The use of FACS for the selection of cell lines with superior productivity characteristics

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Synopsis

- Why use FACS in bioprocess development
- Issues regarding use of FACS
- **Affinity Matrix Surface Capture** assay for superior producers
- Single cell sorting for clonal cell line development
- Functional and phenotypic characteristics that cells may be selected on
- Safeguards employed when using FACS for therapeutic protein production
Cell sorting by FACS

Flow cell: Hydrodynamic focusing and vibration by a transducer produces a stream that breaks into droplets

Interrogation by laser beam, signal processing and sort decision. If droplet contains cell fluorescing green, droplet will be charged and the cell sorted

Electronic delay until droplet reaches break-off point. A charge is then applied if appropriate

Charged droplet containing cell of interest is deflected by electrostatic field into suitable receptacle
Key technologies

- **Instrumentation:** More robust than ever. Experience has allowed design to be improved.
- **Computers:** Benefits from the massive increase in processing power seen over the past 20 years.
- **Lasers:** Large water cooled lasers have been replaced with small, compact solid-state lasers. Optical fibres can deliver the light to the contact points.
- **Probes:** There are about 20 different fluorochromes that can be used. Possible to conjugate to monoclonal antibodies.
- **Multivariate:** Up to 14 different parameters can be measured simultaneously. 4-5 more realistic.
History and evolution

- First flow cytometer appeared in 1947 (Gucker)
- 1950s sheath flow principle demonstrated and first Coulter Counters appeared
- 1970 first commercial flow cytometers
- **Fluorescent Activated Cell Sorting** ‘FACS’ was coined in 1969 (Hertzenberg) and first commercial FACS machine appeared in 1974 manufactured by Becton-Dickinson
- 1977 Coulter EPICS series appeared. Argon laser, multiparameter, data storage, printer
Why use FACS in bioprocess development?

- Established, well supported technology
- Mammalian and microbial cells can be sorted
- Deals with cells on an individual basis, allowing potentially interesting sub-populations to emerge
- Versatile. Can be used for analysis of bioreactor contents or can physically sort cells of interest
- Can select cells on well determined physiological or functional criteria
- Can be sanitized, used aseptically
- Sorts as many single cells in a day as conventional cloning
FACS design issues

- Ease of set-up and use
- Stability and reliability
- Flexibility and adaptability
- Efficient use of resource
- Sanitisation
- Ability to sort aseptically
- Ability to perform single cell sorting
- Assay reagents must be animal cell component free
- Beneficial to our operations
The cuvette based sorting flow cell

In this case sorting single cells into a 96 well plate

A wide variety of different receptacles are possible
The sort drawer protects the sort samples, opened during sorts and closes if a blockage is detected protecting the integrity of the sorted cells.
Interchangeable sample lines

Sample lines can be easily changed
Sanitisation using caustic solutions possible
Disadvantages

- Can only provide a snapshot of a cell at a particular moment
- Correlation between measured variables and what it means at 20,000 litre scale
- Open system, contaminations can still occur
- Cell death during sorting can lead to poor recovery
- Not much scope for automation during sample preparation and analysis
- Careful controls needed
- Day-to-day variability
- Complexity of system means inevitable downtime if repair/maintenance required
- Expensive to buy and maintain
Rapid enrichment of high producing sub-populations

- Number of flow cytometric-based assays developed that can identify and isolate high producing recombinant cell lines
- Affinity-matrix surface capture (AMSC) method for identifying high producing cells in transfectant pools [based on method of Holmes and Al-Rubeai (1999)]
- Fluorescence signal correlated to amount of secreted antibody
- Sort using FACS to enrich for high producers within population
AMSC Assay: construction, capture and detection

- Capture molecule (biotinylated Protein A)
- Neutravidin bridge
- Cell
- Intracellular antibody
- Biotinylated-cell surface
- Captured, secreted antibody
- Free, fluorochrome-labelled detection antibody

EP1415158 A, Lonza
AMSC Assay: construction, capture and detection

- **EP1415158 A, Lonza**

Diagram:
- **Cell**
- **Neutravidin Bridge**
- **Biotinylated Protein A**
- **Secreted Antibody**
- **Biotinylated-Cell Surface**
- **Fluorochrome-labelled Detection Antibody**
Analysis of AMSC-labelled GS-CHO cells producing a recombinant antibody

Fluorescent signals for antibody-producing GS-CHO cells were substantially higher than for non-producing cells.
Does high fluorescence correlate with high productivity?

- Cell populations sorted from high and low signal regions
- Sorted populations expanded and specific production rates (SPRs) determined
- The mean SPRs from the two regions are different
  - \( p = 0.05 \)
- High fluorescence correlates with high productivity
- Sorting enriched the population for high producers
Conventional vs. AMSC-cloning: static culture

- Distributions of productivities from the two methods are similar
- The mean values for antibody concentrations from the two methods are equal
  - $p = 0.17$
Conventional vs. AMSC-cloning: fed-batch, suspension culture
Impact on time-lines of using AMSC-cloning approach

- Both methods generated a panel of cell lines having similar productivities
- Enrichment of transfectant pool before cloning has shortened screening step
  - Time saving = 9 weeks
Conclusion (1)

- Cell line construction and selection methods were run in parallel using a conventional protocol and a flow cytometric protocol
- Cytometric method enriched cell populations for high producers
- For the key selection stage, evaluation in fed-batch suspension culture, mean antibody concentration and mean specific production rate were similar
- The productivities of the panels of candidate cell lines were generally similar
- AMSC-cloning method reduces timeline by 9 weeks
- Potential for further improvement if qualify FACS for single cell sorting
Single cell sorting for cloning

- One approach to qualifying FACS for single cell cloning
- CHO cells stained with fluorescein based dye
- Machine set-up for single cell sorting. Strict criteria for sorting droplets containing only 1 cell
- Test sort performed with fluorescence beads. 1 bead **must** be in each well of a 96 well plate for set-up to be verified
- Fluorescein stained cells then sorted into 96 well plate. Examined under a fluorescent microscope
- Repeated about 5 times in a session
- At end of session. Test sort performed using beads. 1 bead in each well of 96 well plate for machine performance to be validated
Single cell sorting: Results summary

- 13 sorting sessions, 67 plates sorted and assessed, 3 operators
- Empty well = 1626
- 1 cell/well = 4330
- 2 cells/well = 23
- > 2 cells/well = 3
- Total wells = 5982

*Frequency of obtaining 2 or more cells/well was 0.7%*
*Frequency of single cell or empty well was 99.3%*
Conclusion (2)

- Clearly demonstrated the efficacy of FACS for single cell sorts
- We believe the clonality achieved is comparable to limiting dilution based methods
- Numerous advantages e.g. speed and selection criteria
Physiological parameters for sorting cells

- Use measurements of innate cell characteristics
- Sort and clone cells on physiological basis of what makes a better ‘protein factory’
- Depends on availability of specific stains
- Stains need to be vital/non-toxic if further biology is to be performed on the sorted cells
- Cells can be sorted on structural or functional characteristics
# Cell physiological and functional parameters

<table>
<thead>
<tr>
<th>PARAMETER</th>
<th>HOW MEASURED</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Size</td>
<td>Forward angle light scatter</td>
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<tr>
<td>Cell Shape</td>
<td>Pulse analysis</td>
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<tr>
<td>Cytoplasmic granularity</td>
<td>Side scatter</td>
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<tr>
<td><strong>DNA Content</strong></td>
<td>PI, DAPI</td>
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<tr>
<td>DNA Base Ratio</td>
<td>A-T/G-C Preferential dyes</td>
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<tr>
<td>Nucleic acid Sequences</td>
<td>Labelled Oligos</td>
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<tr>
<td>Chromatin Structure</td>
<td>Fluorescence following denaturation</td>
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<tr>
<td>RNA content</td>
<td>Acridine orange</td>
</tr>
<tr>
<td>Total Protein</td>
<td>Acid dyes</td>
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<tr>
<td>Basic proteins</td>
<td>Acid dyes/high pH</td>
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<tr>
<td>Surface/Intracellular Antigens</td>
<td>Fluorescent Antibodies</td>
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<tr>
<td>Surface sugars</td>
<td>Fluorescent Leptins</td>
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<tr>
<td>Lipids</td>
<td>Nile Red</td>
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<td>Receptors</td>
<td>Labelled ligands</td>
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<td>Membrane Integrity</td>
<td>PI</td>
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<td>Endocytosis</td>
<td>Labelled Target molecules</td>
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<tr>
<td>Apoptosis</td>
<td>Annexin V, 7AAD</td>
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<tr>
<td>Cytoskeletal Organisation</td>
<td>Phalloidin</td>
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<td>Cytoplasmic/mitochondrial membrane potential</td>
<td>Cyanine dyes</td>
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<td>Calcium Flux</td>
<td>Indo-1</td>
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<tr>
<td>Intracellular pH</td>
<td>SNARF</td>
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<tr>
<td>Gene Expression</td>
<td>GFP, RFP, YFP</td>
</tr>
<tr>
<td>Organelles (ER, Mitochondria, Golgi)</td>
<td>Fluorescent Probes</td>
</tr>
</tbody>
</table>
Bulk sorting populations on physiological parameter

- Left hand panel shows cells stained prior to sorting
- Right hand panels show stained cells following sort
Conclusion (3)

- There are many physiological and functional characteristics that we can use as a basis to sort on.
- New information from comparative genomic and proteomic studies are giving us more handles as to what makes a better ‘protein factory’.
- FACS can sort and clone cells according to a range of parameters not possible with other methods.
Safeguards for use of FACS for therapeutic protein production

- Dedicated room for cell sorting, cleaned before start of the project
- One project at a time to eliminate chance of cross contamination
- Sample lines on machine changed prior to project
- Machine cleaned with bleach, rinsed with detergent and sterile water. Sanitised by flushing through with ethanol
- Where possible components sterilised by autoclaving
- Fluids used in the system sterilised by autoclaving or filtering through 0.22 μm
- Test sorts performed to verify sanitisation of the system
- Tubes and exterior surfaces swabbed with ethanol
- Personal protective equipment
In summary

- FACS is very amenable technology for bioprocess development
- Improvements in instrumentation have addressed issues affecting use of FACS for developing cell lines for therapeutic protein manufacture
- AMSC assay for the selection of high producing cell lines
- Verified single cell sorting. Controls and safeguards necessary for this
- No comparable methods give the range of physiological parameters to select cells on
- *By integrating these aspects of FACS techniques, it is possible to directly clone cells that possess favourable attributes*
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