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# Single cell sorting and cloning

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**Abstract**

Cell sorters now allow the selection of cells and other bodies according to a range of quite diverse criteria. The additional refinement that allows the sorting of individual cells based on these criteria has seen application in many fields of research. Single cells may be sorted for microscopy, for culture and for genetic analysis by way of single cell PCR (polymerase chain reaction). In practical terms, in the setting up of an instrument for single cell sorting, there are additional requirements to ensure that each detected event is indeed a single cell or body, that this cell can be reliably sorted via saline droplet, separate from its fellow travelers, that the aiming of the droplet deflection is sufficiently precise to find the target vessel and that the cell will be undamaged on arrival. Among the diverse reported applications of the technique, two fields which have benefited greatly are lymphocyte development and haemopoiesis. In the former case, the analysis of gene rearrangements in lymphocytes, both in the pre- and post-antigenic phases of development, has been enabled by the combined technologies of single cell sorting and PCR. It is argued that such experiments could not have been done without that partnership. In a similar way, the single cell sorting technique has been found to be the perfect way to demonstrate precursor/progeny relationships between haemopoietic cells and, further, to demonstrate rigorously the effects of particular cytokines on the haemopoietic system. 

*Keywords:* Single cell sorting; Cloning; Flow cytometry; Fluorescence-activated cell sorting; Polymerase chain reaction; Hematopoiesis

## 1. Introduction

Single cell sorting as a refinement of flow cytometric or fluorescence-activated cell sorting has been used for the selection and isolation of individual cells for microscopy, for culture and, more recently, for genetic analysis by single cell PCR. While the full analytical power of multi-parameter flow cytometry can be applied to the selection of the cells, both by the fluorescent tagging of cell surface molecules and by the insertion of fluorescent genetic markers, the operation can also be as simple as randomly selecting representatives of a cell line for re-cloning. This random selection of individuals may be thought of as an alternative to limiting dilution techniques.

Most of the instrumentational groundwork for single cell sorting was established in the late 1970s as an incremental development of cell sorter electronics and sort logic. In the intervening years, these enhancements have been incorporated into commercial cell sorters and the technique should now be regarded as routine. If it is not so regarded, the reason may be the difficulty of verifying the success of the cell sorting component of the experiment. When sorting cells in bulk, it is usually possible to extract a small sample of the product for re-analysis, whereas, for example, a single cell sorted into a small culture well may fail to proliferate for a

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number of reasons even if it does find its way to the targeted culture medium.

In practical terms, although a slightly higher degree of precision may be necessary, but for a few additional requirements, the configuring of a cell sorter to sort individual cells is similar to that for all flow cytometric sorting. The selection of sort criteria or 'gating' may be affected, as may the setting of sort logic, i.e. number of deflected droplets and the handling of coincidences. Also, the vessel chosen for receiving the cells (e.g., a well of a micro titre plate) will typically present a smaller target, thus necessitating more accurate aiming of the sorted streams.

## 2. Sorting criteria and the selection of cells

Most single cell sorting applications select cells on the basis of some specific fluorescent marker. This may indicate surface phenotype, as with monoclonal antibody staining, some genetic marker or gene product, marked by a variant of the FACS-Gal technique or by a fluorescent protein, or even a cell-physiological state. However, the specific fluorochrome or fluorochromes are only a part of the full set of criteria defining the cell population to be sorted; cell morphology and viability will also typically be included. When sorting single cells, it may be more important to ensure that each one is viable or at least intact. Indeed, specific fluorescence may not be a criterion at all, for example in the case of the re-cloning of a cultured cell line by the selection of random individuals. Whatever criteria are chosen, more stringent gates may be called for than would be used for bulk sorting since accurate specification of the cells is usually more important than maximising cell recoveries. Also, in order to ensure that each object sorted is a single individual, additional care must be taken to detect multi-cell clumps or attached debris.

Two or more cells in a clump or even flowing so close together as to be seen by the sorter as a single entity must be identified. The best tool to use is pulse processing which is available in most commercial instruments. By this method, the shape of the pulse obtained at one or more of the optical detectors upon transit of an object can help identify that object as a single cell or clump. Because the shearing effect of the sheath flow within a sorter nozzle tends to orient doublets and larger clumps lengthways along the flow (Gray et al., 1979; Lucas and Pinkel, 1986), such an object will be marked by a greater pulse width. The pulse width provides information that cannot be gained from any combination of the standard parameters like forward light scatter or side scatter that derive from the pulse height.

An alternative to setting more stringent gates is the 'indexed sorting' scheme as proposed by Dean et al. (1985). In this method, the individual cells are selected from random positions within a wider extent of gate boundaries, but the actual data values for each cell, indexed to the well into which it was sorted, are stored in a separate file. This allows correlation of the measured flow cytometric parameters with the assay results from each well after they are obtained. An indexed sorting option is offered commercially by Becton Dickinson with their FACS Vantage CloneCyt system.

## 3. Precision sorting in the face of random cell transit times

Cells do not traverse the saline stream in phase with the droplet generation oscillations of the sorter. To cope with those that arrive near the edge of a droplet period, one may sort two droplets in the hope that the cell will be pulled into one droplet or the other. In bulk sorting, this will maximise recovery. Furthermore, if another sortable cell is flowing sufficiently closely, the droplet package may be further extended to include it. On the other hand, since foreign cells (those not inside the current sort window) must be excluded from the sort, if such a cell is too close in the flowing stream to a cell that is in the sort window, the sort request may be aborted in order to maintain purity. In single cell sorting, every close cell, even one of the same type and falling inside the sort window, must be discriminated against in order that a single sort event does not result in the sorting of multiple cells. The argument against using two or more droplet sort packages is that cells that travel in the sheath stream at or close to the droplet boundaries may snap the stream prematurely so the charging pulse is not fully applied or not applied at all to the targeted droplets. When

sorting individual cells, it is usually more important to ensure that each attempted sort event will be successful rather than to maximise recovery efficiency. Hence a 'phase gating' technique may be employed. By this technique (Merrill et al., 1979), only cells in phase with the central portion (normally the 2nd and 3rd quarters) of a droplet period are eligible for sorting. Obviously, 50% of otherwise eligible cells will be excluded thereby, so if such cells are rare it may be necessary to trade off the assured success of each sort event against potentially more individual cells sorted. The use of phase gating requires extra precision by the operator in setting the droplet breakoff timing for the sorter; errors of more than 1/4 droplet period will result in lost cells, as compared to two-droplet sorting, where an error of up to 1/2 droplet period is allowable. To make fullest use of the 1/10th or 1/16th droplet breakoff setting precision in modern commercial cell sorters, performance of a droplet delay matrix, in accordance with the manufacturer's instructions, is recommended.

## 4. Destination of cells

### *4.1. Aiming accuracy*

Very few applications test the limits of accuracy with which a standard cell sorter can deposit a single cell. The accuracy of all commercial cell sorters is certainly sufficient for sorting into standard 96-well plates and even into Terasaki plates where the culture medium presents a 3–4 mm target. However, in single cell sorting, aerodynamic effects are important. In bulk cell sorting at high sort rates, the deflected droplets follow each other into the target vessel in an apparently continuous stream, thus stabilising the trajectories. When sorting individual saline droplets where there is no such slipstreaming effect, the aerodynamic drag on each droplet causes a deceleration which will affect its trajectory (Tyrer and Kunkel-Berkley, 1984). Using equations from that report, it is possible to calculate this deceleration at more than 20 *g* which would, in current unmodified cell sorters, leave the droplets floating downwards at a low terminal velocity before reaching the target. The uncertainty in trajectory is reduced if the sorted cells travel downwards at an angle closer to vertical. A minor modification to the cell sorter, re-positioning the waste stream catcher off centre, can allow a vertical trajectory for the sorted cells. It is also more important to avoid air currents in the flow chamber when sorting slower moving individual droplets. Combination of vertical trajectory and positioning the target well or slide closer to the sorter nozzle can result in very accurate positioning (Stovel and Sweet, 1979).

### *4.2. Verification*

Provided the optical shape of the target vessel allows it, microscopy may provide direct verification of successful sorting. Flat bottomed wells of a 96-well plate are optically suitable but present a large field in which to find the deposited cell. Further, a suitable time delay between sorting and microscopy must be allowed for the cell to settle from the top surface of the culture medium to the well bottom. Although their optical properties may not be as good, round bottomed wells work to locate the cell and any subsequent progeny (Boezeman et al., 1997). Terasaki wells are optically suitable, the bottoms present a sufficiently small field and the settling time is shorter given the shallower medium. Cells may be located by inverted microscope 1 h post-sorting (see, for example, Baird and Simmons, 1997).

### *4.3. Microscope slide*

As a further development of the originally reported technique of single bead sorting onto glass slides, Alberti et al. (1984) described means of preserving cells, sorted while viable, for microscopy. A 1:100 dilution of the sheath medium prevented lysis as the medium of the deposited droplet dried out and a thin layer of newborn calf serum aided adherence to the slide. Kanz et al. (1986) demonstrated the use of poly-L-lysine spots on an otherwise water-repellent slide to constrain the sorted drop and obviate the need for dilution of the sheath medium.

### *4.4. Culture*

Among the first practical applications for single

cell sorting was the selection of cell hybrids. Parks et al. (1979) selected antibody-producing hybridomas based on their surface expression. The low level expression was amplified by marking the cells with antigen-coupled fluorescent microspheres. While there may be no universal relationship between the level of surface antibody expression and antibody production, particular hybridomas may show a positive correlation (Marder et al., 1990).

The question of the possible adverse effects of flow cytometry or cell sorting in general on cell viability or function has been addressed elsewhere (Ashcroft and Lopez, 2000). Single cell sorting in particular should not be more damaging. In fact, given the aerodynamic deceleration of single sorted droplets mentioned above, their softer landing in the culture medium might tend to reduce that potential cause of damage. On the other hand, a badly directed single droplet that does not find the medium will dry out almost immediately and the contained cell will lyse. This flags the susceptibility of the process to operator error. The proposition that sorted single cells may be happier in culture in one particular well type than another has not been systematically tested.

### *4.5. PCR amplification*

Zhang et al. (1992) sorted single human sperm for PCR whole genome amplification. McHeyzer-Williams et al. (1991), Brezinschek et al. (1995) and Kantor et al. (1995) reported early success in sorting for single cell PCR in studies of B-cell antibody repertoire. Bertram et al. (1995) point out that the PCR itself provides an internal verification of the lower limit of successful sorting, finding a success rate of at least 96.5%. In a later, more detailed description of the procedure, Kantor et al. (1997) suggest a (reversible) modification to the cell sorter to allow it to sort vertically downwards in order to target the very small volumes (4 μl) of lysis buffer at the bottom of Eppendorf tubes. They further suggest a two-stage sorting; first in bulk and then for single cells, to enhance the selection purity. The lower cell concentration and consequently slower sort rate for the single cell sort also reduces the incidence of doublets.

## 5. Applications of single cell sorting.

A plethora of diverse applications have benefited from the ability to select individual cells in an automated way, for example sorting of cybrids (Schaap et al., 1982), sperm (Zhang et al., 1992), slime mold (Browne and Williams, 1993), cardiac myocytes (Diez and Simm, 1998), bacteria (Caron et al., 1998) and bacteria as carriers of DNA sequences (Perret et al., 1998). However, it is instructive to consider in more depth studies of cellular development and differentiation which have experienced perhaps the greatest impact from the advent of the technique.

### *5.1. Lymphocyte development*

Analysis of lymphocyte development relies heavily on the use of clones. This is the only way in which a population can be derived such that all members contain the same set of gene rearrangements at the heavy and light chain loci for B cells or the alpha and beta loci for the majority of T cells. This maxim applies to lymphocyte development prior to antigenic challenge when gene rearrangement is occurring at the T-cell and B-cell receptor loci, and post-antigen when isotype switching and V gene somatic hypermutation occur in antigen specific B cells. In the past, clonal analysis usually meant analysis of cell lines, either tumours that arose from early in development such as Abelson pre-B cell lines or hybridomas that were derived at various times of the immune response. There are clearly problems in relying on cell lines. Foremost among these is that only a very small fraction of the population of interest can be induced to transform or fuse, thereby raising concerns about how representative the cell actually is. Second is the loss of knowledge about the origin of the cell such as which developmental compartment it arose from. Third is the possibility that the phenotype of the cell altered during the transformation and therefore no exact in vivo developmental counterpart will be found.

The ideal approach to analyse lymphocyte development, both in the pre- and post-antigenic phases, is one which combines clonal analysis with molecular biology. The polymerase chain reaction

(PCR) in combination with single cell sorting has made this possible. In this segment of the review, we will consider three examples whereby single cell sorting has facilitated analysis of lymphocyte development. Indeed, in each of these cases, it could be argued that the insights arising from these experiments were entirely dependent on the use of the technology; there was no other way of doing the experiments.

Analysis of lymphocyte development is in many respects the analysis of immunoglobulin (Ig) or T-cell receptor (TCR) gene rearrangement. Early studies of cell lines showed that gene rearrangement occurred in an ordered manner and that progression along the increasingly refined developmental pathways was at many stages entirely dependent on the rearrangement status of the Ig heavy and light (or TCR $\alpha$ and $\beta$) loci. Bulk analysis of lymphocyte populations, no matter how refined, could never fully answer several of the basic questions surrounding lymphocyte development. For example, most models of Ig gene rearrangement predict that a single cell would rearrange both IgH alleles to be DJ and then proceed to join V segments. First one allele would undergo V to DJ rearrangement and then the second only if the first was non-productive. Thus any individual surface Ig positive B cell should contain only a single functional heavy chain and a single functional light chain. The same obviously applies to the alpha and beta loci of T cells. But how can this be examined at the population level where the cellular association of alleles is lost? Clearly it cannot and requires examination of single cells. For this purpose, single cell sorting has been ideal. It allows partitioning of complex lymphocyte populations using several parameters and then examination of the rearrangement status of both alleles within any given cell from a particular subset. An elegant example of this approach is the work of Ehlich et al. (1994) in correlating the developmental progression of B-cell precursors with gene rearrangements at the heavy chain locus. Examination of single cells from defined populations allowed the determination of sub-populations enriched for DJ rearrangements at both alleles, those containing productive IgH rearrangements and identified a novel population containing predominantly non-productive VDJ rearrangements at both alleles. This work involved the amplification of IgH loci from genomic DNA purified from single sorted cells using a collection of primers able to recognise all gene elements. Similar analyses have been conducted by Melchers and colleagues using essentially identical techniques but also with some surprising results (see, for example, ten Boekel et al., 1998).

Clonal analysis of primary cells provides an enormous advantage in studying the B lymphocyte response to antigen. In this approach, the immunizing antigen is rendered fluorescent by conjugation to a fluorescent protein such as allophycocyanin (APC) (McHeyzer-Williams et al., 1991). Four colour fluorescence analysis then can be used to partition lymphocytes from the immunized animals such that precise sub-populations of antigen reactive B cells can be examined. Once identified, these cells can be sorted singly, RNA extracted and cDNA synthesised using a protocol based on that of Kantor (Kantor et al., 1997). By establishing cDNA libraries from single cells, potential distortions due to some B cells, such as plasma cells, containing considerably more RNA than others is removed. From such single B-cell cDNA libraries, immunoglobulin gene rearrangements can be amplified by PCR and the genetic basis of the immune response characterized. In the particular example considered here, the antigen is the hapten 4(hydroxy-3-nitrophenyl)acetyl. This antigen has the property that, in particular strains of mouse, the immune response is dominated by a single pair of Ig heavy and light chain variable regions. Thus genetic analysis requires the amplification of only these two V gene segments. DNA sequencing of the PCR amplified V gene segments without a DNA cloning step in between allows somatic mutations to be identified unambiguously. Clearly degenerate V gene primers could be used in the PCR to broaden the analysis to other immune responses which are not so genetically restricted. This would be true particularly for immune responses to protein antigens. This approach has been used to analyse successfully exceedingly small populations of B cells responding to antigen. For example, it has been possible to identify and isolate by flow cytometry antigen specific B cells at a frequency of 1 in $10^5$ over a period of 250 days after a

single immunization (Smith et al., 1994). Over the entire range of cell types studied, 30–70% of sorted single antigen specific B cells have given specific PCR products (for example, Smith et al., 1997). This range of success most probably reflects the type of B cell being analysed and its specific mRNA expression level. Thus success is more likely from antibody forming cells and less so from memory B cells. It is not a result of sorting inaccuracy and failing to get the cell into the tube (Kurokawa et al., 1999).

Finally, a recent example highlights again the power of combining single cell isolation from defined populations with powerful molecular techniques to produce a most unexpected result. By isolating RNA from single activated CD4+ T cells, Burakoff and colleagues (Hollander et al., 1998) have been able to show that the IL-2 genes in such cells are allelically excluded. That is, one allele is silent and the other expressed. In this case, single cell sorting was required to isolate cells in such a way that mRNA could be isolated and cDNA synthesised. By using a genetic polymorphism in the IL-2 gene and this approach, which is not unlike the one described above for antigen specific B cells, the expressed allele could be amplified specifically by PCR and the polymorphism between alleles detected.

### *5.2. Haematopoiesis*

With the development of the clone sorting system, it became possible to unequivocally identify the phenotype of the single cell that self renewed or gave rise to differentiated progeny. Visser et al. (1984) sorted single murine stem cells into Terasaki wells and assayed for colony growth. They saw the technique as a replacement for limit dilution and sorted arrays of one, two, four, etc. cells per well. Since the demonstration by Kannourakis et al. (1988) that primitive haemopoietic cells selected by single cell sorting showed the same pattern of differentiation in culture as cells selected by other means, there have been a large number of studies of cultured human stem cells. Discovery of the significance of CD34 in the human system has also added impetus (see Ema et al., 1990; Andrews et al., 1990, and subsequent reports from these groups). In this regard, Sca-1 is the CD34 counterpart in the murine system. Smith et al. (1991) sorted single murine stem cells, selected by lineage markers, Thy-1 and Sca-1, into microtitre wells, but then injected the well contents into mice with a view to subsequently detecting B-cell, T-cell and myeloid progeny.

That this is the perfect experimental system to demonstrate precursor/progeny relationships is further evidenced in the case of the common stromal/haemopoietic stem cell which was finally determined by Waller et al. (1995). The difficulty of finding unequivocal proof for this relationship is evidenced by the earlier work of Huang and Terstappen (1992, 1994).

There is a further benefit of placing a single cell into the culture system. Without other possibly factor-producing cells in the system it then may be rigourously demonstrated that particular cytokines can act to induce proliferation (Xiao et al., 1992; Bernstein et al., 1991, and, in the murine system, Williams et al., 1992).

## 6. Commercial instrumentation

Becton Dickinson Immunocytometry Systems (San Jose, CA, USA) currently offer their ‘CloneCyt’ option for FACS Vantage cell sorters, which replaces the earlier ‘ACDU’ (automated cell deposition unit). Like its forerunner, the CloneCyt allows sorting of one or more cells per well into a wide range of standard microtitre plate formats including the common 96-well plate and 24-well Terasaki plates. User-defined, non-standard plates or microscope slide arrays are also possible. Sorting now in a serpentine pattern of well selection, rather than in the former ‘line feed, carriage return’ pattern, the sorting time, when limited by plate movement and not the appearance of sortable cells, has been decreased to about 2 min for a 96-well plate. The additional ‘IndexSort’ option allows acquisition of data on each cell correlated with the well number into which it is deposited.

Beckman Coulter (Fullerton, CA, USA) offer their ‘Autoclone’ device with EPICS cell sorters. This device sorts into a range of standard microculture plates including 96-well plates and 24-well Terasaki plates. The pattern of deposition is uniquely serpentine for half the wells at a time because of space constraints in the cell sorter’s collection area When

half the wells have been sorted, the plate is rotated for the remaining half. The current Autoclone sorts by charging all unwanted droplets which are deflected to waste while the droplets containing the wanted cells are uncharged and allowed to travel downwards to the target wells.

Cytomation (Fort Collins, CO, USA) have developed by far the fastest single cell deposition unit for their MoFlo cell sorters. The 'CyCLONE' can sort 96 wells in less than 50 s. The CyCLONE accepts up to 1536-well plates as well as the more common varieties and also allows user-defined plates and microscope slide arrays.

In all of these currently offered devices, different sort criteria and numbers of cells sorted may be chosen for various arrays of wells on each plate. The fact that the instrumentation has now gone through several cycles of development over many years points to the maturity of the technique and should give users confidence in its reliability.

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