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Mitochondrial membrane potential identifies cells with high recombinant protein productivity

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ABSTRACT

Development of cell lines for biotherapeutic protein production requires screening large numbers of clones to identify and isolate high producing ones. As such, stable cell line generation is a time- and resource-intensive process. There is an increasing need to enhance the selection efficiency of high-yielding clonal cell lines for cell line development projects by using high throughput screening of live cells for markers predictive of productivity. Single cell deposition by fluorescence activated cell sorting (FACS) is a commonly performed method for cloning to generate cell lines derived from a single recombinant cell. We have developed a novel strategy to identify higher productivity cells at the FACS step by leveraging a simple viable cell staining method that detects mitochondrial membrane potential (Ψm), a key indicator of cellular metabolic activity. We chose a dual-emission dye (Mito-ID, Enzo Life Sciences) that fluoresces green and orange in living cells with the intensity of the orange fluorescence being dependent on the cells Ψm status. Using available clonal cell lines with known productivity, or stable transfectant pools, we evaluated Ψm of cell populations with Mito-ID dye. We determined that the intensity of the Ψm fluorescent signal correlates with the known fed-batch titers of the producer clones, and that cell sorting based on an optimal Ψm staining intensity selectively enriches for higher producing clones from nonclonal transfectant pools. These clones are phenotypically stable for recombinant protein production. Furthermore, the strategy has been successfully applied to identifying higher producing cell lines for a range of antibody molecular formats. Using this method, we can combine an enriching step with the cloning step for high producers, thereby saving time and resources in cell line development.

1. Introduction

Biopharmaceutical development and manufacture are lengthy and complex processes focusing on large-scale production of recombinant proteins. Among the numerous challenges is the need for selection of high producing cells from a very large population of stably transfected cells with diverse productivities that are generally created by random integration of the expression vector (Wurm, 2004). Moreover, to ensure reproducible product quality, regulatory authorities require that a production cell line must be derived from a single cell progenitor (Harmonized Tripartite Guideline, 1998). These clonally-derived cell lines are often referred to as 'clones' and that is the term used here. The commonly performed methods for cloning to generate cell lines include serial limiting dilution (Puck and Marcus, 1955), isolation of colonies from semisolid media typically using ClonePix colony picking (Roy et al., 2017), and single cell deposition by fluorescence activated cell sorting (FACS) (Evans et al., 2015). Although vector engineering and selectable marker strategies can be used to favor transfectants producing the protein of interest (Zhu and Hatton, 2016), screening and selection of high-yielding clonal recombinant cell lines to be used for biomanufacturing remains an extensive process.

A group of FACS and magnetic bead selection techniques allow identification and selective enrichment of producing cells based on immobilization of the desired secreted proteins on or close to the cell surface. These methods include cold-capture (Brezinsky et al., 2003; Pichler et al., 2009), matrix-aided surface capture (Bohm et al., 2004; Holmes and Al-Rubeai, 1999) and gel microdrop technology (Hammill et al., 2000; Powell and Weaver, 1990). However some of the methods can display poor correlation between fluorescent signal intensity and relative cell productivity due to diffusion of secreted product and subsequent binding to nearby non-producer cells (Kumar and Borth, 2012). Some FACS enrichment techniques are facilitated by co-expression of a cytoplasmic fluorescent reporter gene like GFP (Freimark et al., 2010; Meng et al., 2000) or non-fluorescent reporter molecules such as CD4,

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CD20 or CD52 that are directed to the cell surface and then used as sorting signals when visualized by viable surface bound fluorescent dyes (Bailey et al., 2002; Cairns et al., 2011; DeMaria et al., 2007). However, such constitutively expressed reporter proteins have the potential to complicate purification and analysis in biotherapeutic manufacturing. Alternatively, transient or inducible transmembrane-linked surface binding displaying good correlation with productivity (Chuang et al., 2014; Helman et al., 2014; Lang et al., 2016) can be used for selective enrichment of high producer cells, but some of these methods require extensive host cell engineering (Chuang et al., 2014; Helman et al., 2014), making them more complex to implement.

Characterization of metabolic activity and growth properties of recombinant production cell lines is important in confirming suitability for the manufacturing bioreactor process. The final yield of protein products is determined by the combination of cell specific productivity and the integral of viable cell number during the process, both of which are driven by cellular biosynthetic pathways. Mitochondria are the central metabolic organelles that regulate energy metabolism, intracellular ion homeostasis, reactive oxygen species production and cell health (Nicholls, 2002; Szabadkai and Duchen, 2008). Mitochondrial membrane potential (Ψm) is an indicator of mitochondrial function and energy metabolism. Previous studies with hybridoma cells have shown correlation between extra-cellular glucose concentration and Ψm, as measured by Rhodamine 123 (Rh123) (Chen, 1988), as well as specific glucose uptake rate and Ψm (Borth et al., 1993). Hinterkorner et al. also demonstrated that when Chinese hamster ovary (CHO) cells were sorted based on Rh123 staining intensity, there was a positive correlation between Ψm and energy metabolism (Hinterkorner et al., 2007).

We hypothesized that the measurement of Ψm may identify recombinant cells with a favorable bioenergetic profile that would consequently produce larger quantities of recombinant protein products. Using clonal cell lines and pools from multiple cell line development projects, we performed a series of FACS based experiments with Mito-ID dye to evaluate Ψm. We determined that the intensity of orange fluorescent signal of Mito-ID dye in the cells correlates with their productivity and that sorting of cells based on optimal Ψm staining selectively enriches for high producer cells. Importantly, high producer clonal cell lines isolated from this process exhibited expression stability.

2. Materials and methods

2.1. Cell culture

An animal component free and suspension-adapted, proprietary CHO cell line derived from CHO-K1 and a selection system for glutamine synthetase (GS) was used. Clonal cell lines and nonclonal stable transfectant pools were generated by transfection and then selected and maintained in proprietary medium supplemented with 50 μM methionine sulfoximine (MSX, Sigma-Aldrich, St. Louis, MO), and 50 mg/L dextran sulfate (Sigma-Aldrich). Suspension cell cultures were grown at 120 rpm on an orbital shaking platform in a humidified incubator set at 37 °C and 6% CO_2. Cells were passaged every 3–4 days. Measurement of viable cell density and viability was accomplished using trypan blue and a ViCell automated cell counter (Beckman Coulter, Brea, CA).

2.2. Fed batch culture

Antibody production was evaluated by fed batch culture in 125 mL Erlenmeyer flasks or 96 deep well plates (96DW). The production cultures grew at 35.5 °C in a humidified 6% CO_2 atmosphere for 14 days unless otherwise mentioned. Shaker speed was maintained at 120 rpm for flasks and 350 rpm for 96DW. Cell density and viability were monitored during cultivation in flasks but not in the 96DW cultures. Custom-made feed was added to the production cultures on days 3, 5, 7, 9, 11 and 13. Antibody titers in the culture supernatant were determined using Protein A biosensors in an Octet QK384 (Barnard et al., 2015) (Pall ForteBio, Fremont, CA).

2.3. Flow cytometric analysis of mitochondrial membrane potential

Status of cellular Ψm was analyzed using the dual fluorescent emission dye Mito-ID (Enzo Life Sciences, Farmingdale, NY). Cells were stained with Mito-ID dye according to the manufacturer's directions. Briefly, 1×10^6 cells were harvested by centrifugation at 300 ×*g* for 5 min, washed with a 1× Mito-ID assay solution and resuspended in Mito-ID detection reagent at a concentration of 1×10^6 cells/mL. The cells were stained for 30 min in the dark at room temperature and analyzed using an LSRII cytometer (BD Biosciences, San Jose, CA). The 488 nm laser was used for excitation, and 525/50 and 582/15 filters were used to acquire green and orange emitted fluorescence, respectively. Bivariate plots of green versus orange fluorescence as well as univariate histogram plots of orange fluorescence were used to analyze Ψm. The orange fluorescent population represents energized cells with active Ψm. Data analysis was performed using FlowJo software (Tree Star, Inc., Ashland, OR).

2.4. Determination of mitochondrial mass

Mitochondrial mass was determined using nonyl acridine orange (NAO, Thermo Fisher Scientific, Waltham, MA), a dye that binds to the inner mitochondrial membrane independent of Ψm (Maftah et al., 1989). Cells were incubated at the concentration of 1×10^6 cells/mL with 500 nM NAO for 10 min in the dark at room temperature, washed twice with cold phosphate-buffered saline (PBS) and immediately analyzed on an LSRII cytometer using a 488 nm laser for excitation and a 525/50 filter for emission. Data analysis was performed using FlowJo software.

2.5. Cell sorting

Bulk cell sorting and single cell deposition cloning based on Ψm was performed using a BD Influx cell sorter (BD Biosciences) as described previously (Evans et al., 2015). For the bulk sort, 20×10^6 cells were harvested by centrifugation. The cells were washed and stained with Mito-ID dye as described above. Based on orange fluorescence intensity, 2.5×10^5 cells from gated fractions were deposited in 5 mL collection tubes containing culture medium. The sorted cells were centrifuged, resuspended in 2.5 mL fresh culture medium and plated in 6-well plates. For single cell cloning, 1×10^6 cells stained with Mito-ID dye were sorted from the Ψm gated fraction by depositing one cell per well into individual wells of 384-well plates containing conditioned medium. All plates were incubated at 37 °C in a humidified atmosphere with 6% CO_2 for outgrowth.

2.6. Cell cycle analysis

One million cells were washed with cold PBS, fixed in 70% ethanol at −20 °C for 2 h, washed again with cold PBS, and resuspended in staining solution containing 40 μg/mL propidium iodide (BD Biosciences) and 0.1 mg/mL RNAse (Thermo Fisher Scientific) in PBS. After 30 min incubation at 37 °C, cells were analyzed on an LSRII. The 561 nm laser was used for excitation and a 610/20 filter was used to acquire the emission fluorescence. Univariate analysis of cellular DNA content reveals distribution of cells in three major phases of the cycle, namely G0/G1, S and G2/M. Data analysis was performed using FlowJo software.

2.7. Intracellular staining for antibody expression level

Intracellular expression of the heavy and light chains of antibody molecules was determined by staining cells with fluorescently labeled antibodies specific for heavy or light chains. Briefly, the cells were

Table 1
Fed-batch culture productivity of high and low producer clones of historical projects MEDI-A and MEDI-B.

Project ID	Producer type	Titer (g/L)	Qp (pg/cell/day)	IVC (10^9 cell hr/L)
MEDI-A	High	4.85	47.8	5848
	Low	1.63	30.3	4285
MEDI-B	High	6.1	53.8	3517
	Low	1.12	15.7	5186

centrifuged, washed with FACS buffer (1% fetal bovine serum in PBS) and fixed with Fixation Medium A (Thermo Fisher Scientific) for 15 min at room temperature. Next, the cells were washed with FACS buffer and stained for 15 min at room temperature with the staining solution comprised of goat anti-human IgG (Fc)-Alexa Fluor 488 (Thermo Fisher Scientific) and goat anti-human kappa-APC (Biolegend, SanDiego, CA) in Permeabilization Medium B (Thermo Fisher Scientific). The stained cells were washed and resuspended in FACS buffer before analyzing in LSRII for the APC and AF488 double positive population. Data analysis was performed using FlowJo software.

3. Results

3.1. Evaluation of mitochondrial membrane potential on clonal cell lines

High and low monoclonal antibody (mAb) producing clonal cell lines (Table 1) from two historical cell line development projects (MEDI-A and MEDI-B) were harvested from culture and stained with Mito-ID to determine their Ψm status. In energized cells, the dye exists as a green fluorescent monomer in the cytosol and accumulates as orange fluorescent aggregates inside the mitochondria. In cells with compromised Ψm, the dye exists primarily as green fluorescent monomers throughout the cytosol and no longer exhibits orange fluorescence in the mitochondria. Flow cytometric analysis revealed no difference in green fluorescence levels but a significant difference in the range of orange fluorescence intensity between the low and high producer clones of both antibodies (Fig. 1A and B). The green fluorescence provides a measure to assess variation in dye loading; no difference in green fluorescence indicates that there is no loss of dye during analysis and the differences in orange fluorescence are due to differences in Ψm status. Therefore, it can be seen that the cell populations in the high productivity clones exhibited a range of Ψm including a significant portion of the population with high Ψm, whereas low productivity clones contained cell populations with primarily low Ψm. The non-transfected host cells also show a range of Ψm overlapping with the higher values observed for the high-expressing clones and reflects the natural heterogeneity of the host cells for Ψm rather than any recombinant protein load. The intracellular staining of the cells for heavy chain and kappa light chain revealed that all the clones have homogeneous antibody expression (Fig. 1C), suggesting that the difference in their titer values is solely due to difference in productivity and that there may be an association between Ψm status and both volumetric

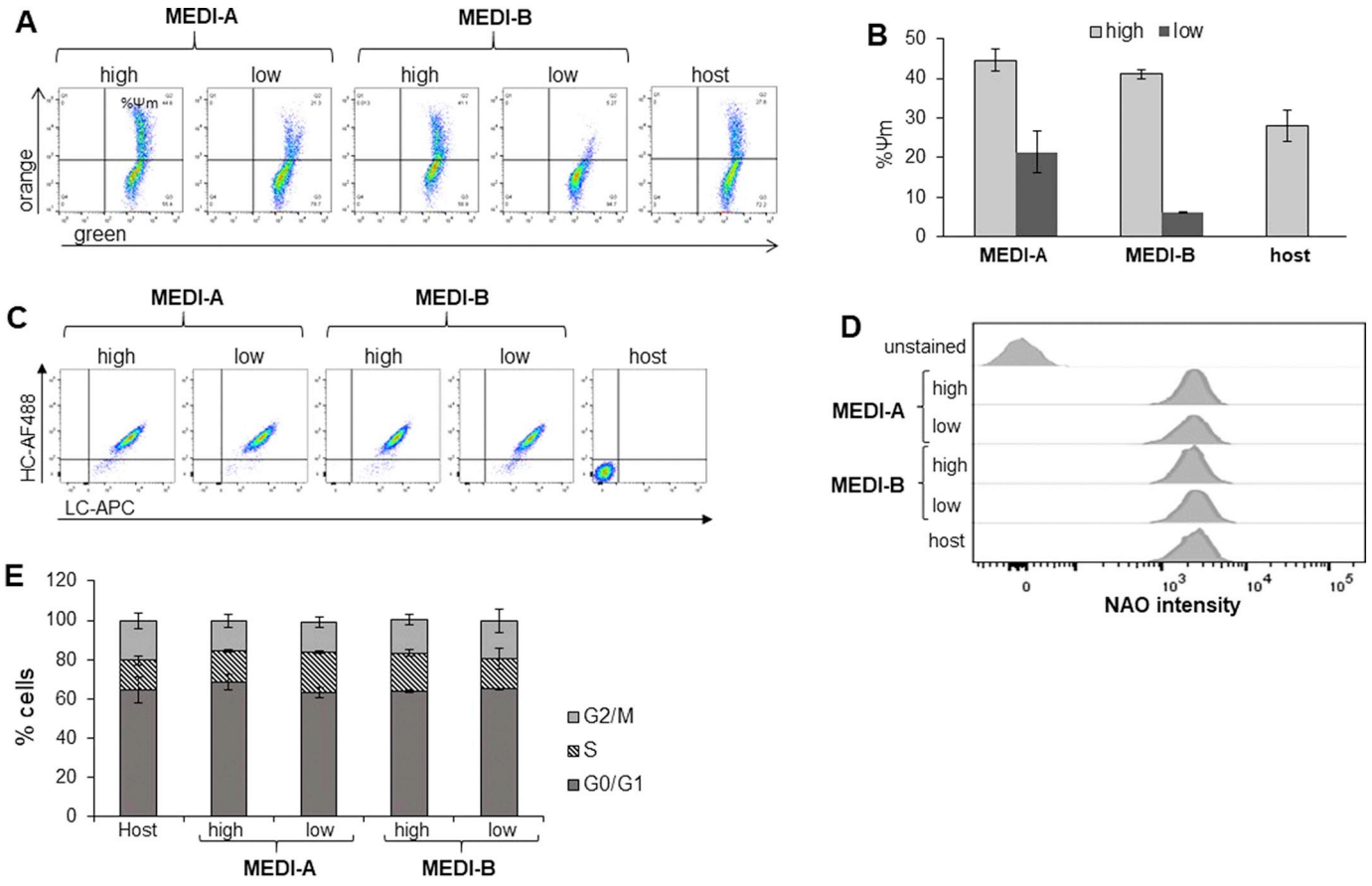

Fig. 1. Correlation of Mito-ID staining for Ψm with productivity in historic clonal cell lines and evaluation of un-transfected host cells. (A) Representative flow cytometry 2-dimensional plots depicting Mito-ID dye staining pattern for green and orange fluorescence in high and low producer clones of MEDI-A and MEDI-B. The top right quadrant of the plots represents cells with high Ψm. (B) Graphical representation of Ψm data showing percentage of cells in the top right quadrant positive both for orange and green fluorescence ($n = 4$). (C) Intracellular staining of light and heavy chains using fluorescence-conjugated antibodies to determine homogeneity of expression. (D) Histogram plots of mitochondrial mass determined by nonyl acridine orange (NAO) staining. (E) Cell cycle analysis using propidium iodide staining of producer clones (n = 4). Data represent mean ± SD. HC, heavy chain; LC, light chain. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

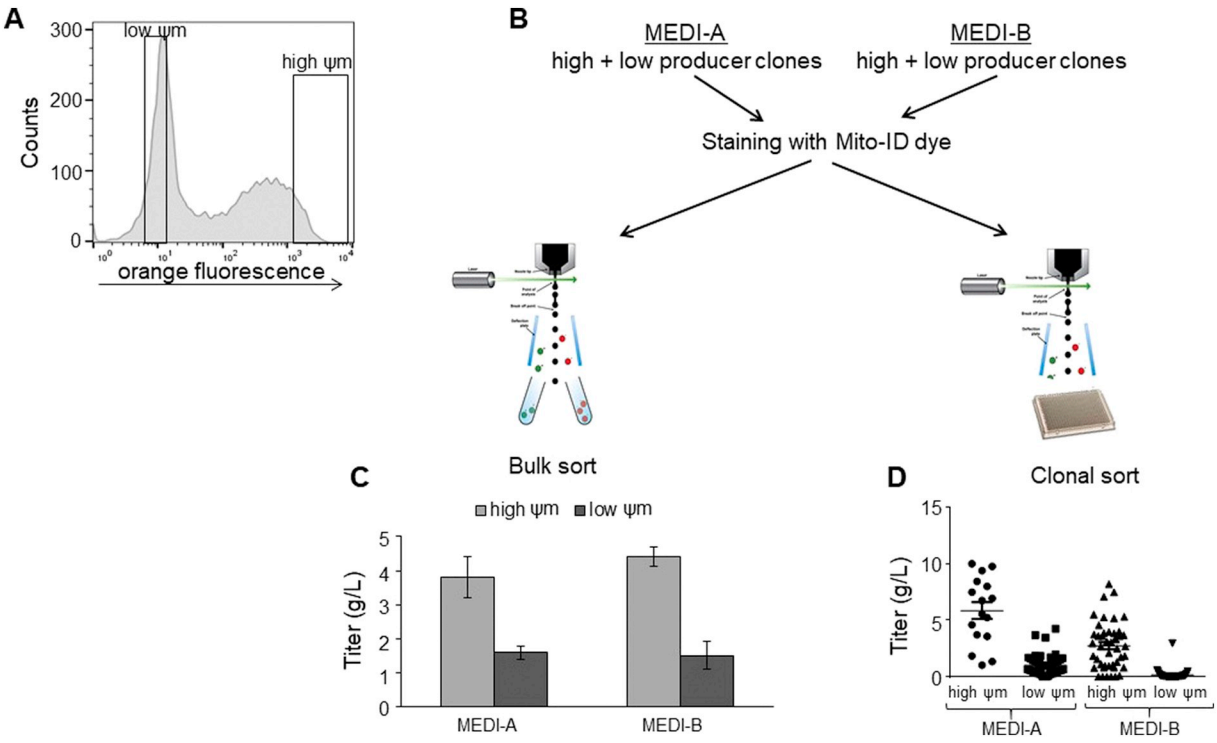

Fig. 2. Productivity of subclones following Mito-ID based cell sorting. (A) Gating strategy employed for cell sorting based on high and low orange fluorescence intensity of Mito-ID staining. (B) An overview of the experiment. The low and high producer clones from each project were mixed in 1:1 ratio and stained with Mito-ID before sorting. (C, D) Fed-batch titer values from bulk (C) and clonal (D) sorted cells using high and low Ψm gates. Data in (C) represent mean ± SD. Each data point in (D) represents an individual antibody expressing subclone.

and cell specific productivity (Table 1). Furthermore, to determine if the Ψm difference observed with the Mito-ID dye results from a difference in the number of mitochondria within each cell, we stained with NAO to measure mitochondrial mass. We found no difference in mitochondrial mass per cell among the different productivity clones (Fig. 1D).

Various studies have reported that Ψm is altered in response to a cellular requirement for more DNA and biomass; as such, Ψm has been found to be higher in the S and G2/M phases of the cell cycle than in G0/G1 (al-Rubeai et al., 1991; Martinez-Diez et al., 2006). Mitochondrial function and activity are closely regulated to fulfill the metabolic needs of the cells and cell cycle regulators such as cyclins, cyclin-dependent kinases and transcription factors play crucial roles in the control of these needs (Lopez-Mejia and Fajas, 2015). Therefore, we sought to assess the distribution of cell cycle phases in the low and high producer clonal cell lines of MEDI-A and MEDI-B in parallel to Mito-ID staining. We found no significant difference in cell cycle phases among the different productivity clones (Fig. 1E), indicating that the difference in their Ψm status is not dependent on cell cycle. We were also able to exclude a correlation of Ψm with growth rate during fed batch production. As shown in Table 1, the IVC was higher for the high expressing and high Ψm clone for MEDI-A, while the IVC for the low-expressing clone for MEDI-B was higher than for the high-expressing, high Ψm clone.

3.2. Cell sorting based on differential mitochondrial membrane potential

To determine whether high producing cells can be enriched based on their Ψm fluorescent signal, we sorted cells based on orange fluorescence intensity following Mito-ID staining (high Ψm and low Ψm gates, Fig. 2A) of mixtures of the two clones expressing MEDI-A and also of mixtures of the two clones expressing MEDI-B (Fig. 2B). Both bulk and clonal sorts were performed, the subpools and subclones were recovered in culture and their productivity was determined in shake flask and 96DW fed-batch cultures respectively. We found that the high Ψm subpools and subclones were more productive than the low Ψm ones for both MEDI-A and MEDI-B (Fig. 2C and D), suggesting a correlation between productivity and the Ψm status of the sorted cells. The diversity of productivity observed in the subclonal populations (Fig. 2D) can be attributed to intraclonal protein expression heterogeneity. It has been reported that gene-expression and productivity can vary significantly among the descendants of a cloned parental cell, even under the same culture conditions (Pilbrough et al., 2009; Tharmalingam et al., 2018).

Next, we evaluated whether high-producing subpools or clones could be enriched from stable transfectant pools using Mito-ID dye staining during sorting. We used two pools from each of three cell line development projects, namely BisX, BisY and ADC. BisX is a hybrid of two different half-antibodies also known as a duet-mAb. The molecule BisY is a difficult-to-express bispecific antibody exhibiting low titer and low specific productivity. ADC (Dimasi et al., 2017) represents an IgG modified for drug conjugation. The initial stable transfectant pools were stained with Mito-ID dye, and the high and low intensity orange fluorescent cells (high Ψm and low Ψm, as shown in Fig. 2A) were sorted into subpools. The resulting newly sorted subpools after subculture and evaluation of fed-batch productivity, revealed significant differences in titer as well as specific productivity between the high and low Ψm populations (Fig. 3A, B and supplementary Fig. 1). Most of the higher Ψm subpools have both higher end titers as well as higher specific productivities. This result supports the positive association between Ψm and cell productivity as observed with the MEDI-A and MEDI-B subpool and subclone samples (as shown in Fig. 2C and D). Interestingly, the differences in cumulative and specific productivities between high and low Ψm sorted subpools were greatest for the 'difficult-to-express' antibody BisY (Fig. 3A, B), while the differences between the high and low Ψm population productivities for the ADC molecule were less dramatic (Supplementary Fig. 1). To determine the intracellular antibody expression pattern of the BisX and BisY subpools,

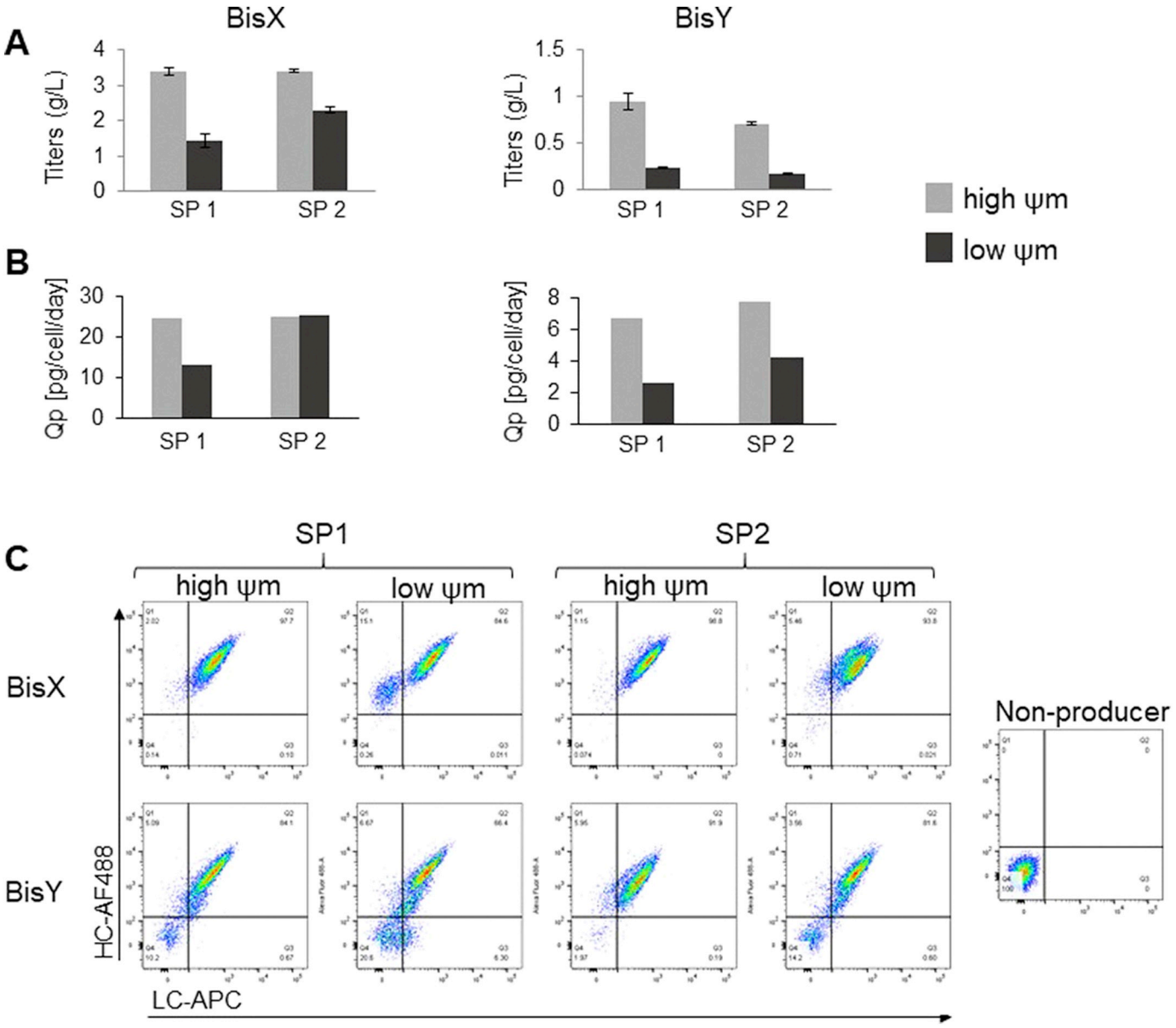

Fig. 3. Association between Ψm and stable transfected cell productivity in Mito-ID stained bulk sorted subpools. Two molecules with varying degrees of difficulty in expression were chosen: BisX (a hybrid of two different half-antibodies also known as duet-mAb) and BisY (a difficult-to-express bispecific antibody). Two stable pools of cells were generated for each of the molecules. The pools were stained with Mito-ID and bulk sorted into "subpools" based on high and low orange fluorescent intensity (Ψm, as shown in Fig. 2A). (A) Fed-batch product titers of the subpools. Data represent mean ± SD ($n = 2$). (B) Final specific productivity of the subpools. (C) Transgene expression pattern of the subpools determined by intracellular staining of light and heavy chains using fluorescence-conjugated antibodies for BisX and BisY subpools after they were maintained in culture for 10 generations. SP, subpool; HC, heavy chain; LC, light chain.

we expanded the subpools for 10 generations and performed flow cytometric analysis of intracellular staining for heavy and light chains (Fig. 3C). We observed differences in homogeneity of transgene expression, with the high Ψm sorted pools trending to have a higher proportion of cells expressing recombinant heavy and light chain (top right quadrant of the bivariate plots, Fig. 3C).

To further investigate the correlation, we worked more extensively with the difficult-to-express BisY pool. First, we performed single cell deposition cloning of Mito-ID dye-stained cells. In this FACS cloning process, four fractions were gated for different Ψm, namely high, medium-high, medium-low and low (Fig. 4A). The medium-high fraction also corresponds to the peak concentration of cells emitting orange fluorescence. Cells from each gated fraction were sorted into 96 individual wells of a 384 well plate at a density of one cell per well. A total of 123 clones recovered and were expanded: 22 of the 96 from the highest Ψm gate, 33 of the 96 from the medium-high gate, 46 of the 96 from the medium-low gate and 22 of the 96 from the low intensity gate. It is notable that the recovery rate of the very high Ψm gated cells was the lowest indicating less robust growth for these cells.

The productivities of the resulting clones were determined after 10 days of fed-batch culture in 96DW. The average titer values indicate that clones isolated from the medium-low and medium-high Ψm gates were more productive than the ones from the low Ψm gate (Fig. 4B). We noticed that the productivities of the clones from the highest Ψm gate were on average lower than those from the medium-high Ψm gate. This was surprising, so the four highest producing clones from each of the four gated groups were expanded and further characterized. NAO staining revealed mitochondrial mass per cell was similar for all the clones irrespective of their Ψm (Fig. 4C), indicating that any Ψm difference observed in the clones is likely due to variation in cellular

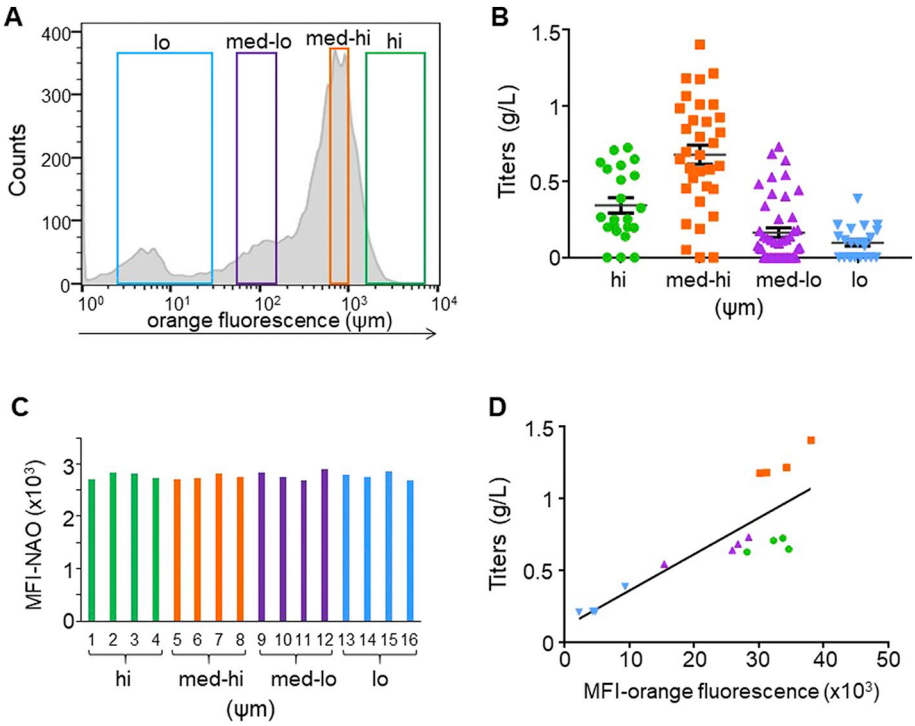

Fig. 4. Correlation between Ψm and cell productivity in cells of a BisY stable pool that was stained with Mito-ID and then FACS sorted by single cell deposition based on orange fluorescence intensity. (A) Gating strategy for high (hi), medium-high (med-hi), medium-low (med-lo) and low (lo) orange fluorescence intensity (Ψm) of Mito-ID stained cells. (B) Product titers of clonal cell cultures from each gated fraction following 10 days of fed-batch culture in 96DW. Each data point is generated using cells expanded from an individual cloned cell. (C) The four highest producing clones identified in the 96DW cultures (shown in Fig. 4B) were analyzed for mean fluorescence intensity (MFI) of NAO staining for mitochondrial mass. (D) The titers of the four highest producing clones from each of the four gated groups were plotted as a function of the Ψm determined after clonal cell expansion.

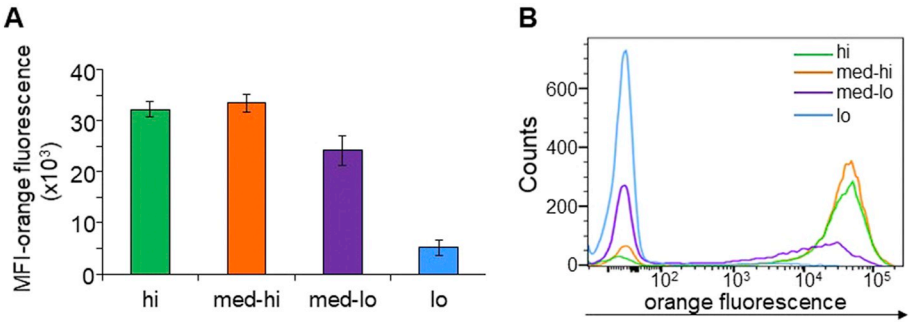

Fig. 5. Stability of Ψm status of clones isolated by Mito-ID staining. (A) The MFI of the orange fluorescence of four top producer clones from each gated fraction of BisY (as shown in Fig. 4A) after being maintained in culture for 30 generations. Data represent mean ± SD (n = 4). (B) Representative flow cytometric histogram plots for the Mito-ID staining of the same clones.

mitochondrial function and activity rather than mitochondrial content. Evaluation of Ψm with Mito-ID dye was also performed for the same clones. When the titer values were plotted against the mean orange fluorescence intensity (MFI) of Mito-ID staining, we again observed a positive correlation (R^2 = 0.692, Fig. 4D), indicating that productivity can be predicted by Ψm status of the cell. Interestingly, the cell lines that originated from the highest orange fluorescence intensity fraction now had Ψm values that overlapped with those from cell lines that originally came from the medium-high Ψm gated fraction. The change in the Ψm relative ranking of the cells may indicate that the very high Ψm cannot be sustained. Too high Ψm is an indicator of mitochondrial hyperpolarization or cellular stress (Korshunov et al., 1997; Murphy and Brand, 1988; Satoh et al., 1997; Vayssier-Taussat et al., 2002). Potential consequences of mitochondrial hyperpolarization include formation of reactive oxygen species (Korshunov et al., 1997; Liu, 1997), reduced ATP utilization (Kaim and Dimroth, 1999) and decreased efficiency of oxidative phosphorylation (Murphy, 1989) leading to disruption of cellular homeostatic balance and growth. We indeed observed 33% less outgrowth and recovery of the highest Ψm clones compared to the medium-high Ψm clones (22 of 96 clones recovered from the highest intensity gate vs 33 of 96 clones from the medium-high intensity gate). High levels of cell stress or mitochondrial hyperpolarization are also not beneficial for cells in the long term and could have put selective pressure on the cells as they expanded from the clones, resulting in a decrease in the observed Ψm (Dimasi et al., 2017; Vayssier-Taussat et al., 2002)(Liu, 1997; Kaim and Dimroth, 1999) (Dimasi et al., 2017; Korshunov et al., 1997; Murphy and Brand, 1988; Satoh et al., 1997).

3.3. Characterization of cell line expression stability

To investigate the stability of the Ψm trait, the four highest BisY-producing clones from each of the four gated groups discussed in Fig. 4 were expanded for 30 generations and evaluated for their Ψm status using Mito-ID staining. We found that the MFI of orange fluorescence followed a similar trend to that of the gated fractions when they were sorted (Fig. 5A). Although we observed that the sorted cells from all the gates except the 'lo' ones gave rise to some cells with different Ψm levels during the expansion period (Fig. 5B), most of the cells from each gated fraction maintained their original Ψm status. The result suggests that the Ψm trait is stable and maintained even after the cells had aged by 30 generations between the analyses. We believe the slight heterogeneity in Ψm profile may have resulted from the inherent plasticity of the CHO nuclear genome or changes to the mitochondrial genome which is in agreement with other published studies that have documented the continuous genetic and phenotypic drifts in cultured CHO cells (Pilbrough et al., 2009; Tharmalingam et al., 2018; Kelly et al., 2017; Ko et al., 2018).

The four clones producing the highest levels of BisY from the medium-high gated group were tested for stability of growth

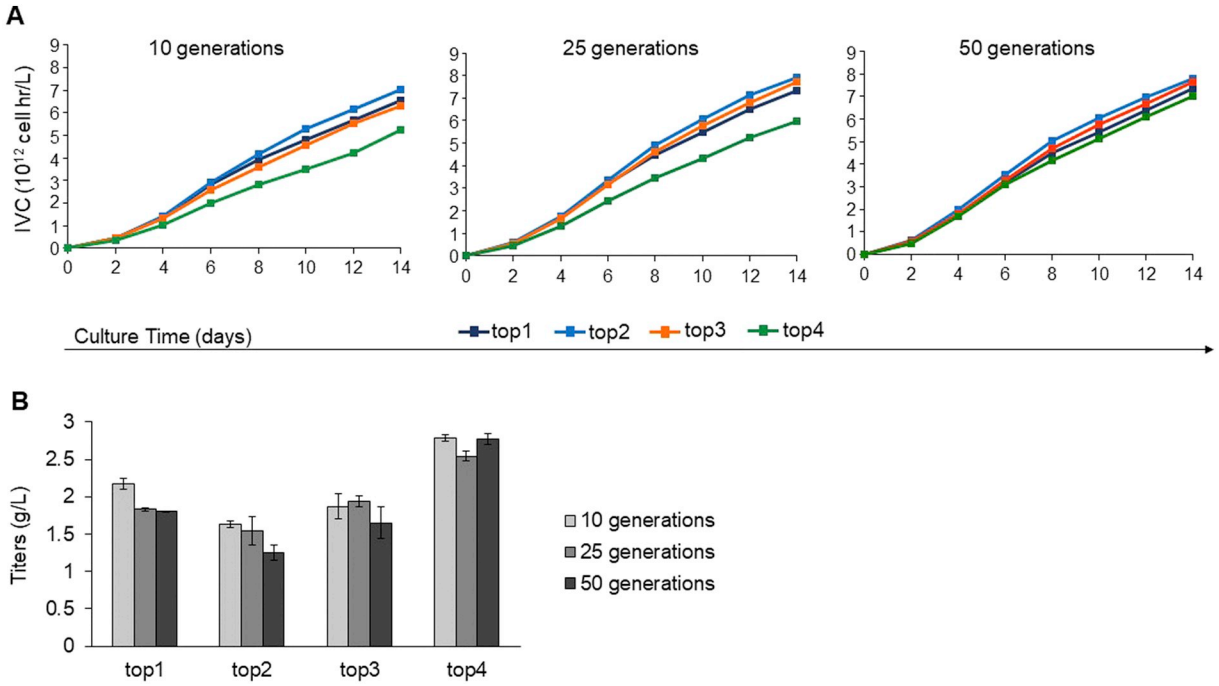

Fig. 6. Expression stability of clones isolated by Mito-ID staining. (A) Growth chart of the four highest producing clones of BisY (from the medium-high group in Fig. 4B) that were analyzed for phenotypic stability at three different generations. IVC: integral of viable cell density. (B) Expression stability of the same four top producer clones was determined by productivity in 14 day fed-batch cultures at 10, 25 and 50 generations. Data represent mean ± SD (n = 2).

characteristics and antibody production. The stability study was performed over a course of 50 generations and all four clones demonstrated consistent cell growth characteristics (Fig. 6A) and antibody productivity (Fig. 6B). Titers in this 14-day process were higher in all passages than was seen in the initial screening 10-day fed batch shown in Fig. 4B because of the longer culture process. These stability data demonstrate that high producing clones isolated using Mito-ID staining are stable for both growth and productivity.

4. Discussion

Determining the most effective method for isolating the highest producing cell lines is one of the primary concerns in the protein drug development process. There has been substantial improvement in the yield of CHO cell based production processes in recent years (Zhu and Hatton, 2016), and yields of over 10 g/L have been reported (Birch and Racher, 2006; Cacciatore et al., 2010). However, clones having this high production potential are typically extremely rare in a population of transfectants, so significant screening efforts are required to identify and isolate them. Moreover, variations in metabolic activity of stably transfected cells warrant an early screening assay to eliminate clones with undesirable energy metabolism. In this study, we describe a simple and feasible method to rapidly isolate highly productive cells for the biomanufacturing process using the single mitochondrial membrane potential parameter, Ψm. The key feature of this approach is the ability to combine the screening for Ψm with the enrichment or cloning step, thereby isolating cells with better productivity. Our results demonstrate that with the exclusion of very high Ψm, status of Ψm in CHO cells correlates with their protein productivity. This allows us to accurately identify and enrich the high producing clones and eliminate the low or non-producer population at the early stage of the FACS cloning step. Moreover, we examined different antibody molecular formats with varying levels of expression, namely mAb (MEDI-A and MEDI-B), a bispecific antibody (BisY), a hybrid of two different half-antibodies (duet-mAb, BisX) and an IgG modified for drug conjugation (ADC). We found that the method has general applicability across different molecule classes and may have its greatest impact with the most difficult-to-express molecules. The dominant role of mitochondria is production of energy via the electron transport chain present in the mitochondrial membrane. Mitochondrial membrane potential is regulated by the components of the electron transport chain (Kadenbach, 2003). Due to enormous energy demand, cells in production phase require enhanced mitochondrial function to meet their energy need. Therefore, it is not surprising to find that cells with augmented Ψm have better productivity.

Our FACS-based strategy shows that stably transfected cells that have medium-high Ψm display enhanced productivity relative to the very high or low Ψm cells. Others have also reported gating cells on Ψm to isolate distinct cell populations with different physiological properties. Sukumar et al. have shown that metabolically robust T-lymphocyte cells can be isolated using a mitochondrial dye TMRM (Sukumar et al., 2016). The T-cells with low Ψm have superior long-term in vivo persistence and greater anti-tumor capacity, whereas, high Ψm is found to be associated with increased cytokine production in a variety of lymphocyte lineages. Therefore, it is most likely that cells need to adjust their Ψm to an optimal level to achieve full production capability. Hinterkorner et al. isolated subclones with higher yield and production rates compared to a parental monoclonal antibody-producing CHO cell line using the mitochondrial dye Rh123 (Hinterkorner et al., 2007). The authors showed a positive correlation between cell-specific glucose uptake rate and Ψm. This method was useful for optimizing energy metabolism by isolating subclones with different physiological properties, such as growth rate, glucose uptake and lactate production. By sorting for Ψm using Mito-ID dye we were able to eliminate clones with undesirable productivity at an early stage of cell line development process and consequently achieved efficient enrichment of high producer clones from heterogenous stable transfectant pools generated from random integration.

Product quality and production stability are as critical as productivity in the cell line development process. A longstanding problem in CHO cell line development has been the increase in heterogeneity of expression levels during expansion of individual clones (Pilbrough et al., 2009; Barnes et al., 2001; Barnes et al., 2006), leading to changes in productivity. Variation in productivity may develop and become

apparent as early as 18 days after cloning. The heterogeneity in expression among the cells of a clone can rapidly become comparable to that of mixed transfectant pools (Pilbrough et al., 2009). We have shown that sorting for cells with moderately high Ψm selects cells that are more homogeneous for transgene expression. This is evident from the higher fraction of positive transgene expressing cells in the subpools selected for high Ψm (Fig. 3C). Therefore, use of Ψm based sorting not only serves to enrich highly productive cells, but may provide a complementary strategy to enrich for homogeneous cells within a phenotypically diverse cell population. Moreover, the high Ψm clones demonstrated consistent antibody productivity over a course of 50 generations indicating their high degree of transgene expression stability.

Mitochondrial function is sensitive to diverse types of cellular insults, including disorder in protein production and associated stress (Rainbolt et al., 2014; Senft and Ronai, 2015; Vannuvel et al., 2013). It would be interesting to investigate whether the decreased productivity of the low Ψm cells is related to problems with protein folding, assembly or secretion, all of which are energy consuming processes resulting in energy depletion and possibly a decreased Ψm. Additionally, we observed that the host cells display a broad range of Ψm, while the low producer cells generally only show low Ψm. The host cell line has no recombinant production load, but is heterogeneous i.e. non-clonal and so the wide Ψm range reflects the natural diversity of this cell population. Hence, it is possible that the high Ψm properties contributed by the progenitor transfected host cell, provide the cellular environment needed for high levels of recombinant protein production, but this requires further study. Future investigations will also delineate the cellular and molecular mechanisms of the correlation between Ψm and cell production load.

Taken together, our present study demonstrates a widely applicable, novel FACS gating strategy based on an optimal, moderately high Ψm as detected by orange fluorescence staining intensity with Mito ID dye. This allows isolation of stable, homogeneous high producing cells. The method identifies and segregates high and low energy cells at an early stage of cell line development process. Using Ψm as a predictive marker of productivity, this technique should provide an easy-to-use, cost-effective and high-throughput tool for the cell line development process for production of recombinant proteins.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jim.2018.10.007.

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