Integrated single-cell analysis shows Pichia pastoris secretes protein stochastically.
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Integrated single-cell analysis shows *Pichia pastoris* secretes protein stochastically.

The production of heterologous proteins by secretion from cellular hosts is an important
determinant for the cost of biotherapeutics. A single-cell analytical method called
microengraving was used to examine the heterogeneity in secretion by the methylotrophic
yeast *Pichia pastoris*. We show that constitutive secretion of a human Fc fragment by *P.
pastoris* is not cell-cycle dependent, but rather fluctuates between states of high and low
productivity in a stochastic manner.

**Keywords:** yeast, heterologous protein secretion, *Pichia pastoris*, clonal selection,
microengraving, single-cell
Since the approval of recombinant insulin in the early 1980’s (1982), the pharmaceutical industry has sought to develop and market drugs that harness the biosynthetic pathways of cells to manufacture therapeutic proteins. There are now 151 recombinant pharmaceuticals approved for human use by either the US Food and Drug Administration (FDA) or the European Medicines Agency (EMEA) (Ferrer-Miralles et al. 2009), and the biologics market is expected to grow 12% this year (Ainsworth 2008). Microbial hosts produce nearly 50% of these biotherapeutics (Ferrer-Miralles et al. 2009). The methylotrophic yeast *Pichia pastoris* has been useful for heterologous protein expression in both biomedical research and industrial production (Cregg et al. 2000; Li et al. 2007). Recombinant engineering has yielded *P. pastoris* strains that produce proteins with homogeneous human N-linked glycans (Hamilton and Gerngross 2007; Jacobs et al. 2009) capable of producing monoclonal antibodies in excess of 1 g/L (Potgieter et al. 2009). These characteristics position *P. pastoris* as an important alternative to traditional expression systems for biopharmaceutical production.

The quantity of protein produced in a batch reactor is a significant determinant in the cost of a biopharmaceutical (Farid 2007). This fact has prompted considerable research on how to maximize protein production and secretion from host cells. The annotated genome for *P. pastoris* strain GS115 will facilitate the identification of genetic factors that govern secretion and enable engineering secretory pathways in a rational manner (De Schutter et al. 2009). Non-genetic factors, however, account for substantial variability among cells and may strongly influence protein production and secretion (Avery 2006). Single-cell, high-throughput screens for protein secretion can thoroughly and efficiently identify cells with altered secretory function and should facilitate strain engineering.

Flow cytometry (FC) can characterize multiple parameters for single cells simultaneously, but analysis of protein secretion by FC requires a physical link between genotype and phenotype—either by surface capture (Mattanovich and Borth 2006; Rakestraw et al. 2006) or surface display (Wentz and Shusta 2007). These methods yield a fixed view of the secretory capabilities of a population of cells at a given time, but secretion is a dynamic process that may change as cells grow, divide, and respond to environmental conditions.
analyses of this process, therefore, require monitoring secretion from single cells (and their progeny) over time.

Microengraving is an emerging method for high-throughput analysis of secreted products from single cells (Love et al. 2006), and has been used for integrative measurements of antibody secretion in B cells over several hours (Story et al. 2008). Here we used microengraving to study the secretion of heterologous proteins from individual clones of \textit{P. pastoris}. These dynamic measurements show that constitutive secretion in this yeast is not linked to the cell cycle, but instead, switches stochastically. This apparent epigenetic phenomenon lasts over several doubling times, but decorrelates over longer periods of culture growth (>24 h).

To quantify secretion of a heterologous protein from individual \textit{P. pastoris} cells, we used microengraving (Fig. 1) and a strain secreting a human Fc fragment under the control of the strong, constitutive GAPDH promoter (Waterham et al. 1997). This promoter was chosen in an attempt to focus on the intrinsic secretory diversity among a population of cells, rather than promoter-induced differences. Additionally, the GAPDH promoter is commonly used for the expression of many proteins (Cregg et al. 2000). Microengraving experiments were conducted for either 1 or 3 h to determine the minimum time required to observe secretion from the maximum number of single cells. A rate of secretion for each cell was determined using a standard curve and the incubation time (Supplementary Fig. 1). The distribution of rates of secretion by single cells, and the frequency of secreting cells (69%, 1 h; 61%, 3 h) were similar for both conditions (Fig. 2B); these results indicate that asynchronous cultures exhibit a steady-state distribution of rates of secretion from single cells. Furthermore, the distribution of the rate of secretion, and the frequency of secreting cells, do not change when a clonal population is sampled at different growth phases.

The doubling time for wild-type \textit{P. pastoris} is on the order of 2-3 h. As such, within 3 h, one would expect most cells to have transitioned through the G2 and M phases of the cell cycle—a period that has been correlated with increased productivity in \textit{Saccharomyces cerevisiae} (Frykman and Srienc 2001). To evaluate this transition specifically, we synchronized cells in G1/S phase and allowed them to proceed to late M phase (Fig. 2A). The fraction of
Figure 1. Microengraving with *P. pastoris* correlates secretion of heterologous proteins with single cells. A single colony of yeast cells was used to inoculate a shake flask, and grown for 1 to 2 days at 25°C. Cells from the shake flask were deposited onto an array of microwells at a density of ~1 cell/well. Microengraving was then conducted to create a protein microarray comprising the secreted proteins captured from each individual well. Secreted proteins were quantified and correlated with the cells present in the corresponding microwells. Scale bar is 50 µm.
Figure 1

Inoculate shake flask with a single colony

Seed microwells with cells from shake flask

Microengraving

Protein microarray

Quantify secreted protein

Cells in microwells

Count cells

Data integration and analysis

81x187mm (300 x 300 DPI)
**Supplementary Figure 1.** Spot assay for conversion of the median fluorescence intensity (MFI) measured for captured Fc secreted by *P. pastoris* into a quantity of protein. **A:** Known concentrations of Fc were incubated in duplicate for 1h on a glass slide previously treated with goat anti-human Ig(H+L) (Zymax). The slide was developed with Cy5-labeled goat anti-human Fc (Jackson) and scanned for fluorescence at 635 nm using a Genepix array scanner (Molecular Devices). **B:** Background-corrected fluorescence intensities for each concentration of Fc were used to construct a standard curve for the rate of Fc secretion (ng*mL⁻¹*h⁻¹). The data were fitted by linear regression (R² = 0.996).
Supplementary Figure 1

A

Rate of Fc secretion (ng/mL⁻¹*h⁻¹)

B

Relative fluorescence units

204x134mm (300 x 300 DPI)
Figure 2. Microengraving with *P. pastoris* shows secretion of heterologous proteins is cell-cycle independent. **A:** Cell cycle arrest of *P. pastoris* using hydroxyurea produces cultures synchronized at G1/S phase. *P. pastoris* cells were treated with hydroxyurea (0.2 M) for 3 h, and then were washed repeatedly with fresh YPD prior to depositing in microwells. Micrographs of cells were taken directly following the release of the cell-cycle arrest and then again 90 min later using 40X magnification. White arrowheads indicate growing daughter cells. Scale bar is 10 µm. **B, C:** *P. pastoris* cells engineered to secrete a human Fc fragment were used for microengraving experiments with incubation periods of either 1 or 3 h. Prior to microengraving, a sample of the yeast cells were synchronized in G1/S phase as shown in panel A. **B:** Histogram of the distributions of rates of secretion for single cells. The threshold for secretion was determined by the background median fluorescent intensity + 2σ. Data shown are representative of three independent experiments. **C:** Secretion rates for synchronized (red circles) and unsynchronized cells (blue squares) obtained during a 1 h experiment as a function of the number of contributing cells per well (min. *n* = 58). Data were fit by linear regression. (*R^2^* sync. = 0.990, *R^2^* unsync. = 0.998) Open features indicate calculated rates for the average contribution per cell in a given well weighted by the global frequency of secretors.
Figure 2

A

B

C

151x243mm (300 x 300 DPI)
individual cells with measureable rates of secretion (59%) was less than that for the asynchronous cultures, and no increase in the median rate of secretion was observed from these cells (Fig. 2B and C). Further analysis of these experiments showed that the average rate of secretion per cell is also consistent for discrete multi-cellular colonies of cells: These groups exhibited no cooperativity or inhibition with regard to secretion regardless of the phase in the cell-cycle or the number of cells present in a given microwell (Fig. 2C). Together, these experiments suggest that secretion by *P. pastoris* does not depend on the stage of the cell cycle.

An unexpected result was that a large fraction of the cells were not secreting detectable quantities of Fc. We next sought to determine if secretion from these unproductive cells would remain ‘off’ permanently, or if secretion might switch ‘on’ stochastically as reported for the intracellular production of proteins in other cells (Kaufmann et al. 2007). We performed serial microengraving (Fig. 3A) to monitor cells and their secretions over ~8h. The average rates of secretion per cell for wells containing a single cell at the beginning of the experiment increased with doubling time (*T_d*) (Fig. 3B). This population-based analysis suggests that the amount of protein secreted by a cell is inversely proportional to its rate of growth. Analysis of the individual events measured, however, reveals that several unique subpopulations contribute to the whole population. Profiles for secretion correlated to single cells at t=0 were classified using self-organizing maps (SOMs) to assess the number of non-degenerate subpopulations (Supplementary Fig. 2). K-means clustering was then performed using the optimized number of bins, and the secretory profiles aligned with auxiliary data on the relative age of the initial cell, the total protein measured from each well, and the number of cells at t=435 min (Fig. 3C). This analysis shows three groups of subpopulations: One group yields no significant secretion over the course of the experiment (35%), one group (32%, two subpopulations) consistently secretes protein, and one group (33%, three subpopulations) exhibits a significant change in their rates of secretion during the experiment.

For all three groups, the cells divided at least once, and there was no significant difference in the average age of the initial cells. These results indicate that non-productive cells remain viable and were not geriatric. The subpopulation of cells that switch from producing during the first measurement to an “off” state further supports the observation that secretion in
Figure 3. Serial microengraving shows that secretion of heterologous proteins by *P. pastoris* is stochastic. **A:** Unsynchronized cells were deposited into microwells. Cells in wells were identified at the start of the experiment and then used to print 4 times serially, each print lasting 90 min. Cells were imaged following the final print and the difference in the number of cells from first to last print was used to calculate doubling time (T\textsubscript{d}). Data from wells containing 1 cell at start and 2 or more cells after 4 prints were subsequently analyzed. **B:** Box plot of the relative amounts of secreted Fc per cell as a function of T\textsubscript{d} (min. \( n = 121 \)). The total amount of secreted protein was determined for each well and then correlated per cell by dividing by the final number of cells in the wells. The tops and bottoms of each box are the 25\textsuperscript{th} and 75\textsuperscript{th} percentiles of the data at each T\textsubscript{d}. The red line in each box is the median. Outliers (red +) are shown. The black line is the linear fit of the data means for each T\textsubscript{d} (\( R^2 = 0.981 \)). Data sets marked with * were subjected to a Student’s t-test; \( p = 9 \times 10^{-10} \). **C:** Multidimensional analysis of single cells over time organized by secretory profile. K-means clustering (\( n = 6 \)) distinguished cells based on their secretory profiles (\( n = 978 \)). Each horizontal line presents data from a well containing a single cell at the start of serial microengraving. Heatmaps (blue) indicate the relative rates of secretion per well during each of the four prints. For each subgroup, the mean temporal profile for secretion is superimposed (red line). The mean fluorescent intensity of an extracellular stain (Calcofluor white) indicative of the age of the parent cell (purple, normalized range 0-100), the total secreted Fc from all four prints (green), and T\textsubscript{d} (orange) are aligned for each well in the subgroups. Red triangles denote the mean value of each parameter plotted. The percentage of events comprising each subgroup is also indicated.
Figure 3

A

Deposit cells

Count cells at start & perform serial microengraving

Print 1: t = 75 min

Print 2: t = 165 min

Print 3: t = 255 min

Print 4: t = 345 min

Count cells after final print

B

Relative secreted protein per cell

<150 173 218 345

Average doubling time (min)

12000

10000

8000

6000

4000

2000

0

C

Relative Age

Time (min)

Total Protein

Final # Cells

Wells with Single Cells (10k)

Min

Max

Relative Rate of Secretion

188x216mm (300 x 300 DPI)
Supplementary Figure 2. Self-Organizing Map (SOM) analysis of data generated by serial microengraving identifies 6 distinct clusters present in the data (panels A-F). These clusters are similar to those identified by K-means clustering using 6 bins (Figure 3, panel C): both the mean profiles of secretion and the number of members in each cluster were consistent for multiple executions. The number of maps was allowed to vary from 4-12 for 50,000 iterations using GenePattern. Red squares denote data means and blue circles are SOM centers.
Supplementary Figure 2

A. Cluster 1 - 182 items

B. Cluster 2 - 71 items

C. Cluster 3 - 72 items

D. Cluster 4 - 369 items

E. Cluster 5 - 194 items

F. Cluster 6 - 90 items

193x237mm (300 x 300 DPI)
these cells is not tightly linked to the cell cycle: These cells divide at least once, but their rate of secretion does not change. Together with the other two subpopulations in this group, the data indicate that constitutive secretion is a semi-stable, stochastic process influenced by non-genetic inheritance. That is, parent and daughter cells appear to have the same secretion phenotype following division, and switch between “on” and “off” phenotypes synchronously. We note that the two subpopulation of cells that appear to remain “on” may switch more frequently than the sampling rate. Interestingly, though, one of these subpopulations does grow more rapidly than the other cells, and even when corrected for the average number of cells per well, secretes more protein than the other populations. This interpretation directly contradicts the correlation between growth and productivity presented in Figure 3B, and highlights the importance of evaluating a population engaged in a dynamic process, like secretion, by tracking changes in time, rather than by average contributions at a particular endpoint.

To determine if the phenotypes for secretion were stable following multiple rounds of division, we recovered single cells that were identified initially to be either high producers or low producers. The cultures grew from single cells to late log phase in liquid culture, and we then re-examined them via microengraving. Both the median rates of secretion and the steady-state distributions of rates of secretion were similar to those of the original parent culture (Fig. 4A); supernatants from the daughter cultures (n = 45) also contained comparable amounts of Fc as assessed by ELISA (Fig. 4B). Similar experiments previously reported for S. cerevisiae showed that dilution of a parent culture, followed by expansion at 20 °C, yielded a profile of secretion comparable to that observed for the parent (Huang et al. 2008). Our results indicate there is no intrinsic defect in the secretory capacity of the non-productive cells, and that fluctuation in the rate of secretion is due to a short-lived, epigenetic event that is diluted over many cell divisions.

Similar to reported results for intracellular protein expression in S. cerevisiae (Kaufmann et al. 2007), our results suggest that secretion of proteins by daughter cells and parent cells changes in a synchronized manner, and that this property leads to the emergence of unique semi-stable populations in a culture. Synchronization of secretion, however, does not extend over many rounds of division from a parent cell (Spencer et al. 2009). It is unlikely that the observed changes in secretion result from changes in expression of the promoter regulating transcription of
**Figure 4.** Daughter cells secrete similar titers of Fc fragment at similar rates regardless of secretion phenotype of parent cells.  **A:** Histogram of the distributions of rates of secretion for single cells in clonal populations selected from a parent culture. Single cells from a clonal parent culture (red line) were identified as either high or low producers and were recovered and grown to late log phase in liquid culture. Single-cell distributions of rates of secretion for these new clonal lines were investigated via microengraving and are presented in duplicate (high producers, blue lines; low producers, green lines). Frequencies of secreting cells were the same for all clones shown (70±5%).  **B:** Plot of secreted Fc fragment titer from liquid cultures following clonal expansion of single cells retrieved from a parent culture. Individual cells were selected based on secretion phenotype in the parent culture (blue, high producers; red, intermediate producers; green, low producers; gray, non-producers), grown to late log phase and supernatants were harvested. Bars represent an average of three replicate measurements of secreted Fc as determined by ELISA. Dashed line indicates the median titer for all 45 samples.
Figure 4

A

Rate of secretion (ng/mL⁻¹·h⁻¹)

Frequency

Parent culture
Low producer 1
Low producer 2
High producer 1
High producer 2

B

Secreted Fc (mg/L·OD₆₀₀)

Daughter cultures

High producers
Medium producers
Low producers
Non-producers

128x152mm (300 x 300 DPI)
the inserted gene: GAPDH is located in a highly transcribed region of the \textit{P. pastoris} genome (De Schutter et al. 2009) and the distribution of rates of secretions observed experimentally for the entire population is representative of a protein that is constitutively expressed (Cai et al. 2006; Waterham et al. 1997). The observed fluctuations, therefore, may result from variations in a post-transcriptional process, perhaps in some part of the secretory pathway itself. Studies are underway to understand the mechanism. We believe these experiments underscore the challenges of optimizing production strains on the basis of genetic engineering alone, and suggest that understanding how to promote long-lived synchronization of non-genetic states will be important for maximizing yield in batch reactors.

**Materials and Methods**

\textbf{\textit{P. pastoris} cell culture.} \textit{P. pastoris} secreting a human Fc under the GAPDH promoter (a gift from GlycoFi, Inc.) was streaked from a frozen clonal stock onto solid YPD (Yeast Peptone Dextrose) media. Colonies were allowed to develop at 25°C for several days. A single colony was used to inoculate 10 mL liquid YPD and the culture was then grown to late log phase (OD$_{600}$ = ~1.0-1.5) at 25°C with shaking at 290 rpm.

\textbf{Cell-cycle arrest of \textit{P. pastoris}.} \textit{P. pastoris} cells were grown in liquid culture to late log phase (OD$_{600}$ = ~1.0-1.5) then treated with hydroxyurea (0.2M) and were incubated at 25°C for 3 h (Day et al. 2004). Cells were harvested by centrifugation and washed 3 times with fresh YPD to remove traces of hydroxyurea and release the cell-cycle arrest.

\textbf{Microengraving using \textit{P. pastoris}.} Microwell arrays containing ~250,000 wells (each 30 µm in diameter) were fabricated in poly(dimethyl-siloxane) (PDMS) as reported previously using photolithography and replica molding (Ogunniyi et al. 2009). PDMS arrays were sterilized by treatment with an oxygen plasma (PDC-32G, Harrick) for 20s and then were submerged in a 1% aq. solution of (3-glycidoxypropyl)trimethoxysilane for 50 min at 80°C. PDMS arrays were washed twice with sterile PBS before depositing cells. \textit{P. pastoris} cells were harvested from liquid culture by centrifugation, resuspended in PBS at a density of 1 X 10$^6$ cells/mL and stained with Calcofluor dye (250 µg/mL) for 10 min at 25°C. Cells were deposited onto the treated microwells arrays by dispensing 500 µL of the cell suspension dropwise onto the array. The
cells were allowed to settle into the microwells for 5 min before excess media was removed and cell deposition was repeated. The surface of the array was dewetted by aspiration and washed with YPD. (Cells cultured in these wells were viable and expanded when left overnight at 25°C.) Glass slides were prepared as described (Ogunniyi et al. 2009) using 25 µg/mL goat anti-human Ig(H+L) antibody (Zymed) as the primary antibody for Fc capture. The array of microwells filled with *P. pastoris* cells was dewetted of excess media and was placed well-side down onto the surface of a treated, dry glass slide. The sandwich comprising the array and the glass slide was held together in a hybridization chamber (G2534A, Agilent Technologies). The entire assembly was incubated at 25°C for either 1 or 3 h. After incubation, the treated glass slide was removed from the surface of the microwell array and was immediately immersed in PBS.

**Interrogation of the printed microarray.** Following microengraving, glass slides were blocked (1% milk/0.05% PBS Tween 20) for 30 min and washed as described (Ogunniyi et al. 2009). The slide was then treated with a solution of goat anti-human IgG secondary antibody (Cy5 conjugate, Jackson ImmunoResearch) at 0.5 ng/mL and incubated for in the dark for 45 min at 25°C. Slides were washed, dried, and then imaged with a microarray scanner (GenePix 4200AL, Molecular Devices) using a 635-nm laser and factory installed emission filters. The laser was used at 70% power and the PMT gain was set at 450; these values were determined to maximize the linear range of detection in these experiments.

**Spot assay for construction of standard curve.** The fluorescence intensity for each individual spot on the engraved protein microarray was converted to a quantity of protein using a standard curve. The standard curve was obtained by constructing a protein array using known quantities of Fc (50, 25, 5, 1, 0.5 and 0.1 ng/mL) diluted in YPD and spotted on a glass slide as treated above. The slide was incubated for 1h, then developed and imaged as described above. Background-corrected fluorescence values were plotted against concentration to determine the linear range of the microengraving assay.

**Microscopy and micromanipulation.** Phase contrast and fluorescence images of the cell-loaded PDMS microarray were acquired using AxioVision software (v4.7.2, Carl Zeiss) and an
automated inverted microscope (AxioObserver Z1, Carl Zeiss) equipped with a Hamamatsu EM-CCD camera. *P. pastoris* cells were retrieved from individual wells using a CellCelector (Aviso) as described (Choi et al. 2009) with optimized settings for yeast cells. Retrieved cells were each deposited into a separate well of a 96-well plate containing 200 µL YPD. 96-well plates were incubated at 25°C for 1-2 days before using the contents of a well to inoculate a larger liquid culture (1-10 mL YPD).

**Serial microengraving using *P. pastoris***. A PDMS microwell array containing ~80,000 wells (each 50 µm in diameter) was treated and loaded with *P. pastoris* cells as above. The array of microwells filled with *P. pastoris* cells was used to generate a protein microarray as described during a 90 min incubation at 25°C. Following the incubation, the entire sandwich comprising the PDMS microwell array and the glass slide containing the protein microarray was submerged in sterile PBS and separated to minimize cell loss. Phase contrast and fluorescence images of the cell-loaded PDMS microarray were immediately acquired and the protein microarray was developed as above. The cell-loaded PDMS microarray was then used 3 times subsequently to produce protein microarrays as described, adding fresh YPD media to the array of microwells in between each microengraving experiment. Following the final microengraving incubation, the cells in the PDMS microarray were restained with Calcofluor (250 µg/mL) for 10 min at 25°C, washed with YPD and phase contrast and fluorescence images were acquired again.

**Data processing and statistical analysis**. Phase contrast and fluorescence images of the cell-loaded PDMS microarray were analyzed for identification of the number of cells in each well using the MabAnalyze program (custom script). Images of the printed microarrays were analyzed using GenePix Pro 6.0 (Molecular Devices). The background intensity for each array was determined from the median of all values measured in regions between individual spots of the array. Spots in the array were identified as positive when the signal-to-noise ratio was greater than 2—that is, when the spot intensity was greater than the sum of the background intensity for the array plus two standard deviations of the values used to calculate the background intensity. Multidimensional data were correlated using MatchBox (custom script). All subsequent data filtering and analysis was performed using Microsoft Excel. Data clustering
by Self-Organizing Maps and K-means analysis was performed using GenePattern (Reich et al. 2006).

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