Multi-parameter flow cytometry technology advances for rare cell analysis and sorting

The study of rare cell populations is important to advance medical diagnostics and therapeutics. For many clinical studies, rare cell counts promise to provide valuable alternate end points; examples are circulating tumour cells in peripheral blood, tumour stem cells, endothelial cells in blood, hematopoietic progenitor cells and their subpopulations, antigen specific T-cells and foetal cells in maternal circulation. Besides representing a powerful diagnostic tool, detection of rare cell populations is also of value to fundamental research in disease mechanism and target identification. With multi-parameter capabilities and a very high analysis rate, flow cytometry is in a unique technological position to address the needs of extremely rare cell analysis. In this review, we describe advances in technology that further enhance the capabilities for rare cell analysis and isolation as well as points to consider for obtaining optimal results from rare cell analysis. Many of these considerations do not only apply to flow cytometry but to other techniques using the counting approaches, eg digital PCR, expression analysis, and proteomics.

Flow cytometry provides multiple data from individual cells. The ability to characterise a population of cells based on individual cells results in population level data far more meaningful than the number of cells that express a particular marker of interest. The distribution of expression of a marker in a population has long been recognised as a useful characteristic. Distribution is being increasingly investigated as a natural mechanism to tune a systemic/population response to a stimulation[1]. This trend towards multiple, simultaneous measurements on a per cell basis has firmly identified flow cytometry as a High Content technology.

Innovation in instrumentation

Flow cytometers are comprised of a fluidic system, an optical system, detection electronics and data acquisition. The fluidic system aligns particles (cells) from an aqueous suspension in a one-dimensional file in a fluid stream. The cells in the stream are illuminated
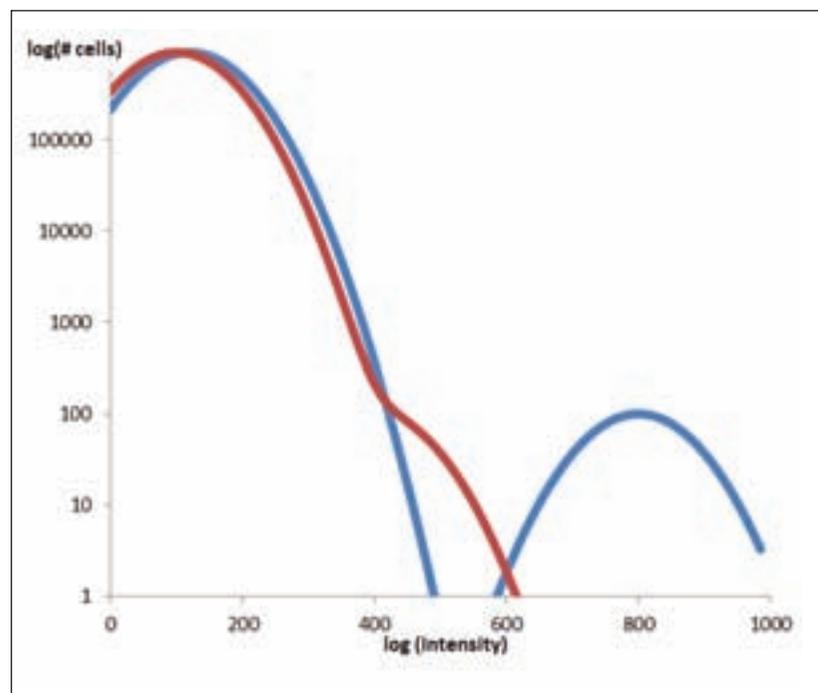
By David Gebhard and Dr Diether Recktenwald

Flow Cytometry

by one or more well-positioned light beams. When a particle traverses the light, a pulse of scattered or fluorescent light is generated and then analysed. Several independent optical measurements are usually performed per cell and stored on a per cell basis in a computer file. Advances in lasers and detectors have reduced the size and increased the performance of modern flow cytometers. About 20 different measurements can be performed simultaneously on a cell with this approach at rates of up to 100,000 cells per second. A recent development extends the number of measurements to about 50 markers simultaneously by using element labels with ICP mass spectrometric detection rather than optical labels. On-going research aims at matching the MS performance by using full spectral analysis of fluorescence[2] but also at higher levels of multiplexing with surface enhanced Raman labels[3]. Measuring more parameters helps to better differentiate between rare cells and background, resulting in an increase of specificity.

Innovation in labels

Fluorescent molecules are the most commonly used labels for cytometric measurements because they can be detected at very low concentrations. Colorimetric dyes are used less in cytometry because of their relatively low sensitivity. Originally, small organic fluorescent molecules such as fluorescein and rhodamine were used by cytometrists. The discovery that algal pigments from photosynthetic light harvesting protein com-

plexes, eg phycoerythrins, phycocyanins and peridinin chlorophyll proteins, can be successfully conjugated as pigment proteins to affinity reagents provided many additional labels for multi-colour fluorescence analysis and extremely high sensitivity detection. Later additional work on fluorescent nanoparticles (eg quantum dots or silica nanoparticles) impregnated with conventional organic dyes has added another group of labels which are still optimised for brightness and non-specific staining. Recently, organic chemists have modified fluorescent polymers from the electronics industry to create extremely bright labels in many different colours, further adding to the fluorescent label repertoire for highly sensitive, multiplexed detection of cellular biomarkers[4].

As mentioned in the instrumentation section above, element labels for detection by ICP MS are another option for cytometrists seeking high levels of multi-parameter detection. These labels are selected from non-radioactive isotopes which do not generally occur in biological systems, eg ^{139}La, ^{141}Pr, ^{142}Nd, ^{143}Nd, ^{144}Nd, ^{145}Nd, and ^{146}Nd. Because ICP MS determines the mass of the element, non-radioactive isotopes of the same element can be used as different labels; Neodymium, for example, has 142 to 146 different stable, non-radioactive isotopes. To make them useful as labels for affinity reagents, they are bound to chelating agents which in turn are covalently attached to the appropriate affinity reagent. The ability of ICP MS to discretely resolve signals based on mass represents a significant solution to the problem of overlapping fluorescence emission spectra. This, in effect, broadens the bandwidth of available data[5].

Another area of potential signal source for cytometry is the use of surface-enhanced Raman spectrometry. Nominally, Raman quantum yield is about one million times lower than typical organic flurochromes. However, signal enhancements of 10^{10} using gold or silver-coated nanoparticles have been reported. At present, consistent enhancement factors required for routine applications have not been achieved[3].

Progress is being made throughout the label areas, promising even better capabilities for rare cell cytometry in the near future.

Innovation in affinity reagents

Monoclonal antibodies, reactive with cell surface markers and conjugated with fluorescein, were used to measure the lymphocyte populations of B-cells, T-cells and the helper and suppressor cell subsets of T-cells with early flow cytometers. In the meantime

Flow Cytometry

	SAMPLE 1	SAMPLE 2	SAMPLE 3	SAMPLE 4
	6	2	6	8
	3	7	1	6
	1	3	5	3
	1	4	5	6
	1	4	6	3
MEAN	2.4	4	4.6	5.2
ST DEV	2.2	1.9	2.1	2.2
		OVERALL	MEAN	4.1
			ST DEV	2.2

more than 300 cellular markers have been assigned a cluster designation (CD number). Antibodies have been complemented with additional affinity reagents such as aptamers, molecular imprints and others. A recent review of progress in the field has been published recently[6]. In addition to affinity reagents for proteins, nucleic acid-based assays also provide increased applications in cytometry. Improvements in amplification technology now allow the detection and counting of single RNA molecules in single cells using flow cytometry[7].

Innovation in software and algorithms
As mentioned above, flow cytometry measure-

Beckman Coulter FC500	<1%	
Beckman Coulter	<0.1%	
BD FACSCanto II	<0.1%	loader <1%
BD FACSVerse	<0.5%	
Guava EasyCyte	<0.2%	
Life Technologies Attune		loader <0.5%
Miltenyi MACSQuant	<0.01%	

Table 2: Carryover specifications, published by the manufacturers for different flow cyometers

ments provide many data numbers per cell. Two measurements per cell can be easily displayed in a dotplot or contour plot. For data sets beyond two numbers per cell, gating is applied; this approach analyses the data, looking at pairs of parameters[8]. Early approaches to cluster analysis have been used to analyse the data, and automation of data analysis is making good progress. More recently, however, more sophisticated bioinformatics approaches are used for the analysis of cytometry data, including those from rare cell subpopulations[9].

Sorting
High end flow cytometers have the capability to sort cells in real time, based on the analysis approach described above. Analysis rates of up to 100,000 cells per second are possible; special thresholding allows even higher cell throughput by only analysing the easily identified subset of interest. This makes flow cytometric sorters the key choice for further characterisation of single rare cells using genomic single cell technologies after sorting, or for selected populations using proteomic analyses[10].

Fluorescence Activated Cell Sorters have long been based on jet-in-air, electrostatic droplet deflection designs. Considerable progress has been made in the speed and accuracy of cell sorting. Micro-fluidic flow cytometers have been under development for a number of years and show impressive progress in miniaturising flow cytometric analysis. The parallelisation of flow analysis to comprise simultaneously operating flow analysis and sorting represents an innovative step to increase the processing of individual cells. Cytonome/ST researchers have demonstrated robust identification of extremely rare target particles. Using Cytonome/ST's existing parallel detection technology and cross contamination-free sample system, the researchers were able to reliably identify 60 fluorescent target particles within a sample of 640 million non-fluorescent particles (corresponding to the identification of one target particle in 10 million total particles) in less than one hour. Parallelisation of analysis and sorting overcomes the limitations of single channel processing and allows direct analysis of large sample volumes (**Figure 5**).
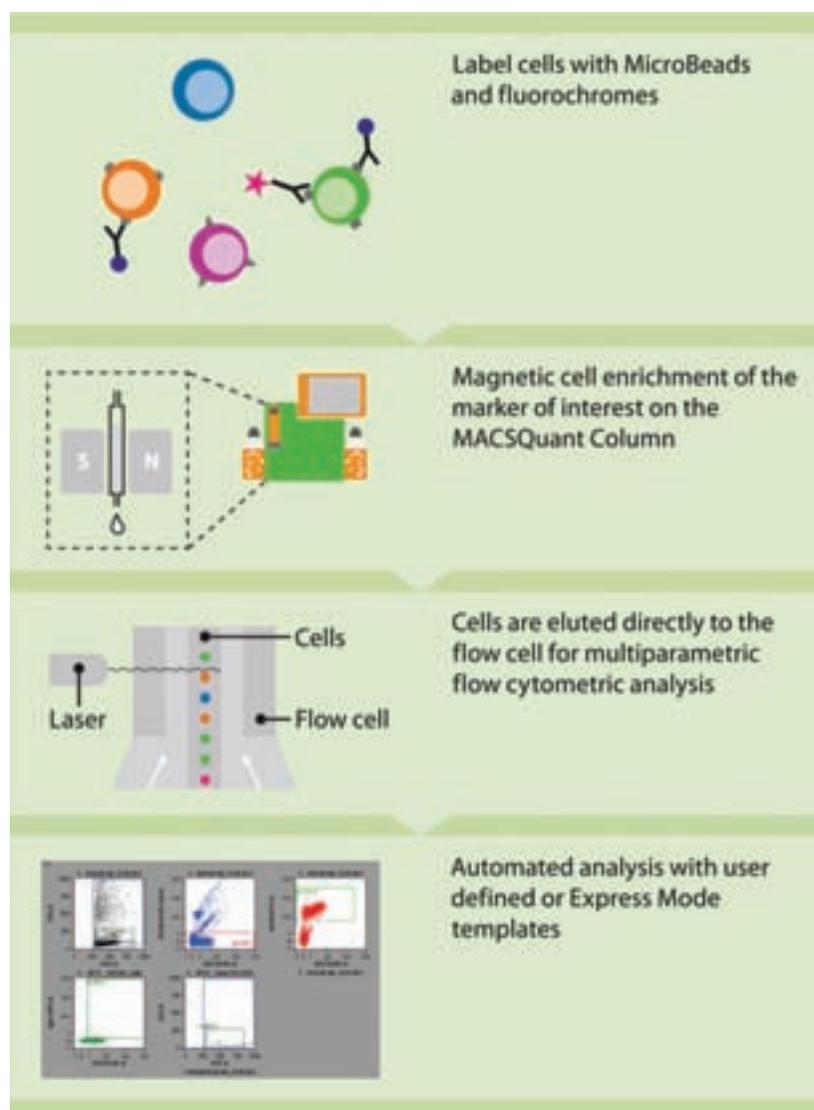
Rare cell analysis considerations
The analysis of very rare cell populations at the level of 10^{-3} or lower requires some special considerations. A very low level of false positives must be achieved by using appropriate markers with generally bright labels as described above. **Figure 1** illustrates the effect of marker brightness.

Using a low intensity marker (red curve) does not resolve a rare population (only a shoulder is observed in the histogram), whereas a substantially brighter marker shows a well separated population which can be measured easily in terms of concentration (number of molecules per cell) and subpopulation count. Precise counting of cells of a rare population is another challenge and requires the understanding of counting statistics. The expected standard deviation is equal to the square root of the actual count during the analysis of the sample. When only four cells are observed, a standard deviation of two or a coefficient of variation of 50% is expected. **Table 1** shows that in the absence of taking this into account, erroneous conclusions can be drawn. The numbers for sample one through four are derived from the same population. However, an inexperienced observer could conclude that there is a more than two-fold increase in count from sample one to sample four. This creates a dilemma when quantitative counts are needed to assess the effect of a treatment on rare cell subpopulation. The low count limitation cannot be overcome by using better markers or labels; but it requires the use of a larger sample volume to increase the cell count in order to increase the precision.

Another important system parameter, which substantially affects rare cell data, is sample to sample carryover. Many cytometry systems exhibit particle carry-over in the 0.1% range, which without special washing steps severely restricts the limit of detection for rare cells. **Table 2** shows carryover values from the manufacturers' specifications for several commercially available flow cytometers.

When carryover has been taken into account, the limit of detection for rare cells is primarily influenced by the selection of specific markers on the cell population of interest, the expression level of those markers, and the non-specific binding properties of the cell labelling reagents. A labelling strategy, which labels most of the cells that are not part of the population of interest ('dump channel'), drastically reduces the effect of non-specific labelling by eliminating the targets for non-specific labelling while at the same time potentially reducing the efficiency of the rare cell detection, ie trading sensitivity for specificity.

The pre-enrichment of rare populations of interest is another way to improve the precision of the data. **Figure 2a** shows the workflow of this approach, **Figure 2b** shows an instrument, which integrates magnetic particle-based pre-enrichment with flow cytometric analysis,

Label cells with MicroBeads and fluorochromes

Magnetic cell enrichment of the marker of interest on the MACSQuant Column

Cells are eluted directly to the flow cell for multiparametric flow cytometric analysis

Automated analysis with user defined or Express Mode templates

Applications for drug development

For several decades, modern pharmaceutical discovery has been driven by the increasing industrialisation of biological assays. This, coupled with significant improvements in chemical synthesis, has lead to the widespread practice of High Throughput Screening, whereby assay densities have increased from plates of 96 wells to 384 and 1536 wells. Rapid biochemical assay formats and analysis combined with rapid chemical design and synthesis have revolutionised Quantitative Structure Activity Relationship studies. Taken together with a concomitant focus on molecular reductionism, driven by advances in genomics technology and sequencing, the result has been an industrial wide focus on target-based discovery. Recently, however, in light of the clear industry wide failure to generate significant New Chemical

Figure 2a
Workflow of single cell rare cell analysis with in-line magnetic pre-enrichment of the rare cell population with the MACSQuant™
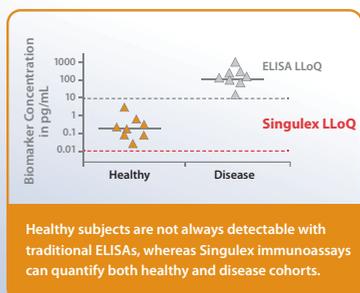
Figure 2b: Photo of the system. *(Graphics for 2a and 2b kindly provided by Miltenyi Biotec)*

Although phenotypic screening can be interpreted to mean different things, it is generally used to imply an assay for a systemic biological response of a cell or organism. Its definition has come to include the analysis of previously undetected correlated changes that can be increasingly detected and statistically rationalised by analysis of high content data. The concept can be viewed as a 'Phenotypic Fingerprint'. It is often encumbered by the perceived requirement to deconvolute the mechanism that results in the observed phenotype. Medicinal chemists and the application of Quantitative SAR require a biochemical target on which to focus. Quantitative SAR, though elegant in concept, can be frustrated by the ruggedness and high dimensional nature of the 'Activity Landscape'[12] and 'Activity Cliffs', where a minor change in structure abrogates activity. Interestingly, the notion of a phenotypic fingerprint has been applied to retrospective analysis of historical HTS data to cluster biologically similar reactivities and relate them to structurally related classes of compounds[13]. The increasing ability of
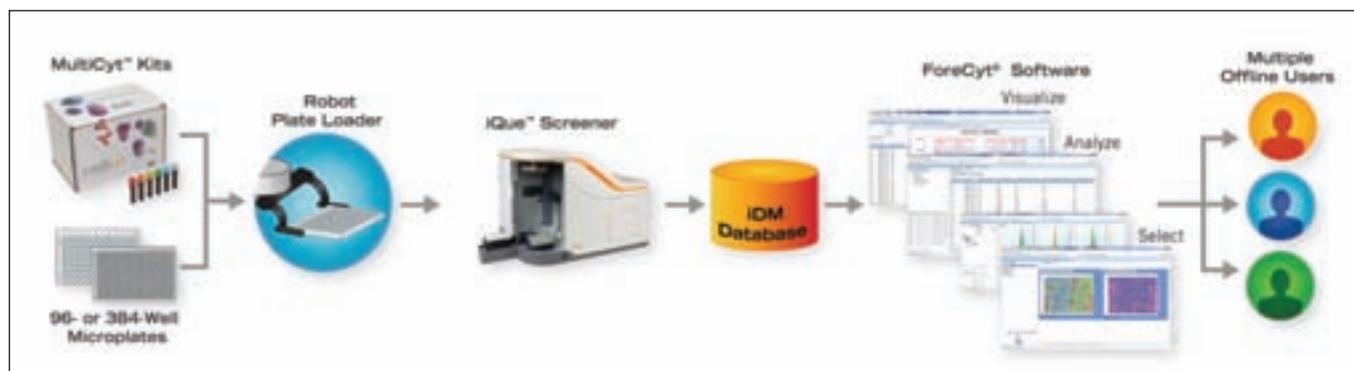
Entities (NCE), there has been a call to redirect screening technologies away from putative targets and engage in Phenotypic screening and Physiologically Relevant assays[11].

high content flow cytometry, often aligned with quantitative image analysis, can provide a significant leverage in this space. The developing tools of high content Phenotypic data, based on single cell analysis, are being increasingly applied to this critical intersection of chemical and biological space. The introduction of High Throughput flow cytometry, as realised in the IntelliCyt HyperCyt and iQUE Screener systems, is an example of the potential of plate-based informatics, integrated automation and high content flow cytometry. The University of New Mexico Center for Molecular Discovery features High Throughput Flow Cytometry as part of the NIH program-NIH Roadmap for the Molecular Libraries Probe programme[14,15] (**Figure 3**).

Highly dimensional data allows investigators to look for and identify nodes, or intersecting points of signal transduction, that occur when a system is perturbed[16]. The very high content data available from single cell analysis can be used to phenotype the cells in a population and simultaneously to assay the status of signal transduction pathways. This can be used to identify the topology of signal transduction network and, more importantly, the state of the network. This has enormous potential in the burgeoning interest of personalised medicine. High Content cytometric data and the techniques of rare cell analysis can identify relapsing tumours and, based on the phospho-signalling status of pathways of interest, can guide physicians to a targeted chemotherapy as well as identify potential targets for future drug development[3].

Similarly to the enabling technologies brought to bear in cytometry. Cytometry can be seen as an enabling technology for sensitive proteomic and genomic analysis. The heterogeneous nature of primary tissues and population response can swamp and confound highly sensitive technologies. There can be too much material to detect a minute signal,

or in a manner similar to wellular/aggregate data, the nature of the signal is itself heterogeneous.

Correlation of transcript levels within individual cells has provided insight and a potent indication of cell types and distinct subpopulations of cells in studies of stem cell differentiation, tumours and populations within differentiated cell types[17,18]. The use of Index Sorting (a high content flow cytometric record of each individual cell that is sorted) and single cell expression analysis represent potent additions to the tool box (Fluidigm/FACS), where the complete correlated record of marker expression can then be interpreted in the context of expression analysis. Genomic analysis of single cells can distinguish between biomarkers or genes with correlated expression or uncorrelated expression in specific cell types or subpopulations – thus clarifying measurements made on the aggregate. A

Figure 3
Workflow of single cell analysis with a modern cytometric system, optimised for automated high-content screening. *(Image kindly provided by IntelliCyt)*
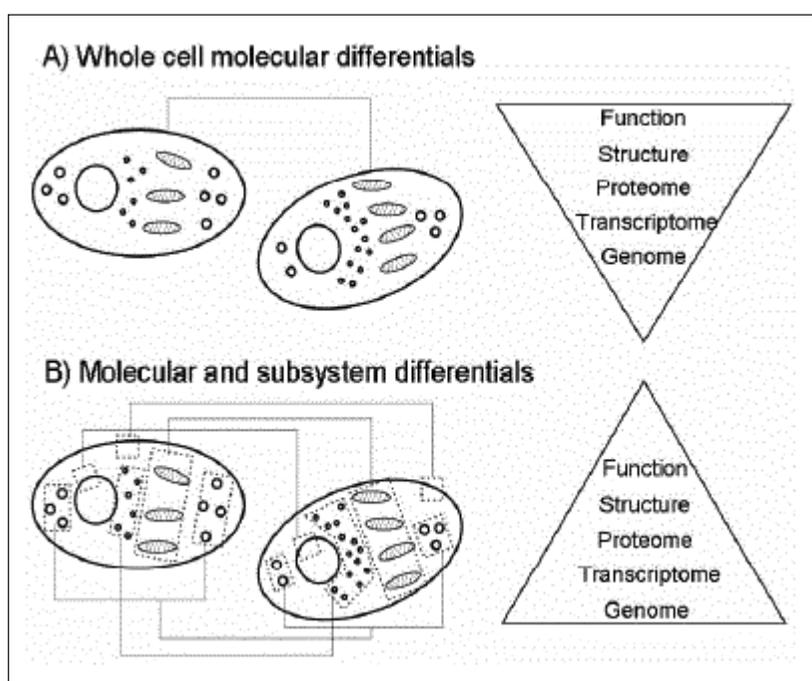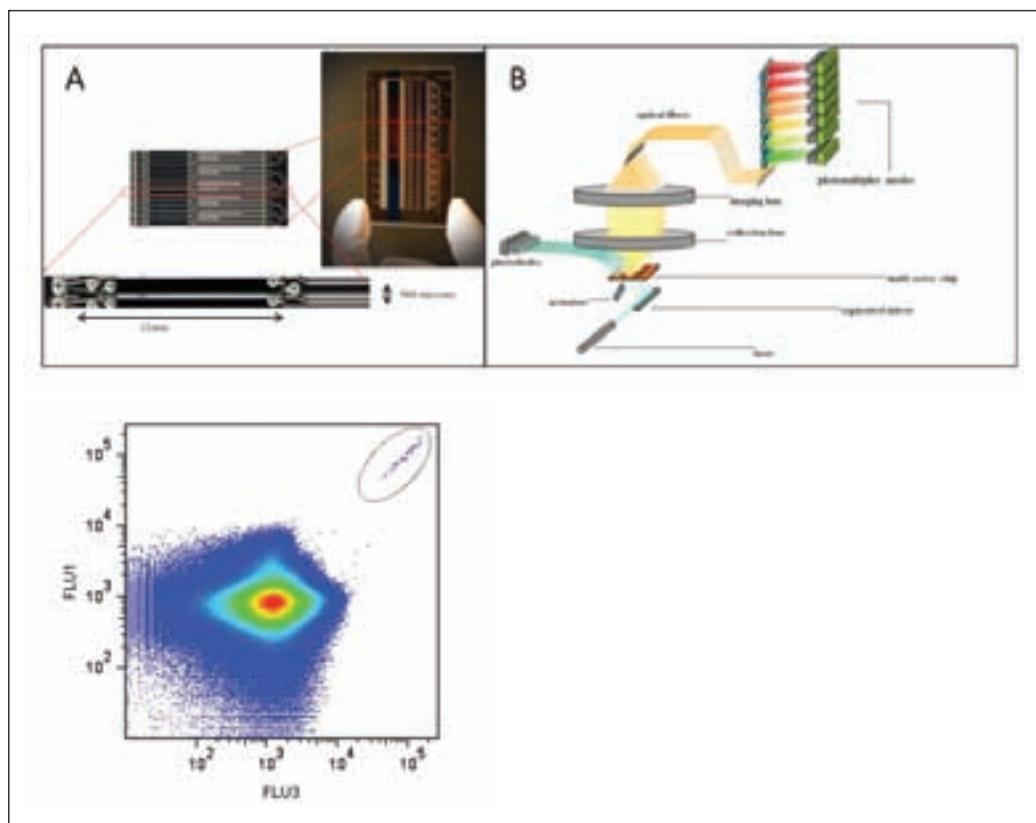
Figure 4

Flow Cytometry

potent illustration of correlated phenotyping and transcript analysis maybe found in the dissection of early immunologic profile changes in vaccine development where previously unrecognised subsets of CD8 T-cells were detected[19]. It is interesting to note that investigators now distinguish transcript analysis on 100 sorted cells as bulk when defining gene signatures and true single cell transcript analysis to define the co-ordinate expression analysis of multiple genes at the single mRNA transcript level to reveal true subsets of cells[20].

A comprehensive proteomic experiment can require milligrammes of protein corresponding to several million cells[18]. The need for large amounts of starting material is required to fractionate the manifold species of proteins that themselves vary in abundance. If a cell type of interest, eg stem cell, tumour, or functional player in a systemic response, is a relatively minor population, flow cytometric characterisation and sorting offer a direct means to highly enrich the population of interest for sensitive proteomic analysis[21,22,23].

The basic technology of creating a 'cytometric address', which has been adopted in Cytometric Bead Array applications to allow for high dimensional multiplexing for soluble analyte assay (bead-based ELISA) (Luminex and others), has also been shown to be a powerful means to multiplex cell based assays by flow cytometry – fluorescent cellular bar-coding[24]. By using combinations of three fluorophores, it was possible to generate 96 distinct cytometric addresses for high resolution multiparameter analysis. A very recent expansion of this technique is the development of cell surface expressing DNA-binding Zinc Finger domains, where sequence specific fluor-conjugated oligonucleotides are used to generate cytometric addresses[25]. This elegant approach to categorising cells takes advantage of the high content nature of flow cytometry and has allowed in-depth large scale analysis of signal transduction, identifying heretofore hard to detect minor co-responding populations.

The combination of atomic mass spectroscopy and single cell analysis as realised in the CyTOF atomic mass cytometer marks the evolution of cytometry into the post-fluorescence era. The expanded spectra of mass cytometry and the clever application of cellular bar-coding has been demonstrated to combine and analyse a matrix of 96 different combinations of stimuli and inhibitors across a number of different cell types and signal transduction pathways[26]. Although the CyTOF technology is an acute case of 'destructive sampling', the insights gained can lead to standard

fluorescence-based FACS sorting experiments for populations of interest that would have otherwise been undetectable and are now accessible for genomic and proteomic analysis.

An unusual example of rare cell analysis is the study of phenotypic differences between genetically identical cells. Analyses of intracellular biochemical signal transduction and networks have been traditionally done by stimulating populations of genetically identical cells. When performed in a well-based platform, the assay reports an aggregate signal. An intermediate wellular response could be a uniform intermediate response, or the strong response of half the population and non-response by the other half. It could also be the result of a heterogeneous response. The contributions of individual cells are obscured. A single cell technology such as flow cytometry can reveal the characteristic contributions of the members of the responding population. A consequence of interpreting data from an aggregated wellular assay is that signalling pathways have come to be viewed and modelled as being finely tuned circuits that behave in a predetermined way[27,28]. Examination of minor differences rather than their general features can reveal novel regulatory mechanisms for diverse cellular processes[29]. The fundamental molecular mechanisms that result in the divergence of expression and function in otherwise identical cells have been suggested to be a fruitful area of investigation. Single cell cytometry and sorting, proteomic and expression analysis will all be required.

The ability of high content single cell analysis to detect, characterise and isolate for genomic and proteomic analysis, as well as to understand the fundamental nature of a population of individuals is the foundation of Cytomics. The cytomics approach reflects the reality that cells and their inter-relationships, and not genes or biomolecules, represent the elementary function units of organisms. Cytomes consist of single cells in various states of proliferation, differentiation and activation. Rational target identification and prosecution based on a cytomic investigation of disease represents a significant change in approach: Top Down rather than Bottom-Up[30] (**Figure 4**).

In conclusion, the technical advancements being made across the spectrum of hardware, software and 'wet-ware' biological tools are making deep and disruptive impacts in many fundamental disciplines within clinical application of cytometry and the whole of drug discovery.   DDW

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Dr Diether Recktenwald currently provides professional expertise on cytometry technology utilisation through Desatoya LLC. Until October of 2012, he served as VP of Advanced Technologies for BD Biosciences, where he evaluated new approaches to Cytometry. Dr Recktenwald has more than 30 years of experience in flow cytometry systems and has published previously on pushing the limits of rare cell analysis. For his scientific education he performed postdoctoral work in Physical Chemistry and Structural Biology at Stanford University after earning a PhD with work on an enzyme mechanism from the Ruhruniversitaet Bochum in Germany.

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