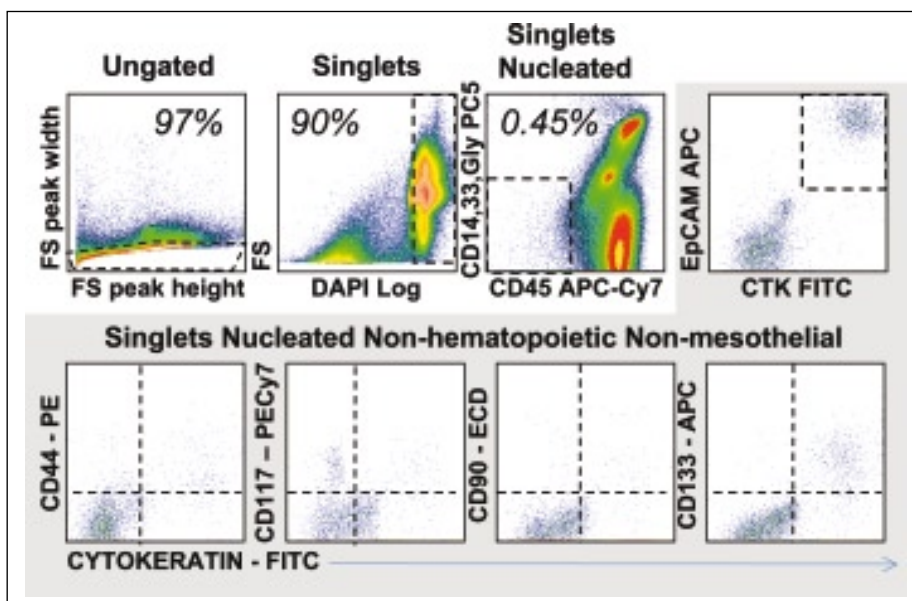


Fig. 3: Flow cytometric gating strategy for identification of cancer stem cells. From left to right: forward light scatter pulse analysis used to identify singlets (eliminating cell clusters); noncellular debris and dying cells eliminated by fluorescent staining (DAPI); subsequent histograms gated on various marker populations (e.g. Cytokeratin positive EpCAM positive tumour cells)

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