New developments in SINGLE CELL separation and analysis technologies

Recent advances of single cell technologies are facilitating the opportunity to discern biological insights within individual cells and providing a means to reveal previously hidden relationships between individual cells within a population. Single cell technologies can be crudely split between those that enable single cell separation and those that enable single cell analysis. From the cell separation perspective, of interest are those new approaches that isolate single living cells from a fluid sample, manipulate cells for image-based selection, utilise infrared laser capture, and those that compartmentalise individual cells into piciloter droplets. The key analysis technologies are those that facilitate amplification for gene expression profiling, transcriptome analysis and sequencing. Miniaturised all-in-one systems that bridge the gap between single cell separation and analysis hold out the promise of greatly simplifying the processing and bringing automation to the investigation of single cell biology. New tools addressing many of the hurdles associated with working with single cells are becoming available at a rapid pace helping build new single-cell applications. The study of single cells is likely to impact our understanding and treatment of human disease over the coming years.

By Dr John Comley

Historically cell populations were assumed to be homogeneous and most biological techniques and assays (eg gene expression profiling) have been performed on populations containing thousands, even millions, of cells. The results obtained from such analysis (eg expression levels) represent an amalgam of the biological status of each cell within the population analysed. The recent advent of single cell analysis techniques has enabled the opportunity to discern biological insights within individual cells and have provided a means of revealing previously hidden relationships between individual cells within a population or to detect subpopulations. Minority, rare cell events, and small changes between individual cells (eg differences in size, protein levels and expressed RNA transcripts) may hold the key to answering hitherto unresolved questions in cancer, stem cell biology, immunology, developmental biology and neurology.

The analysis of single cell biology presents significant challenges including the amount of starting material available, the separation and isolation of single cells, platform sensitivity and sample throughput. Most single cells are commonly manipulated as an unordered, unsorted, heterogeneous

Figure 1: Main reason for using a single cell versus an average of a pooled population of many cells

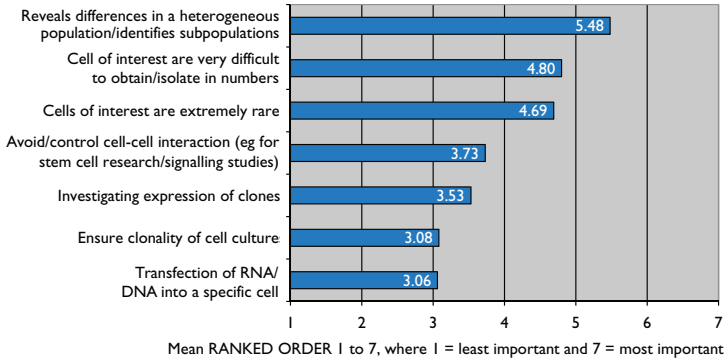


Figure 2: Scientific research area where interest in single cell technologies best fits

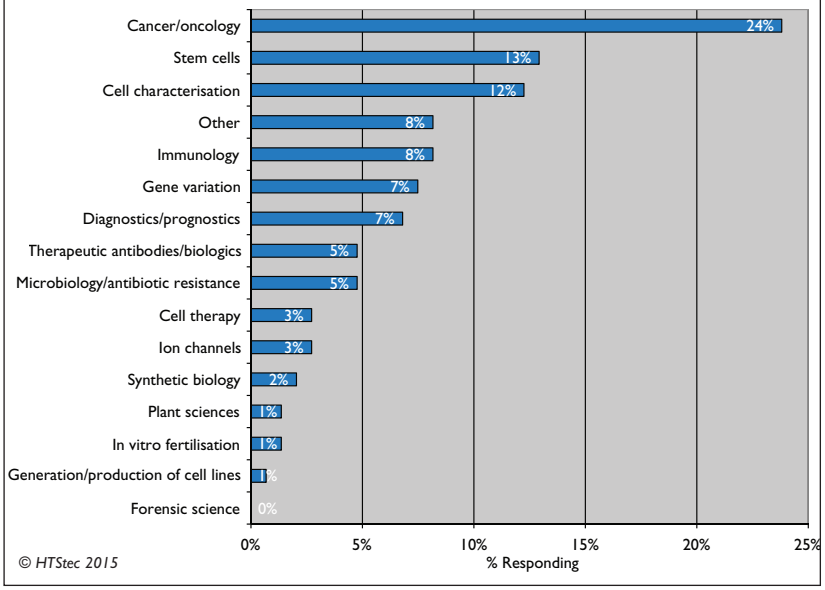
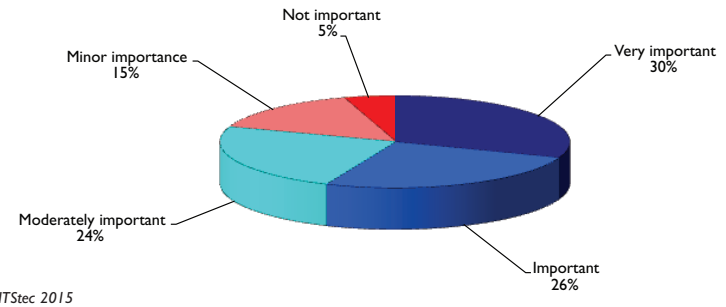


Figure 3: Importance placed on the use or application of single cell technologies



cell assembly often after lengthy periods of cell culture, labelling and isolation. Sample handling is probably the biggest bottleneck owing to the difficulty of analysing large numbers of single cells and conducting studies with sufficient experimental power to unravel statistically meaningful effects at the single cell level. New single cell platforms facilitating separation and analysis, and in some cases both, that potentially overcome these technology limitations are rapidly being developed and are proving of increasing interest to researchers, particularly in the area of single cell genomics.

In October 2014 HTStec undertook a market survey on single cell technologies[1] mainly among research labs in pharma, biotech and academia. The main objective of this survey was to gain insight in the application requirements, market opportunities, unmet needs and demand for single cell technology-related products. In this article highlights from this market survey are reported and the findings are discussed together with vendor updates on single cell separation and analysis technologies.

Why use single cells?

Intracellular differences in a heterogeneous population was ranked the most important reason for using a single cell versus an average of a pooled population of many cells. This was followed by cells of interest that are very difficult to obtain/isolate in numbers; and then cells of interest that are extremely rare. Ranked least important was transfection of RNA/DNA into a specific cell; and ensures clonality of cell cultures (**Figure 1**).

Where interest in single cells best fits

The scientific research area where survey respondents' interest in single cell technologies best fits was oncology/cancer (24% of respondents). This was followed by stems cells (13% of respondents) and cell characterisation (12% of respondents). All other areas had less than 10% of all respondents (**Figure 2**).

How single cells technologies are regarded

Most (31%) survey respondents placed high importance on the use or application of single cell technologies today with a further 26% scoring it important and 24% moderately important. That left 15% scoring it of minor importance and only 5% not important today (**Figure 3**).

Most (50%) survey respondents rate the value of specific information derived from single cell technologies (versus a pooled population of many cells) highly. This was followed by 36% rating it very

highly, and then 12% medium, 1% low and 0% none (**Figure 4**).

Most (41%) survey respondents' views on whether information derived by single cell technologies can be compared to alternative approaches was 'yes it can – but unlikely to show >50% complementary'. This was followed by 28% of all respondents who thought 'no – both are telling us different things'. Only 13% thought 'yes it can – they should be 100% complementary' (**Figure 5**).

Single cell technologies used today

FACS/flow cytometry was rated the separation technology currently most used to isolate single cells from tissues or liquid culture today. This was followed by random seeding or liquid dilution into microplates; manual cell picking; and then microfluidic/lab-on-a-chip devices. Least used was capillary-based isolation (**Figure 6**).

The analysis technologies survey respondents have most applied today to single cells (ie >50% use) were fluorescence microscopy, FACS/flow cytometry, brightfield microscopy and PCR/qPCR. Least applied by survey respondents to single cells were mass cytometry and label-free mass-based sensor (**Figure 7**).

The sources most used to isolate single cells were adherent cell cultures (62% using) and suspension cell cultures (55% using). The next most used sources were clinical samples (46% using); and fresh tissues (31% using). Least used were formalin-fixed tissues/sections (11% using) and embryos (7% using) (**Figure 8**).

Downstream work on isolated single cells

The majority (62%) of survey respondent's downstream work on isolated single cells mainly involves immediate destruction of cells to extract/purify cellular components for analysis. The remaining 38% of survey respondents mostly undertake further maintenance/culture of the isolated living cell under optimum conditions (**Figure 9**).

Applications where single cells have been applied

Gene expression profiling was the application survey respondents have most already applied/investigated with single cells. The next most applied/investigated applications by survey respondents were RNA/DNA sequencing; biomarker discovery, validation and/or screening; and then single cell heterogeneity; or cell line development. All other applications had been applied/investigated

Figure 4: Value of specific information derived from single cell technologies

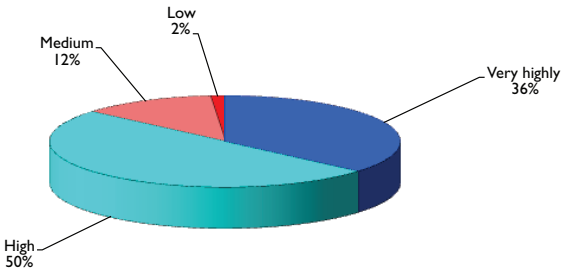


Figure 5: Can information derived by single cell technologies be compared to alternative approaches

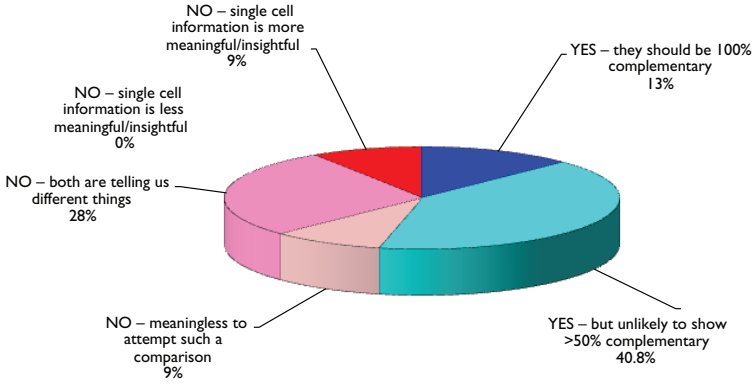


Figure 6: Technologies currently used to isolate single cells from tissues or liquid culture today

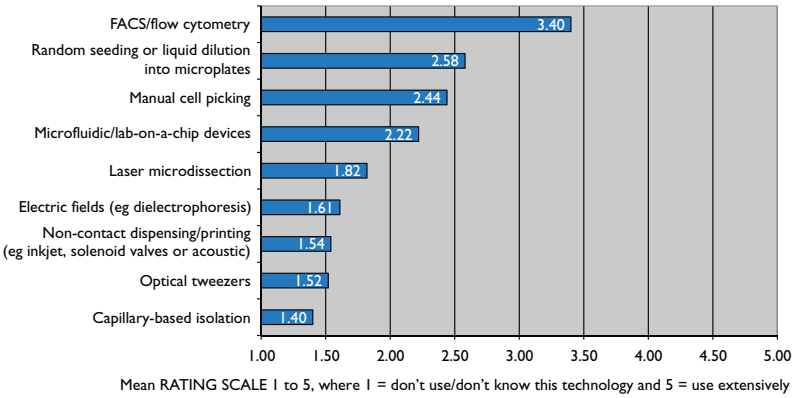


Figure 7: Analysis technologies applied to single cells today

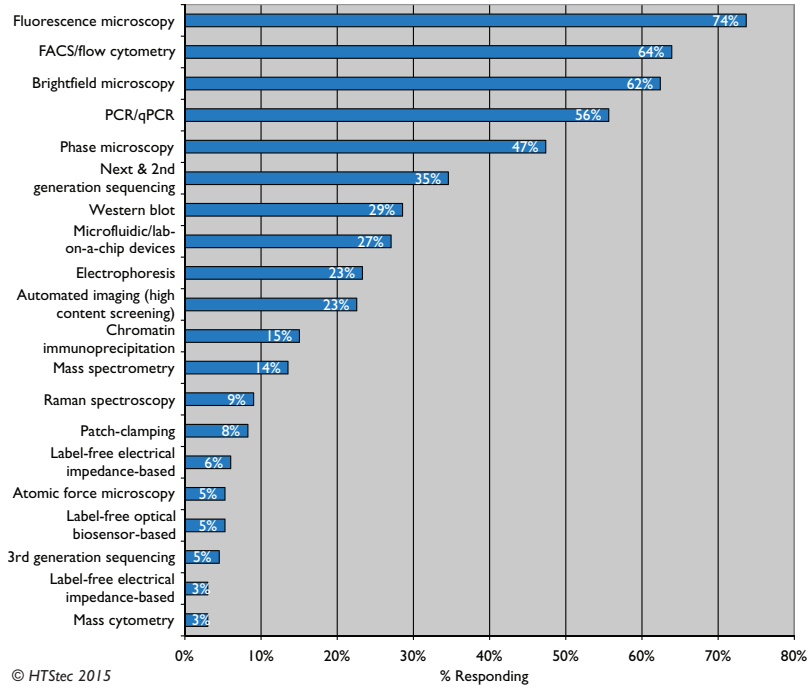


Figure 8: Sources Used to isolate single cells

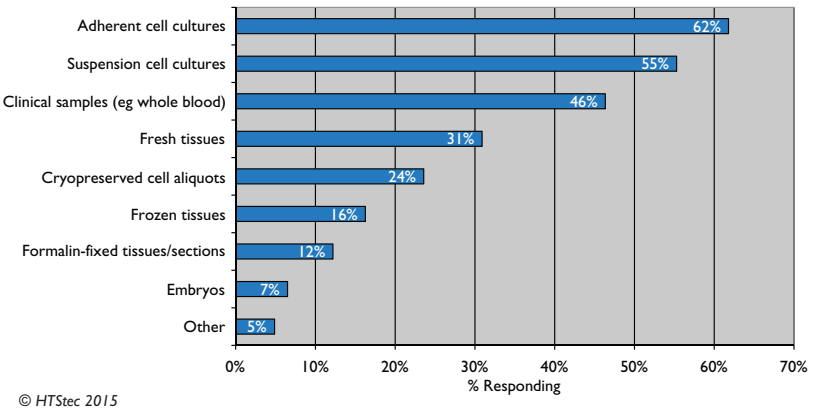
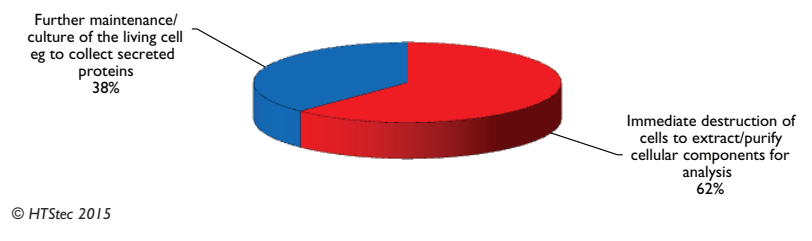


Figure 9: What downstream work on isolated single cells involves



by less than 25% of survey respondents. Least applied/investigated applications by survey respondents were generating human embryonic stem cells and vaccine development (**Figure 10**).

Function of single cell enabling technology of greatest importance

The ability to pick a single cell with a specific phenotype from a heterogeneous cell mixture was rated the function of a single cell enabling technology of greatest importance to their research. This was closely followed by the ability to analyse the genetic content of a single cell; ability to separate/transfer single cells into desired locations (eg the wells of a microplate); and then ability to separately investigate every cell from a heterogeneous mixture. Rated least important were the ability to inhibit or control cell-cell interaction; and ability to collect secreted proteins, enzymes or metabolites from a single cell (**Figure 11**).

Vendor updates on single cell separation and analysis technologies

Advanced Cell Diagnostics (www.acdbio.com) is focused on spatially-preserved single-cell expression analysis. New single-cell sequencing and expression analysis techniques are uncovering important cell-to-cell variations in gene expression which have been masked by traditional techniques that analyse bulk tissue or cell populations. However, these new techniques require isolation of single cells from their native context, resulting in loss of information on the spatial relationship of the analysed cells. Spatially mapped expression data at the single-cell level is crucial to understanding cellular organisation, clonal evolution and cell-to-cell interactions in complex tissue, eg how intra-tumour heterogeneity contributes to tumour progression and resistance to targeted therapy. Mapping RNA expression to single cells is possible with traditional RNA *in situ* hybridisation (ISH) techniques, but it has been hampered by the limited performance and high technical complexity of these methods. RNAscope is a new RNA ISH technology developed by Advanced Cell Diagnostics in recent years. It addresses the long-standing challenge of poor signal-to-noise ratio of traditional ISH by employing a unique probe design strategy that amplifies the signal by thousands of fold without also amplifying the background. It achieves single molecule detection under standard bright-field and fluorescence microscopy. Advanced image analysis software allows quantitation of transcript levels in spatially-preserved individual cells. Multiplex detection allows up to

Figure 10: Applications where single cells have been applied

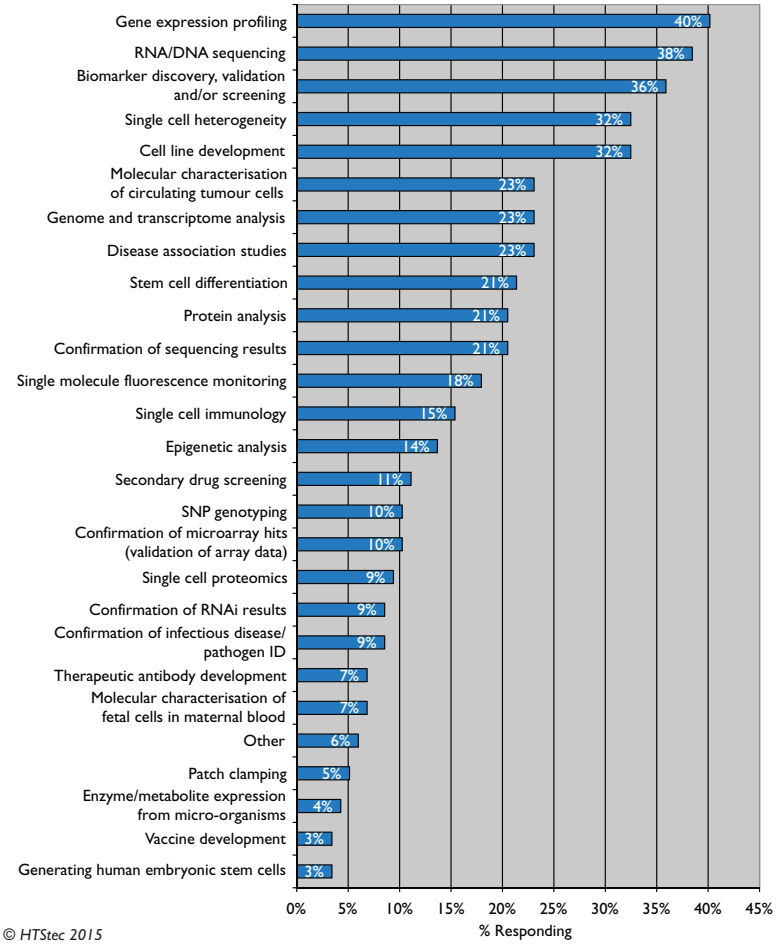
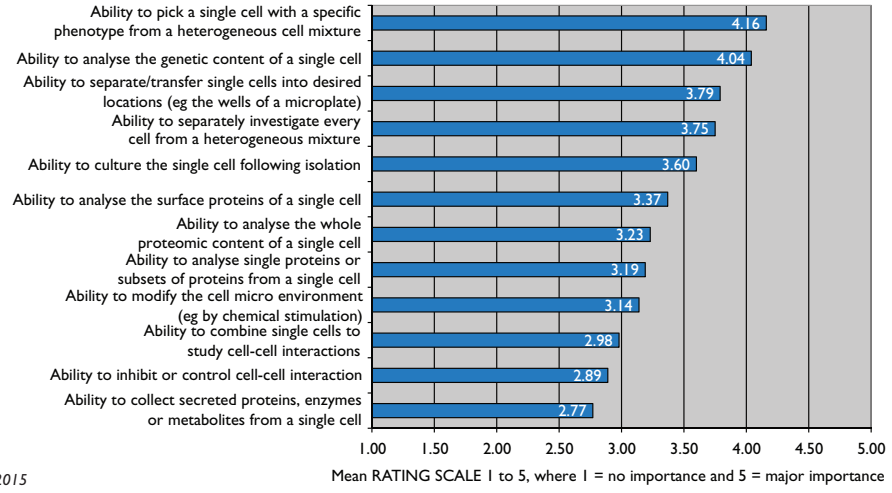


Figure 11: Functions of a single cell enabling technology of greatest importance



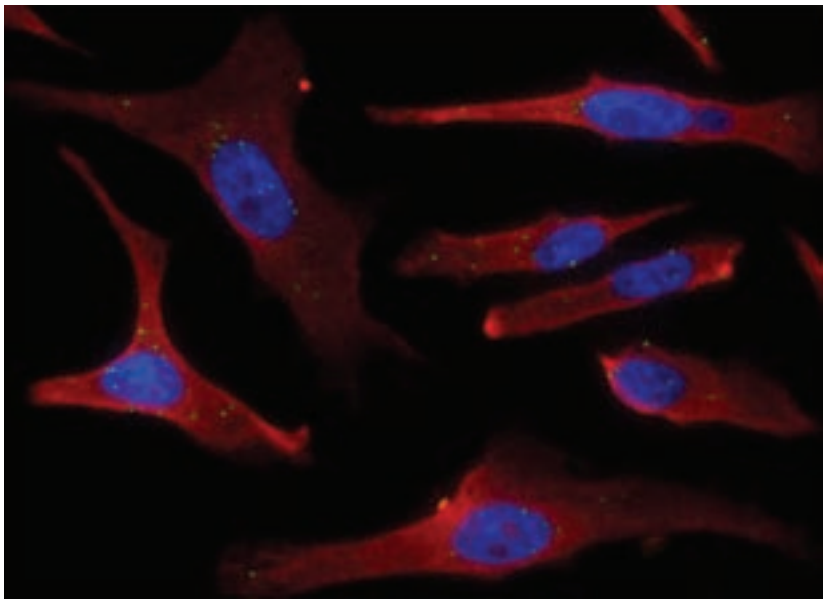


Figure 12: Example of single cell HER2 mRNA detection in HeLa cells using RNAscope® probe and signal amplification system from Advanced Cell Diagnostics. A probe set to 18S rRNA was used as internal control for RNA detection. Nuclei were counterstained with DAPI (blue). Original magnification, x40

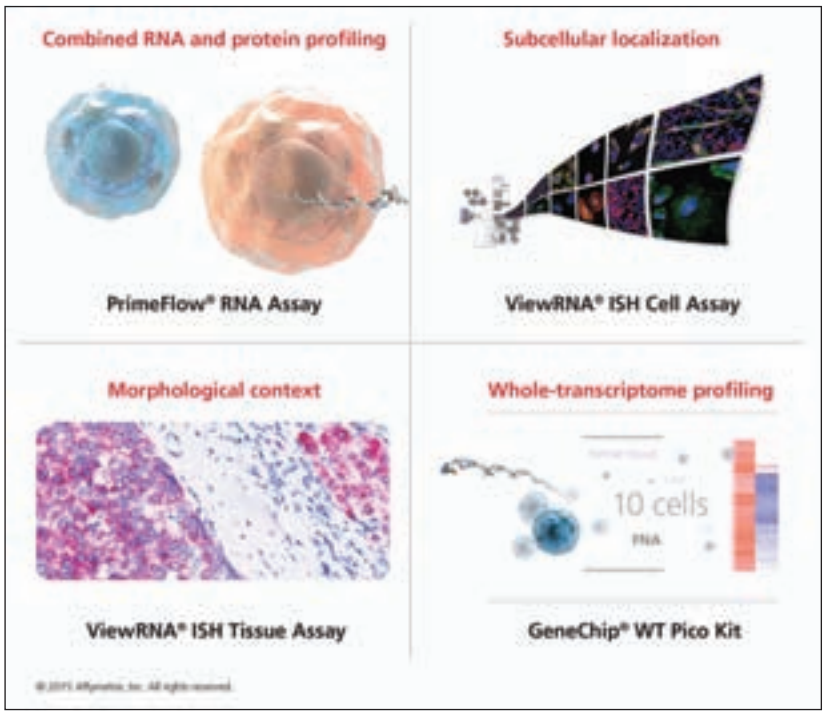


Figure 13: Affymetrix single cell platforms for revealing gene expression heterogeneity from whole-transcriptome to single transcripts

four RNA species to be mapped to single cells simultaneously. The latest advances in this technology include improved robustness and fully automated assays on existing automated slide staining systems. It should prove invaluable to the rapidly evolving field of single cell biology (**Figure 12**).

The ability to analyse gene expression from single cells is providing biological insight at the highest resolution possible. **Affymetrix** (www.affymetrix.com) platforms enable researchers to study single cells from whole transcriptome to single transcripts. Affymetrix's GeneChip® WT Pico Kit (WT Pico Kit) is a microarray target preparation solution designed for whole-transcriptome profiling from as little as 100pg of input RNA, as few as 10 cells. It is compatible with the majority of sample types, including cultured cells, fresh/fresh frozen, or formalin-fixed paraffin-embedded (FFPE) tissues, and whole blood. WT Pico Kit ensures strand specificity is preserved, which maximises coverage of the whole transcriptome for gene-, exon-, and alternative splicing-level analysis as well as long non-coding RNA analysis. The ViewRNA™ ISH Tissue assays, utilising branched (b)DNA signal amplification technology, allow users to quantitatively detect specific mRNA or long non-coding (lnc)RNA transcripts within the morphological content of their sample, directly from FFPE tissues. These stained tissues may be visualised using bright-field or fluorescent microscopy, and can achieve single molecule sensitivity for any transcript in any tissue. The ViewRNA® ISH Cell assays enable simultaneous single-cell visualisation of up to four RNA transcripts, or one microRNA (miRNA) and two additional RNA transcripts, with single-molecule sensitivity. Under equivalent imaging conditions, the ViewRNA ISH Cell assay is 100 times brighter than traditional FISH, creating at minimum a 2-3 times higher signal-to-noise ratio. With PrimeFlow™ RNA Assay, scientists can now reveal the dynamics of RNA and protein expression simultaneously within millions of single cells. PrimeFlow™ RNA Assay enables detection of up to three RNA transcripts combined with intracellular and cell surface antibody staining using a standard flow cytometer to generate multi-parametric, high-content data while maintaining single-cell resolution in heterogeneous cell populations (**Figure 13**).

Beckman Coulter's (www.beckmancoulter.com) MoFlo Astrios EQ sorter technology is a logical solution toward characterising and selecting for single cell targets no matter how complex the task may be. The system is designed to support up to 52-parameter sort decisions with the ability to collect

singlet events into 96, 384 or even 1,536 well-plates without a single electronic abort. The Astrios EQ and EQs achieve this through a series of unique technologies. The first being the electronic threshold and event-rate parameters are independent meaning every event is accounted for in the system and not wasted. This is particularly useful where very high acquisition rates are needed during rare-event detection modalities. Secondly, a patented dual enhanced Forward Scatter option provides a unique way of fingerprinting sample profiles based upon their scatter characteristics. Once identified, a target event can be selected for multi-parameter single cell deposition and when coupled with the aid of robotic integration; automation-ready workflows accelerate and maximise walk-away single cell collection. In addition, the Astrios' unique R-Theta Arm technology allows Best-in-Class precision when translating well-to-well position ensuring the highest plating efficiency possible. Post-acquisition computational analysis using a powerful Index Sort GUI provides the confidence to fully characterise each single cell event before committing the acquisition to downstream processes including Targeted Genome Editing and Clonal Expansion for drug-target interaction (**Figure 14**).

Figure 14
Beckman Coulter's MoFlo Astrios high-speed six-way sorter for selecting single cell targets

The isolation of single cells is prerequisite for most applications in the field of single-cell analysis. Isolation is often performed manually or by using extensive laboratory equipment[2]. Single-cell printing, developed at **cytena** (www.cytena.com) and

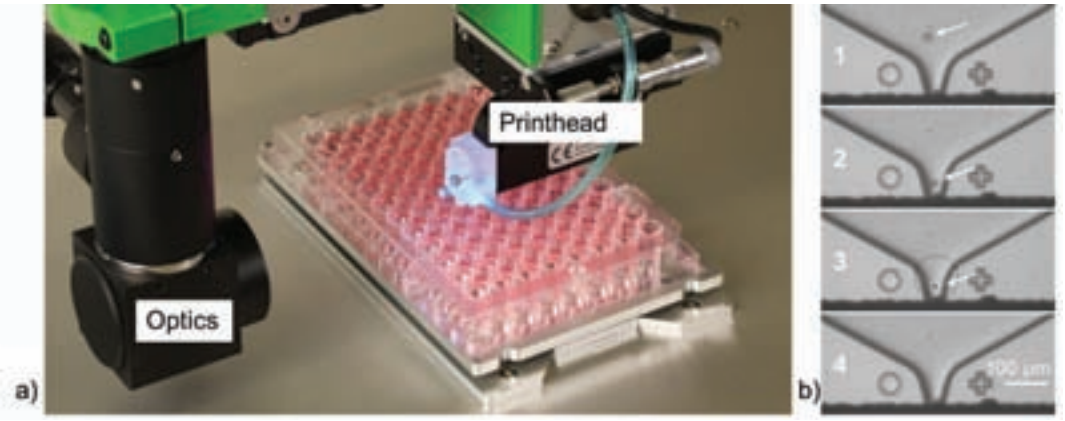


Figure 15
cytena's Single-Cell Printer (SCP). a) The system employs high resolution optics, inkjet-like printing and an automated object recognition algorithm to detect cells in a nozzle of a dispenser chip before dispensation. Droplets containing exactly one single cell are printed into a well plate. b) Image-based proof of single cell deposition of a CHO cell. An individual image series is stored for each event. 1: before the single cell event; 2: at the event, the cell is in the nozzle; 3: at the event with image processing overlay; 4: after the event, the cell has left the nozzle

the University of Freiburg, is an innovative tool for the isolation of single living cells from a fluid sample. Similar to an inkjet printer, the single-cell printer (SCP) generates free-flying microdroplets, which are delivered in rapid succession to any substrate (eg microtiter plates). The use of a microscope camera and computer-assisted automatic image processing enables the system to print droplets containing exactly one cell, while droplets with more or without cells are removed by a pneumatic shutter system. The image data is stored and assigned to each position or well. The sample is processed inside a single-use disposable printing cartridge, which avoids the risk of cross-contamination. The system is easy to use compared to other automated systems such as FACS and it fits in standard laminar flow benches for sterile works. The contact-free printing approach is not limited to specific substrates and is used for a wide range of applications. In monoclonal cell line development, clone recovery rates for CHO, HEK and L292 cells of around 80% were observed. In single-cell genomics, direct PCR of DNA from single cells and whole genome amplification of single-cells has been demonstrated. Single-cell printer prototypes are available to pilot customers and technology partners through cytena's early access programme (**Figure 15**).

Fluidigm (www.fluidigm.com) has several systems that focus on the study of single-cell genomics and proteomics, but its C1™ and Polaris™ systems are particularly relevant in the fields of single-cell separation and analysis. Fluidigm's C1 system can rapidly and reliably isolate, process and prepare individual cells for genome and transcriptome

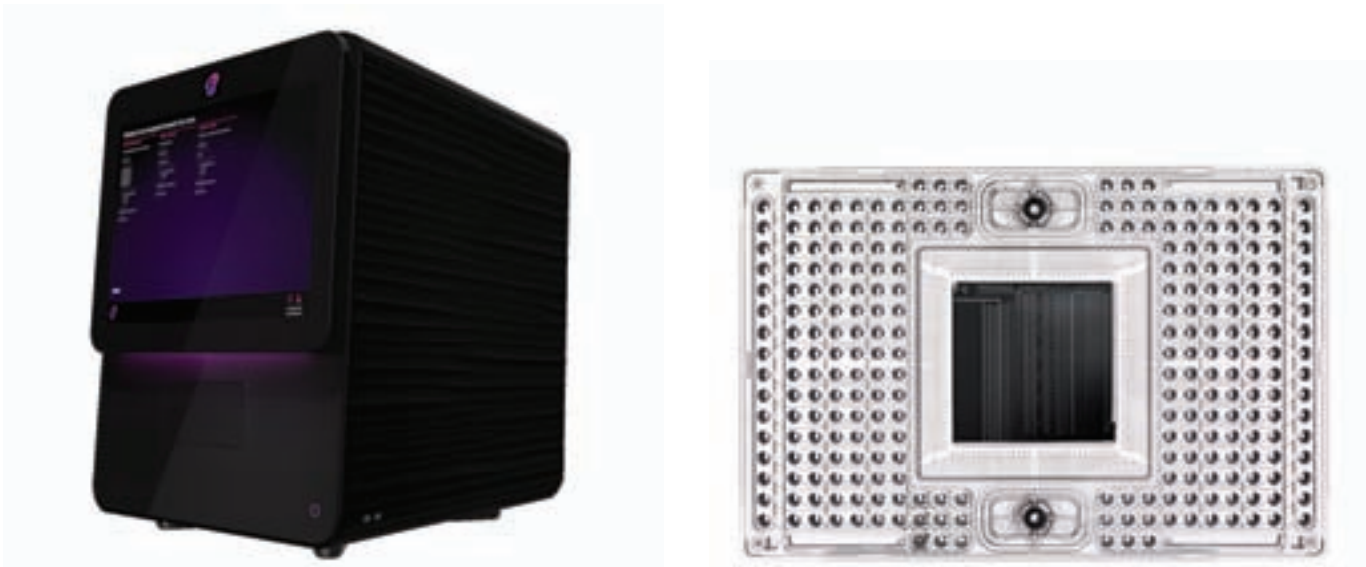


Figure 16: Fluidigm's Polaris system (left) and associated chip (right) with precisely designed integrated fluidic circuit

analysis. Using just the C1, researchers can isolate cells, stain them and prepare templates for sequencing or qPCR providing researchers with the ability to survey the tissue landscape/heterogeneity to understand disease. C1 allows scientists to understand the diversity of cell types within the normal tissue and define unique characteristics (using a transcriptional/gene expression profile) to differentiate and categorise each cell type. By sampling normal and disease tissue, researchers use the C1 to compare the type and distribution of cells and quantify different cell types as the disease progresses. Fluidigm's newly announced Polaris system integrates cell selection, isolation, dosing, culturing and molecular preparation into a single workflow, thereby enabling researchers to directly correlate gene expression with environmental conditions and phenotypic information. Researchers can recreate the cell's natural milieu and modify the cell's environment using different nutrients, stimuli or drugs, then measure changes in the gene expression/transcriptome to see how the cell has responded to different conditions. This allows researchers to see which biological pathways are activated and determine the functional role of each cell type in disease progression. Polaris also allows researchers to determine drug response/efficacy (either singular or combination drugs). They can expose the target cell type to different combinations of drugs and at different dosages to measure the range of cell responses and identify which ones are the most effective in shutting down or activating the appropriate pathways (**Figure 16**).

GE Healthcare (www.gelifesciences.com) pioneered random-primed Phi29 DNA polymerase-based isothermal amplification with the illustra TempliPhi Rolling Circle Amplification (RCA) kit in 2001, which amplifies the entire sequence of a circular template, eliminating the need for plasmid preps. Then, in 2003, the illustra GenomiPhi kit for whole genome amplification, by the process of Multiple Displacement Amplification (MDA), was launched. Since that time, the team at GE have been working to improve on these products to meet the needs of the growing Genomics market, especially Next-Generation Sequencing. Just recently, GE introduced illustra Single Cell GenomiPhi, (http://www.gelifesciences.com/GELS/campaigns/illustra-single-cell-genomiphi) specifically formulated and designed for the emerging field of Single Cell Genomics. Researchers in this area are challenged with not only the issues related to handling of individual cells and the DNA from them, but with the template concentration requirements of most downstream analysis methods; for instance, from 100 up to 2,000 nanograms of genomic DNA is required for whole genome sequencing on illumina and Ion Torrent platforms. Any less, and allele representation, full coverage, low sequence bias and alignment efficiency suffers. These demands have been considered in the development of illustra Single Cell GenomiPhi. With this improved kit, the six picograms of DNA from single human cells can be reliably amplified for successful analysis by a wide range of techniques including whole genome or exome sequencing, targeted resequencing, SNP arrays, comparative genome analysis, genomic engineering (eg CRISPR, TALEN) and more (**Figure 17**).

Figure 17: The genome of a single cell can be reliably analysed, following amplification with the GE Healthcare illustra Single Cell GenomiPhi kit

The Echo® 525 Liquid handler from **Labcyte** (www.labcyte.com) offers unparalleled performance at nanolitre volumes. Using only acoustic energy to transfer samples and reagents, Echo systems avoid any risk for cross contamination or sample carryover. This results in comparatively higher precision and accuracy throughout its transfer volume range (25nL to 1uL) for assays in 96-, 384- or 1536-well formats. Researchers conducting single cell analysis can use the Echo 525 liquid handler to miniaturise lysis and amplification following the separation of single cells into a microplate well. After amplification, the Echo 525 system can be used to prepare miniaturised

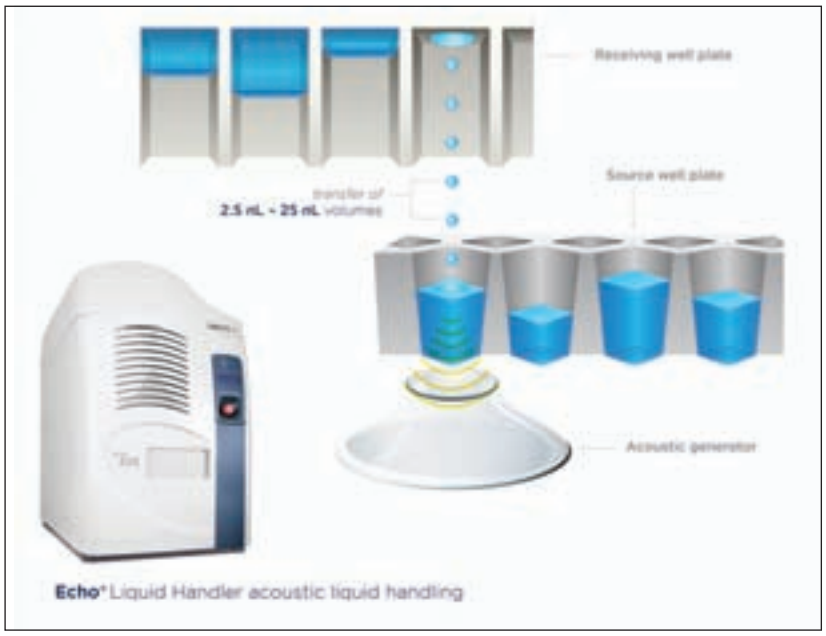


Figure 18: Diagram of acoustic liquid transfer process. Labcyte Echo 525 liquid handlers transfer 25 nanolitre droplets of samples and assay reagents from microplate wells using only acoustic energy. Droplets are rapidly transferred to build larger volumes in seconds. Transfer without the use of tips, capillaries or similar devices eliminates sample carryover to assure great accuracy and precision across its transfer volume range (25nL to 10µL)

libraries for next-generation sequencing. Our users have demonstrated four-fold reductions of the post-separation lysis and amplification volumes, and up to 100-fold reductions of library preparation reactions. The cost and time savings from miniaturisation achieved with Echo 525 liquid handlers greatly increases the efficiency and throughput of any single cell analysis programme (**Figure 18**).

Typically, conducting single-cell genomic analysis is challenging because the amount of genomic DNA or RNA present in a single cell is very limited. **QIAGEN**'s (www.qiagen.com) REPLI-g® products enable whole genome amplification (WGA) or whole transcriptome amplification (WTA) from single cells with highly uniform sequence coverage and superior fidelity. REPLI-g is based on our unique Multiple Displacement Amplification (MDA) technology – an isothermal amplification, using the enzyme Phi 29 polymerase. The enzyme delivers up to 1,000-fold higher fidelity compared to Taq DNA polymerase-based methods. Proven in the research community by many citations is its REPLI-g Single Cell Kit for WGA from single cells. For WTA, the REPLI-g WTA Single Cell Kit provides uniform amplification of transcripts – including low-abundance transcripts – from just a single cell. For correlating genomic status with the transcription pattern, QIAGEN provides the REPLI-g Cell WGA & WTA Kit, which enables uniform WGA and WTA in parallel reactions from very small samples (25-1,000 cells). Brand new to the family are the REPLI-g Single Cell DNA Library Kit and the REPLI-g Single Cell RNA Library Kit. Both kits provide a streamlined workflow for single-cell NGS library construction with high fidelity. The kits leverage QIAGEN's REPLI-g MDA technology and the ligation efficiency of its GeneRead library construction technology. The high WGA or WTA yields and high ligation efficiency of the library construction reagents remove the need for PCR-based amplification, thereby eliminating PCR-related bias and errors. The entire procedure generates high-quality sequencing libraries within a few hours (**Figure 19**).

Figure 19: Single cell genomics by QIAGEN. Get the most out of a single cell with QIAGEN's REPLI-g® products

Advanced cell sorting and enrichment methods have been responsible for a large body of productive research, but they produce as a final product heterogeneous cell sub-populations. DEPArray™ and Ampli1™ technologies from **Silicon Biosystems** (www.siliconbiosystems.com) allow researchers to continue promising lines of investigation by enabling isolation, recovery and molecular characterisation of single, targeted cells of interest, such as CTCs. The DEPArray™ system uses a dielectrophoretic array cartridge to hold suspended cells in a grid pattern for image-based selection. Single cells of interest are moved to a collection reservoir, without flow or friction, by microelectrode-mediated manipulation of dielectrophoretic fields. Isolated cells remain intact and live cells remain viable. Any cell that can be identified by fluorescent markers or probes can be isolated on the DEPArray™ system, and morphological features, such as size and circularity, can be included in cell selection criteria. The Ampli1™ WGA Kit ensures balanced amplification of genomic DNA from single cells. Using site-specific digestion and single-

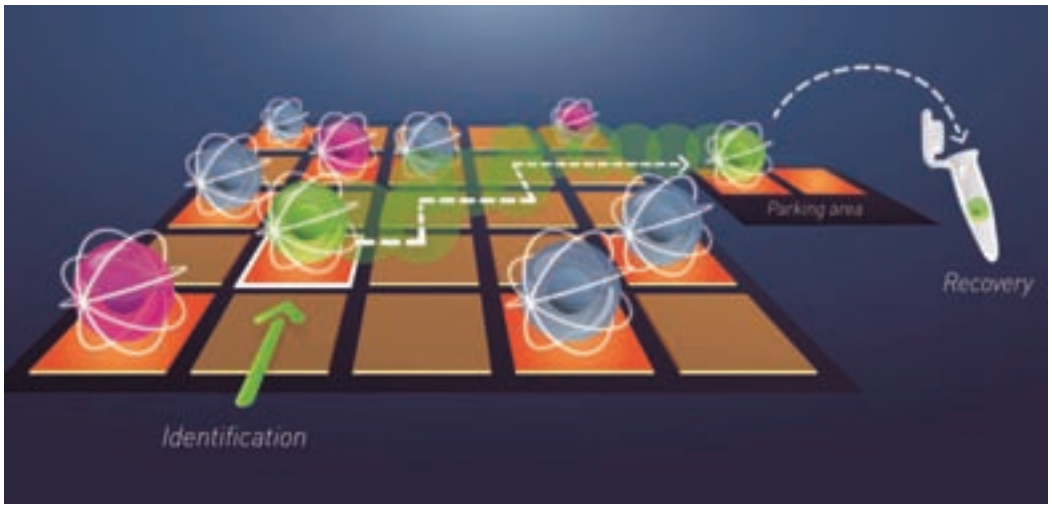


Figure 20
Representation of single cell selection with DEPArray™ technology from Silicon Biosystems. Dielectrophoretic cages hold cells in a grid pattern for image-based interrogation. The single cell selected for analysis is separated, isolated and recovered by software-controlled dielectrophoretic force manipulation

primer amplification, the Ampli1™ WGA Kit produces a library of approximately 19 million fragments representing the entire genome. DEPArray™ and Ampli1™ technologies have enabled researchers to establish reliable workflows for isolation and genetic analysis of single cells. Using these workflows, researchers have applied qPCR, CGH, CNV and gene-specific point mutation analysis by NGS to the analysis of bone marrow, breast, prostate, lung and colorectal cancers, uncovering relationships between structural chromosomal changes and specific mutations, establishing genetic heterogeneity between individual CTCs from the same patient, and comparing the gene mutation status of primary tissues against CTCs (**Figure 20**).

Sphere Fluidics (www.spherefluidics.com) is an established Life Sciences Tools company commercialising single cell analysis systems for therapeutic discovery. The company has patented novel biochip systems that automatically process millions of miniaturised single cell (or molecule) tests in picodroplets (ie small compartments of a picolitre volume). One particular strength of this approach is it traps secreted or released proteins and biomarkers which are normally very difficult or impossible to measure. Single cell analysis, cloning and isolation techniques are critical for biopharmaceutical discovery and development. Conventional techniques offer partial solutions performing some of the following functions: high-throughput screening, protein secretion assays, rapid cell sorting, single cell dispensing to microplates and monoclonality verification. Sphere Fluidics is currently selling research instruments, mainly for the academic market, which do many of the above processes. These are sold with a range of compatible biochips (eg Pico-Gen™: a picodroplet production biochip) and specialist chemicals (eg Pico-Surf™: biocompatible surfactants which stabilise the picodroplets). The company is also now developing Cyto-Mine® for industrial markets – initially biopharmaceutical discovery and development. Cyto-Mine® will be the first integrated device specifically designed to automatically perform all of these functions in a single system. Compared to conventional approaches, this technology is fast, sensitive, integrated and miniaturised – offering tremendous cost-savings. As the device requires a number of biochips, these will now be integrated into a Cyto-Cartridge™. The instrument will later be launched in other markets including: bioproduction (synthetic biology), stem cell and cell therapy engineering, single cell disease research and single cell diagnostics and prognostics (**Figure 21**).

Figure 21
Sphere Fluidics Cyto-Mine®: the single cell analysis and monoclonality assurance system

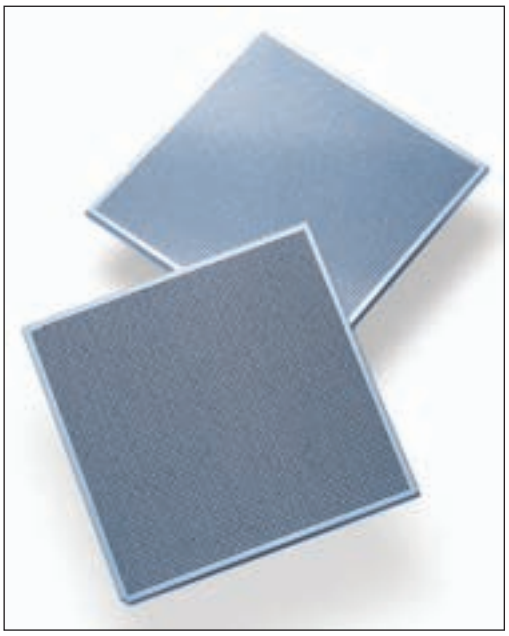


Figure 22
Wafergen Biosystems SmartChip™ technology provides unbiased isolation of up to 1,800 individual cells of various type and size

Thermo Fisher Scientific (www.thermofisher.com) offers integrated solutions for genomic research using precious or limited samples. Scientists can use the Applied Biosystems™ Arcturus™ Laser Capture Microdissection (LCM) System, with proprietary infrared (IR) laser capture capability, to collect single cells or small groups of cells. Since the IR laser operates at much lower energy than UV lasers, investigators can isolate small numbers of cells with minimal impact on DNA, RNA or protein integrity. Thermo Fisher Scientific also offers the PicoPure™ and Paradise™ nucleic acid purification kits, optimised for use with frozen and formalin-fixed, paraffin-embedded (FFPE) LCM samples, respectively. And once nucleic acids are extracted, sequence information can be obtained using Ion™ AmpliSeq™ panels. Ion AmpliSeq technology is a targeted PCR-based, next-generation sequencing technology that facilitates analysis of sequences from 10ng of material or less. Investigators have a wide range of Ion AmpliSeq panels they can choose from: from whole exome, whole transcriptome and targeted cancer panels, to custom-designed panels that satisfy their particular targeted sequencing needs. Together, these integrated products offer the tools necessary for facilitating microgenomics investigations.

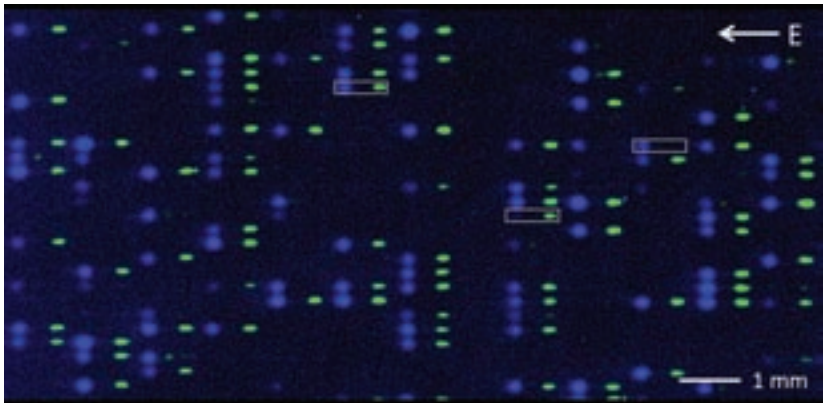


Figure 23: A portion of a Zephyrus Biosciences' zWest™ chip is shown where the analysed cell population expresses two protein markers (green and blue). Single cells are settled into an array of microwells, lysed and SDS PAGE is performed on each single cell lysate. Many of the cells express both markers as shown in the highlighted box labelled #1. However some cells express only the green marker (#2) or only the blue marker (#3). Approximately 1,000 single cells are analysed in parallel for each sample

WaferGen Biosystems (www.wafergen.com) is developing a new single-cell analysis platform that will dramatically increase the pace of biological discovery. With improvements in cell isolation, cell selection and throughput, researchers will gain enhanced control in single-cell experimental design. Leveraging SmartChip™ technology, its new platform provides unbiased isolation of up to 1,800 individual cells of various type and size. After isolation, researchers will be able to select which cells to analyse using RNA sequencing. The platform will also have the ability to process up to eight samples simultaneously, providing a new method to interrogate cell type and condition combinations. Its early access partners are examining multiple disease states in diverse cell types, including tumours and developing neurons. The research community is striving to develop a deeper understanding of the impact of cellular heterogeneity. WaferGen Biosystems' open and scalable single-cell platform will drive the next wave of biological discovery and clinical advancement (**Figure 22**).

Zephyrus Biosciences (www.zephyrusbio.com) is commercialising a single-cell western blotting system that enables simultaneous measurement of multiple proteins regardless of their location in a cell. The Zephyrus Z1™ instrument and zWest™ chips perform SDS-PAGE on thousands of single cells in parallel. The technology is compatible with standard western-validated antibodies, allowing researchers to measure protein expression at the single-cell level for both surface proteins and intracellular targets such as transcription factors and phosphorylated proteins that are difficult to measure using flow cytometry. The Zephyrus Z1™ is used to study heterogeneity in differentiated stem cell populations, tumour heterogeneity in dissociated tumour samples, and intracellular signalling pathway activation for phosphorylated

Table 1: Comparison of the single cell technologies supported by vendor offerings*

	SINGLE CELL SEPARATION				SINGLE CELL ANALYSIS						
SINGLE CELL TECHNOLOGY VENDOR:	Cell Sorting	Cell Selection	Cell Isolation	Cell Dispensing	ISH Cell Assay	Genomic Analysis	Transcriptome Analysis	Amplification	Next-Gen Sequencing	Western Blotting	Cell Culture /Drug Assays
Advanced Cell Diagnostics					✓	✓		✓			
Affymetrix					✓	✓	✓	✓			
Beckman Coulter	✓	✓		✓							
Cytena			✓	✓							
Fluidigm		✓	✓	✓		✓	✓	✓	✓		✓
GE Healthcare						✓		✓			
Labcyte								✓	✓		
Qiagen								✓	✓		
Silicon Biosystems	✓	✓	✓					✓			
Sphere Fluidics			✓	✓							✓
Thermo Fisher Scientific		✓	✓			✓	✓	✓	✓		
Wafergen Biosystems		✓	✓						✓		
Zephyrus Biosciences										✓	

* This table is based only on the information highlighted in the vendor supplied snapshots printed above.

Table 2: Some feedback from survey respondents on unmet needs in single cell technologies

- Better method of cell selection and maintenance of clonal population.
- Better throughput for separation and analysis techniques.
- Better, non-toxic long term cell-tracking markers, better analysis platforms.
- Cost-effective, integrated isolation and dispensing of single cells.
- Easy data management tools for single cell experiments.
- Integration of single cell analysis systems with molecular characterisation by mass spectrometry.
- Isolation of cells from complex small tissues (eg blood vessels) without using culture conditions.
- Isolation of single circulating tumour cell that can adequately separate cancer cells from white bloods cells and return viable cells, not coated with magnetic beads.
- Less complex instruments, possibility to analyse RNA and protein expression profile within just a single cell in high throughput mode.
- Multifunctional way to perform experiment only on the cell you want, without affecting the rest of the population.
- On a practical level what is needed is to get the costs down, so more scientists would be able to attempt/incorporate single cell technologies into their research.
- One major concern is the lack of quality control standards for the different single cell genome and transcriptome amplification technologies, in particular when it comes to real clinical samples such as circulating tumour cells.
- Poor reliability. Massive amplification required to get anything, and it is of much lower complexity than what the cell originally had.
- Possibility to streamline capture, manipulation and analysis without dilution in a large volume of medium (eg contained in an Eppendorf tubes or MTP well).
- Proteomic analysis at single cell level.
- Single cell epigenetics.
- Single cell nuclear conformation studies.
- We are at a watershed moment in the sense that many technologies need to be combined to achieve a reliable platform. Advances in nanotechnology, laser technology, mass spectrometry, computing and biological research are all conspiring towards a viable cost-effective platform. There needs to be greater understanding of how each process manipulation might bias readout and the true significance of the data observed at present.

targets that cannot be accessed with flow cytometry. The Z1™ system can also be used to validate protein expression of RNA targets discovered in single-cell RNA-seq or gene expression studies. The company is a spinout from UC Berkeley and has an exclusive licence to technology that was recently published[3]. In addition to addressing applications in immunology, stem cell and cancer research, Zephyrus recently developed a Z1™ assay capable of measuring surface or intracellular protein target expression in single FoxP3-positive regulatory T cells (Tregs); a measurement that enables characterisation of Tregs and may facilitate the development of novel immune checkpoint inhibitor therapies (**Figure 23**).

Discussion

Table 1 summarises the single cell technologies supported by vendor offerings reported in the above updates. Single cell vendor offerings have been classified broadly into those that enable single cell separation and those that enable single cell analysis. From the cell separation perspective of interest are those new approaches that isolate single living cells from a fluid sample (cytena, Fluidigm), those that manipulate cells for image-based selection (Silicon Biosystems), those that use IR laser capture (Thermo Fisher Scientific), and those that compartmentalise individual cells into piciloter droplets (Sphere Fluidics). The most prevalent analysis technologies reported are those that facilitate amplification for genomic analysis, and also transcriptome analysis and sequencing (Advanced Cell Diagnostics, Affymetrix, Fluidigm, GE Healthcare, Labcyte, Qiagen, Silicon Biosystems, Thermo Fisher Scientific and Wafergen Biosystems). It is noteworthy that a

number of vendors are developing integrated technology platforms that aim to bridge the gap between single cell separation and analysis (eg Fluidigm, Silicon Biosystems, Sphere Fluidics and Wafergen Biosystems). These miniaturised all-in-one systems hold the promise of greatly simplifying the processing and analysis, but also bringing automation to the investigation of single cell biology and helping build new single-cell applications.

Table 2 lists some of the feedback we obtained from survey respondents on unmet needs that they feel limit single cell technologies and their exploitation today. These comments give weight to the viewpoint that single cell technologies are currently immature and for the most part the utility of available tools does not adequately meet many research needs on single cells. However, based on the interest we have found around this topic, as exemplified by the big response we got to the survey, it is reasonable to conclude that expectations are very high in this rapidly evolving field, particularly around the key single cell analysis application of gene expression profiling. New tools addressing many of the hurdles associated with working with single cells are becoming available at a rapid pace and the future study of single cells is likely to impact our understanding and treatment of human disease. DDW

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