THE ISOLATION OF SENESCENT POPULATIONS OF *S. CEREVISIAE* (BAKER'S YEAST)

by
James J. Claus

Submitted to the Department of Chemistry
in Partial Fulfillment of the Requirements for the
Degree of

MASTER OF SCIENCE
in Biochemistry

at the

Massachusetts Institute of Technology
June 1995

Signature of Author _____ 5/30/95
Department of Chemistry
May 12, 1995

Certified by _____ 5/26/95
Leonard Guarente
Thesis Co-Supervisor

Certified by _____ 5/21/95
William H. Orme-Johnson
Thesis Co-Supervisor

Accepted by _____
Dietmar Seyferth
Chairman, Departmental Committee on Graduate Students

THE ISOLATION OF SENESCENT POPULATIONS OF *S. CEREVISIAE* (BAKER'S YEAST)

by
James J. Claus

Submitted to the Department of Chemistry on May 12, 1995
in Partial Fulfillment of the Requirements for the Degree of
Master of Science in Biochemistry

ABSTRACT:

Saccharomyces cerevisiae (baker's yeast) is a unicellular eukaryote with a finite life span defined by the number of times a cell divides. A certain mean and maximum life span are characteristics of a given strain, but vary widely from strain to strain, indicating genetic components are involved in longevity. To characterize the changes that take place as cells age and to examine the molecular and genetic mechanisms responsible for such changes, it is desirable to have a means of isolating senescent populations of *S. cerevisiae*.

It is shown here that individual cells can be stably labeled with a biotin/avidin/fluorescein complex, and that this label is not generally transferred from a mother cell to its daughter cell during the course of cell division. Thus a population of labeled mother cells may be cultured for a period of time and then separated from their descendants using a fluorescently-activated cell sorter (FACS). The FACS technique gives relatively low yields, and is a tedious process, but purity is excellent.

In a related technique, mother cells are labeled with a biotin/avidin/ "paramagnetic bead" complex, which allows cells to be sorted magnetically. While the FACS must sort each cell one by one (with a throughput of ~2500 cells/second), the magnetic sorter effectively sorts all cells simultaneously, allowing a suspension of 10^8 cells to be sorted in 10 minutes versus 10 hours with the FACS. Purity is very good, but it remains to be seen whether it is equal to the purity achieved by the FACS.

Possible applications of these techniques include screening for long-lived mutants and biochemical characterization of *S. cerevisiae* populations of different "age". A high degree of conservation of basic cellular processes has been demonstrated in recent years between yeast and mammalian cells. This suggests that the study of yeast aging is likely to yield significant insights into the cellular aging of higher eukaryotes, organisms which are less amenable to experimental work.

Thesis Co-Supervisors: Leonard Guarente
Professor of Biology

William H. Orme-Johnson
Professor of Chemistry

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER ONE: INTRODUCTION

BACKGROUND

Saccharomyces cerevisiae (baker's yeast) is a unicellular eukaryote which divides asymmetrically to give a mother cell and a daughter cell[1]. The daughter cell originates as a small "bud" protruding from the cell wall of the mother, and gradually grows in size. At cytokinesis the mother is still larger than the daughter, allowing the two to be unambiguously identified. *S. cerevisiae* exists in both haploid and diploid forms, with haploids of opposite mating-type conjugating to form diploids and diploids undergoing meiosis and sporulation to yield haploids[2]. Both the haploid and diploid forms may be stably maintained by the vegetative (mitotic) reproduction described above.

THE METRIC OF AGE IS REPRODUCTIVE CAPACITY

The life span of a yeast cell is finite; individual mother cells eventually cease mitotic division and frequently lyse shortly thereafter[3]. Calendar time plays little role in this process; rather, Müller and coworkers have shown that life span defined as "the total number of cell divisions completed" is essentially invariant under a range of conditions that produces wide variations in the chronological measure[4]. A certain mean and maximum life span are characteristics of a given strain, but vary widely between strains, demonstrating that longevity is determined at least partially by genetic components[5].

---

[1]Strathern, J.R., Jones, E.W., and Broach, J.R., eds. (1981) The Molecular Biology of the Yeast *Saccharomyces*: Life Cycle and Inheritance. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory;
Strathern, J.R., Jones, E.W., and Broach, J.R., eds. (1982) The Molecular Biology of the Yeast *Saccharomyces*: Metabolism and Gene Expression. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

[2]Herskowitz, I. (1988) Life Cycle of the Budding Yeast *Saccharomyces cerevisiae*. *Microbiol. Rev.* **52**:536-553

[3]Mortimer, R.K., Johnston, J.R. (1959) Life Span of Individual Yeast Cells. *Nature* **183**:1751-1752

[4]Müller, I., Zimmermann, M., Becker, D., Flomer, M. (1980) Calendar Life Span Versus Budding Life Span of *Saccharomyces cerevisiae*. *Mech. Ageing Dev.* **12**:47-52

[5]Sacher, G.A. (1978) Evolution of Longevity and Survival Characteristics in Mammals. In: The Genetics of Aging. Schneider, E.L. (ed.) 151-168. New York: Plenum Press.

Experimentally, life spans of individual yeast cells are determined using a microscope equipped with a micromanipulator[6]. Virgin daughters (cells which have never budded) are deposited at isolated locations on an agar slab, and their growth is monitored hourly. Once a virgin cell has produced a mature bud, the bud is plucked away and the virgin cell is now a one-generation-old mother cell, and so forth. A cell is deemed to have reached the end of its life span when it has produced no buds for a period of 24 hours.

As demonstrated by Pohley, with each passing cell cycle there is an exponential increase in the probability that a mother cell will fail to produce another bud[7]. This exponential rise in the age-specific death rate is exhibited by many aging metazoan populations, most notably humans[8]. Interestingly, baker's yeast does not senesce clonally; descendants of old mothers eventually recover as great a life expectancy as daughters from young mother cells[9,10]. Superficially, this "resetting" of life spans in descendants while mother cells senesce resembles the (unknown) process by which aging metazoan organisms endow their young offspring with a full life span[11].

MORPHOLOGICAL AND OTHER CHANGES ARE ASSOCIATED WITH AGING

Each cell division results in the formation of a raised ring of chitin (known as a "bud scar") on the mother cell and a more diffuse circular structure (known as a "birth scar") on the daughter cell. The number of bud scars on a cell is thus an indication of the number of daughters

---

[6]Mortimer, R.K., Johnston, J.R. (1959) Life Span of Individual Yeast Cells. *Nature* **183**:1751-1752

[7]Pohley, H.-J. (1987) A Formal Mortality Analysis for Populations of Unicellular Organisms (*Saccharomyces cerevisiae*). *Mech. Ageing. Dev.* **38**:231-243

[8]Arking, R. (1991) The Biology of Aging. 41-44. Englewood Cliffs, New Jersey: Prentice Hall.

[9]Johnston, J.R. (1966) Reproductive Capacity and Mode of Death of Yeast Cells. *Antonie van Leeuwenhoek J. Microbiol. Serol.* **32**:94-98

[10]Müller, I. (1971) Experiments on Ageing in Single Cells of *Saccharomyces cerevisiae*. *Arch. Mikrobiol.* **77**:20-25

[11]Jazwinski, S.M. (1993) Genes of Youth: Genetics of Aging in Baker's Yeast. *A.S.M. News.* **59**:172-178

that the cell has produced. Cell size increases approximately linearly with each cell division[12,13], and it is observed that a cell at the end of its life span quite commonly has a diameter five times as large as when it was a virgin[14]. Similarly, generation time can be expected to increase about six-fold over the course of a life span, with minimal increases early on and precipitous increases a few generations prior to mitotic arrest[15,16]. Jazwinski has noted that this phenotype of increased generation time is dominant in yeast; daughters of old yeast cells initially display generation times which are typical of the mother cell, but after about three divisions acquire the shorter generation time typical for young cells. Jazwinski postulates that this is indicative of the presence of a "senescence factor" in old mother cells, which is lost by gradual degradation or dilution in daughter cells.

Daughters of older cells often have a decreased proliferative potential, but a full life span is recovered in grand-daughters or great grand-daughters[17]. Close to the end of the life span, the daughters of aged cells are often elongated, misshapen, and display a drastically decreased proliferative capacity[18]. Finally, older cells show a marked decrease in the ability to mate[19].

SIMILARITIES TO THE HUMAN DIPLOID FIBROBLAST (HDF) MODEL OF CELLULAR SENESCENCE

Hayflick originally showed that HDFs cultured serially have a finite life span *in vitro*, as measured by the number of population doublings

---

[12]Johnson, B.F., Lu, C. (1975) Morphometric Analysis of Yeast Cells IV. Increase of the Cylindrical Diameter of *Schizosaccharomyces pombe* During the Cell Cycle. *Exp. Cell Res.* **95**:154-158

[13]Egilmez, N.K., Chen, J.B., Jazwinski, S.M. (1990) Preparation and Partial Characterization of Old Yeast Cells. *J. Gerontol. Biol. Sci.* **45**:B9-17

[14]Unpublished results.

[15]Mortimer, R.K., Johnston, J.R. (1959) Life Span of Individual Yeast Cells. *Nature* **183**:1751-1752

[16]Egilmez, N.K., Jazwinski, S.M. (1989) Evidence for the Involvement of a Cytoplasmic Factor in the Aging of the yeast *Saccharomyces cerevisiae*. *J. Bacteriol.* **171**:37-42

[17]Austriaco, N., Kennedy, B., Personal communication.

[18]Unpublished results.

[19]Müller, I. (1985) Parental Age and the Life-Span of Zygotes of *Saccharomyces cerevisiae*. *Antonie van Leeuwenhoek J. Microbiol. Serol.* **51**:1-10

undergone before a cell strain stops dividing[20]. The similarities between aging in yeast and aging in HDFs is striking. HDFs increase in size as the number of population doublings rises, and the generation time lengthens[21]. Furthermore, in fusions the senescent phenotype is dominant; in heterokaryons formed from a young fibroblast and a senescent fibroblast, initiation of DNA replication is inhibited in the young nucleus[22,23,24]. Microinjection into young fibroblasts of polyA+ RNA derived from old cells duplicates the inhibition of DNA synthesis observed in heterokaryons[25], strongly suggesting the presence of a senescence factor such as that postulated to exist in yeast by Jazwinski. Indeed, several such senescent cell derived inhibitors (SDIs) have recently been cloned[26].

WHY STUDY AGING IN YEAST?

As discussed above, aging in yeast is superficially very similar to aging in human diploid fibroblasts. Moreover, it has been demonstrated that in many areas of basic cellular function the similarity between yeast and mammalian cells extends down to the molecular level[27]. Amazingly, it is not unheard of to have yeast proteins which will function in place of their mammalian counterparts and vice versa.

---

[20]Hayflick, L. (1965) The Limited *in vitro* Lifetime of Human Diploid Cell Strains. *Exp. Cell. Res.* **37**:614-636

[21]Sherwood, S.W., Rush, D., Ellsworth, J.L., Schimke, R.T. (1988) Defining Cellular Senescence in IMR-90 Cells: A Flow Cytometric Analysis. *Proc. Natl. Acad. Sci. USA* **85**:9086-9090

[22]Pereira-Smith, O.M., Smith, J.R. (1982) Phenotype of Low Proliferative Potential Is Dominant in Hybrids of Normal Human Fibroblasts. *Somatic Cell Genet.* **8**:731-742

[23]Norwood, T.H., Pendergrass, W.R., Sprague, C.A., Martin, G.M. (1974) Dominance of the Senescent Phenotype in Heterokaryons Between Replicative and Post-Replicative Human Fibroblast-Like Cell. *Proc. Natl. Acad. Sci. USA* **71**:2231-2235

[24]Stein, G.H., Yanishevsky, R.M. (1979) Entry into S Phase is Inhibited in Two Immortal Cell Lines Fused to Senescent Human Diploid Cells. *Exp. Cell Res.* **120**:155-165

[25]Lumpkin, C.K., McClung, J.K., Pereira-Smith, O.M., Smith, J.R. (1986) Existence of High Abundance Antiproliferative mRNA's in Senescent Human Diploid Fibroblasts. *Science* **232**:393-395

[26]Hensler, P. Personal communication.

[27]Herskowitz, I. (1985) Yeast as the Universal Cell. *Nature* **316**:678-679

Cyclin E, the TATA box binding factor TFIID[28], various transcriptional activators[29], and the yeast RAS2 protein[30] are all examples.

Experimentally, yeast is a very tractable model system. *S. cerevisiae* has excellent classical genetics, a rapid growth rate, and a range of molecular genetic techniques virtually as powerful as are available in any prokaryote[31,32]. Cloned genes can be disrupted and replaced with selectable markers at will, mutations constructed *in vitro* can be inserted into the genome (replacing the wild-type copy), and mutations occurring in known genes *in vivo* can be plucked from the genome and analyzed. In short, yeast represents an easily studied model system that has a good probability of yielding pertinent insights into the aging of more complex, less experimentally tractable organisms.

To characterize the changes that take place as cells age and to examine the molecular and genetic mechanisms responsible for such changes, it is desirable to have a means of isolating senescent populations of *S. cerevisiae*. For this reason, we set out to develop such a method.

---

[28]Buratowski, S., Hahn, S., Sharp, P. and Guarente, L. (1988) Function of a Yeast TATA Element-Binding Protein in a Mammalian Transcription System. *Nature* **334**:37-42

[29]Kataoka, T., Powers, S., Cameron, S., Fasano, O., Goldfarb, M., Broach, J., Wigler, M. (1985) Functional Homology of Mammalian and Yeast RAS Genes. *Cell* **40**:19-26

[30]Guarente, L. (1988) UASs and Enhancers: Common Mechanism of Transcriptional Activation in Yeast and Mammals. *Cell* **52**:303-305

[31]Bostein, D., Fink, G.R. (1988) Yeast: An Experimental Organism for Modern Biology. *Science* **240**:1439-1443

[32]Struhl, K. (1983) The New Yeast Genetics. *Nature* **305**:391-397

CHAPTER TWO: SORTING BY FACS

BACKGROUND

Our original hope for using a fluorescently-activated cell sorter (FACS) was to be able to sort *S. cerevisiae* cells directly based on the number of bud scars per cell, using a method suggested by K. Madura[1]. Such a method would obviously be desirable since the number of bud scars on a cell is an unequivocal measure of the cell's age. In pilot studies, Calcafluor White was used to stain the bud scars of an exponentially-growing *S. cerevisiae* culture (strain BKY14C), which was then sorted by FACS into several classes of cells, as characterized by magnitude of "forward scatter" (a measure of size) and "fluorescent intensity"[2]. Ideally, all bud scars would be stained identically and "background" staining of the cell as a whole would be uniform among all cells and negligible compared to the staining of even a single bud scar. In such a case we could write

$$I = ni + k \qquad (k \leq i) \qquad [1]$$

where I is the measured fluorescent intensity of a cell, n is the number of bud scars present on the cell, i is the fluorescent intensity due to a single bud scar, and k is the fluorescent intensity due to background staining. In this ideal case one would expect "fluorescent intensity" to correlate very well with number of bud scars. However, when the actual sorted cells were examined by fluorescence microscope, there appeared to be little correlation between the degree of fluorescence and the number of bud scars present. Rather, the sorted "high fluorescence" class contained cells with seemingly random numbers of bud scars but strong background fluorescence, while the "low fluorescence" class contained cells again with apparently random numbers of bud scars but weak background fluorescence. In other words, the fluorescent intensity of a cell as measured by the FACS was being primarily determined by the degree

---

[1]Kiran Madura, personal communication.

[2]See Appendix 1 for a schematic diagram and detailed explanation of the FACS instrument.

of background fluorescence and not by the number of bud scars present. In terms of equation [1] above, k >> i (and k varied largely between cells). In this case, the measured fluorescent intensity is a very poor predictor of the number of bud scars, making it impossible to sort the cells effectively.

In an attempt minimize background staining and hence enable effective sorting, Calcafluor concentrations and staining times were varied, as was the temperature at which the cells were stained. In no case could strong staining with low background be predictably obtained. In fact, bud scars on strain BKY14C most often appeared as dark spots on a more highly fluorescent background, a situation completely incompatible with effective sorting based on the number of bud scars. A switch to strain PSY142 yielded marked improvements in bud scar staining with lower fluorescent background and bud scars standing out as bright "rings", but staining was still unpredictable from one time to the next, as has been lamented by other researchers[3]. These difficulties led us to eventually abandon our attempts to sort *S. cerevisiae* based on number of bud scars.

FLUORESCENT LABELING OF THE CELL SURFACE

Although never experimentally tested, it had long been assumed with regard to mitotic division in *S. cerevisiae* that the wall of the daughter cell is synthesized *de novo* while that of the mother cell retains all or at least part of its identity[4]. It occurred to us that if this were indeed the case, then stable fluorescent labeling of the cell walls of a yeast population at a given time t_0 should allow us at any later time to unambiguously identify the cells which were present in the culture at the original time t_0. Such cells could then be sorted away from their non-fluorescent descendants using the FACS, thus yielding a pure population of old cells.

---

[3]Pringle, J. R., "Staining of Bud Scars and Other Cell Wall Chitin with Calcafluor" in Guthrie, C., Fink, G. R., eds. (1991) Guide to Yeast Genetics and Molecular Biology. San Diego, CA: Academic Press.

[4]Mortimer, R.K., Johnston, J.R. (1959) Life Span of Individual Yeast Cells. *Nature* **183**:1751-1752

There are several conditions which are critical to the efficacy of such a proposed method. These conditions are met, as is discussed below.

CELL SURFACE LABEL IS STABLE AND IS NOT TRANSFERRED TO DAUGHTERS

Label must not be transferred from mother cell to daughter cell during cell division. Non-transfer of label supports a hypothesis of *de novo* cell wall synthesis in daughter cells, and provides a tag which unambiguously identifies the progenitors of the culture. The cell label must be maintained sufficiently well on the cell surface to allow cells that have grown for many generations to be clearly distinguished. The label should not break down excessively with time, nor should the turnover of stained components in the cell membrane result in excessive loss of label. Furthermore, the label must be relatively resistant to bleaching, because each cell must be sorted several times by the FACS in order to obtain senescent cells.

To examine these concerns, NHS-LC biotin (Pierce) was used to label the cell walls of a population of yeast cells obtained from an exponentially growing culture. NHS-LC biotin is a water-soluble, activated succinimidyl ester derivative of biotin which undergoes nucleophilic attack by primary amines (such as lysine side chains) to yield a product in which the attacking amino group is covalently attached to the biotin group[5]. Following the biotinylation, the same cells were incubated with fluorescein-conjugated avidin (Pierce) to fluorescently label the previously modified proteins. The avidin-biotin interaction is the strongest known non-covalent biological recognition between protein and ligand ($K_d = 10^{-15}$ M) and is unaffected by most extremes of pH and other denaturing conditions[6]. These biotinylated, avidinylated cells were then examined using a fluorescence microscope and found to exhibit a highly uniform, bright green fluorescence.

An aliquot of the fluorescent cell solution was transferred to rich liquid media and grown at 30° C for six hours. During this time the

---

[5]Product literature. Pierce Scientific Supply. Rockford, Illinois.
[6]Green, N. M. (1975) Avidin. *Adv. Protein Chem.* **29**:85-133

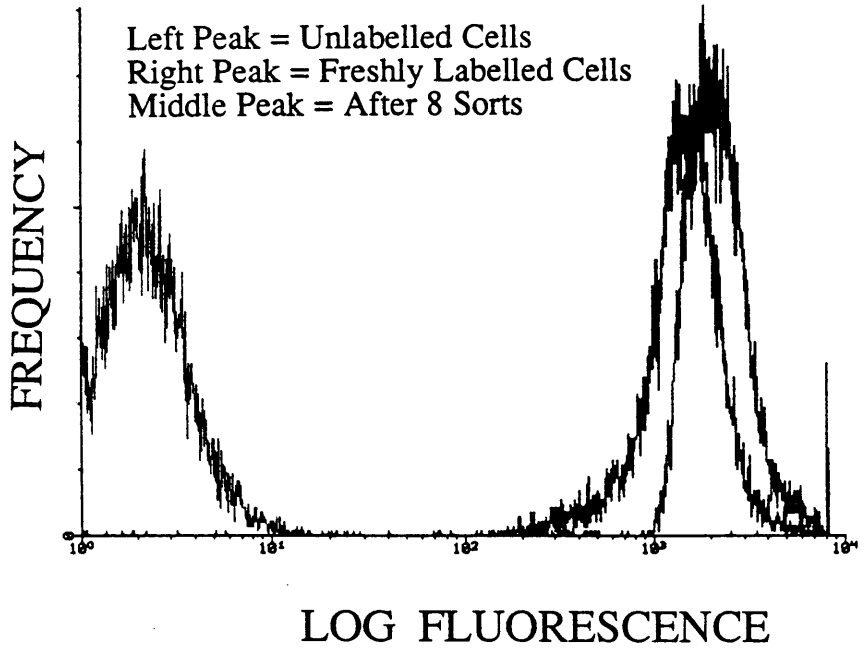
vessel containing the cells was wrapped in aluminum foil to prevent possible bleaching of the fluorescein by ambient light. Following this incubation, the cell culture was again examined under the fluorescence microscope and was found to be a mixture of fluorescent and non-fluorescent cells. Fluorescent cells were judged to be as bright as newly stained cells, and non-fluorescent cells appeared completely devoid of label; in no case were "faintly fluorescent" cells or cells with a non-uniform fluorescent pattern observed as might be expected if label were transferred from mother cells to daughter cells. Given this initial positive result, additional cells were labeled and this time grown at 30° C for 24 hours. Examination of these cells again showed a small fluorescent population and a much larger non-fluorescent population. Overall fluorescence did appear to have decreased relative to freshly stained cells, perhaps due to gradual degradation of the fluorescein with time or turnover of proteins in the cell wall. Despite the decrease in fluorescence, labeled cells were still clearly distinguishable from unlabeled cells.

To examine bleaching due to laser interrogation of cells during FACS sorting, a population of labeled cells was prepared by biotinylation/avidinylation. The cells were passed through the FACS, and the fluorescence intensity distribution was recorded for the population. The same cells were then passed through the FACS an additional seven times, each time recording the fluorescence intensity distribution for the population. A small decrease in fluorescence intensity was observed with each additional passage through the FACS. However, comparison with an unstained population shows that the fluorescent cells are still clearly distinguishable even after eight passages through the FACS machine. These data are presented in Figure1.

PURITY IS VERY HIGH

Extreme purity is desirable for many applications of a technique for senescent cell isolation. To test purity obtainable by the FACS under optimal conditions when sorting fluorescent/non-fluorescent *S. cerevisiae*, a mixture of 20% labeled and 80% unlabeled cells was

FIGURE 1: Negligible bleaching occurs during FACS sorting. Freshly labelled *S. cerevisiae* cells were passed through the FACS eight times, causing a minimal reduction in fluorescent intensity. Sorted labelled cells remain easily distinguishable from unlabelled cells.



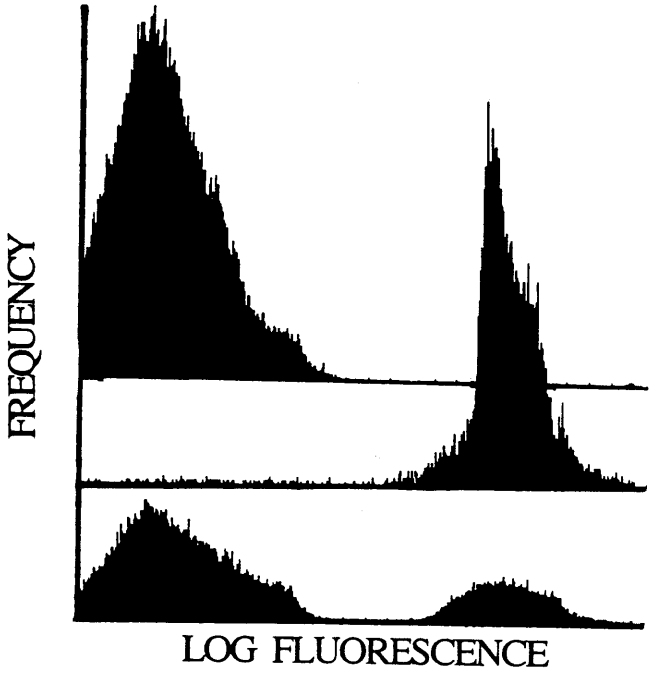


FIGURE 2: Sorting on the FACS gives high purity. From top to bottom are the non-fluorescent cell population after one sort, the fluorescent cell population after one sort (99.1% pure), and the mixed population prior to the sort (20% fluorescent cells). Purity could be increased further by additional sorts.

prepared and sorted using the FACS. With a single sort, labeled cells were enriched to 99.1% of the population, and higher purity would be obtainable with subsequent sorts. These data are shown in Figure 2.

THE LABELING PROCEDURE IS NOT EXCESSIVELY TOXIC

The labeling procedure must not be excessively toxic. Toxicity is indicative of detrimental changes in the cells, unrelated to aging. Extreme toxicity affects our ability to even obtain senescent cells, while more moderate toxicity complicates the analysis and characterization of senescent populations by leaving us wondering whether observed impairments or changes in cell function are due to aging or simply to the effects of the labeling procedure.

To examine possible toxic effects of cell labeling, life spans were determined for 40 cells subjected to the biotinylation/avidinylation procedure and 40 cells serving as a negative control. Unfortunately, the labeling procedure does appear to result in early death for some fraction of the labeled cells, although the maximum life span is unaffected. These data are shown in Figure 3.

To explore the origin of this effect in greater detail, life spans were determined for 40 cells that had undergone the battery of washings and incubations in buffer that comprise the biotinylation-avidinylation procedure, but which were not actually biotinylated or avidinylated. Life spans were also evaluated for 40 cells serving as a negative control. These data are shown in Figure 4. The results suggest that the early deaths observed in the biotinylation/avidinylation experiment are not due to the biotinylation/avidinylation itself, but rather result from the stresses of 11 washes and a lengthy period of incubation in moderately basic buffer (pH 8), some fraction of which is at room temperature. Modifications of the labeling procedure may reduce this toxicity to some degree, but in any case the effect on mean life span is not severe. Furthermore, the toxicity appears to manifest itself in a rash of early deaths, while leaving the subsequent pattern of death due to aging relatively unchanged (as evidenced by the lack of change in

FIGURE 3: Effect of Biotinylation/Avidinylation on the Lifespan of Individual S. cerevisiae Cells

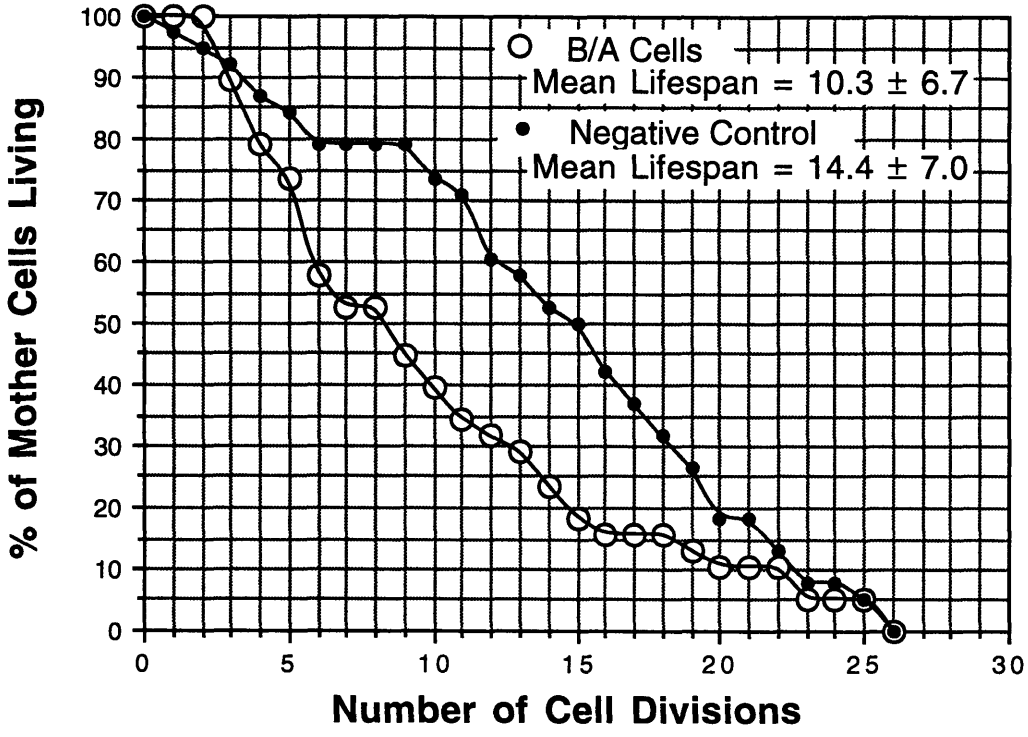
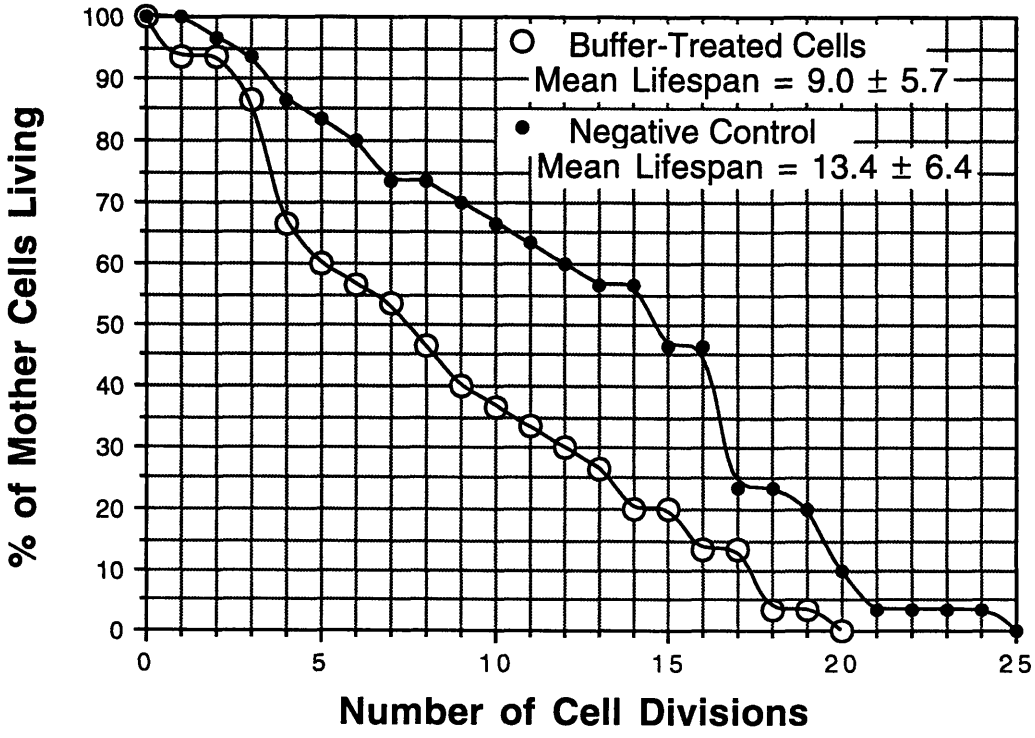


FIGURE 4: Effect of Buffer Treatment on the Lifespan of Individual S. Cerevisiae Cells



the maximum life span). It can be surmised that cells which are not killed prematurely enjoy a normal aging process.

THE FACS SORT ITSELF IS NOT EXCESSIVELY STRESSFUL

FACS sorting must have a negligible effect on the life span if senescent populations are to be reliably obtained, because each cell must be sorted several times when isolating cells of advanced age. To illustrate the need for several sorts, suppose one wished to isolate 50,000 cells with a mean age of 32 generations. If one allowed an initial 50,000 cells to divide 32 times, one would end up with the task of sorting 50,000 fluorescent cells out of a population of $50,000 \times 2^{32}$ total cells (about 200 trillion cells). At roughly 10 million cells per hour, it would require 2284 years to sort so many cells. Obviously it is much more efficient to sort at 8 generations, place the cells into fresh media, sort again at 16 generations, place the cells into fresh media, and so on. This requires four sorts of $50,000 \times 2^8 = 12.8$ million cells each, a task that can be completed in a number of hours as opposed to a number of millennia.

Life spans were determined for 30 cells which had been sorted on the FACS and for 30 cells serving as a negative control. Sorting cells using the FACS appeared to have no measurable effect on life span. These data are shown in Figure 5.

ISOLATED CELLS ARE ADVANCED IN AGE

A preliminary experiment was carried out to show that fluorescent cells re-isolated after being allowed to grow freely for a period of time are indeed advanced in age (as demonstrated by a decrease in the number of cell divisions remaining before mitotic arrest). Labeled cells were grown in rich media for 20 hours and then sorted on the FACS and life spans determined for 30 cells. Life spans were also determined for 30 cells serving as a negative control. Hemocytometer counts of cell density before and after the 20-hour period of growth indicated a 3,300-fold increase in cell number. If we assume that all cells divide at the same rate, then the labelled cells must divide once for every doubling of the population. A 3,300-fold increase in cell

FIGURE 5: Effect of FACS on the Lifespan Of Individual S. cerevisiae Cells

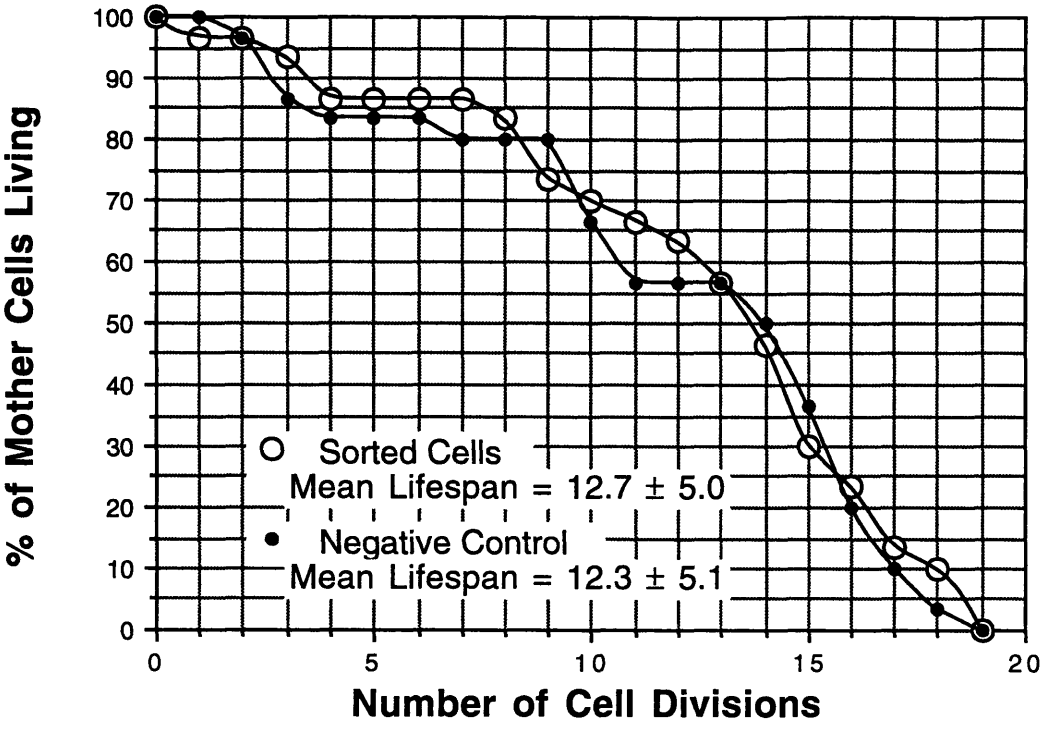
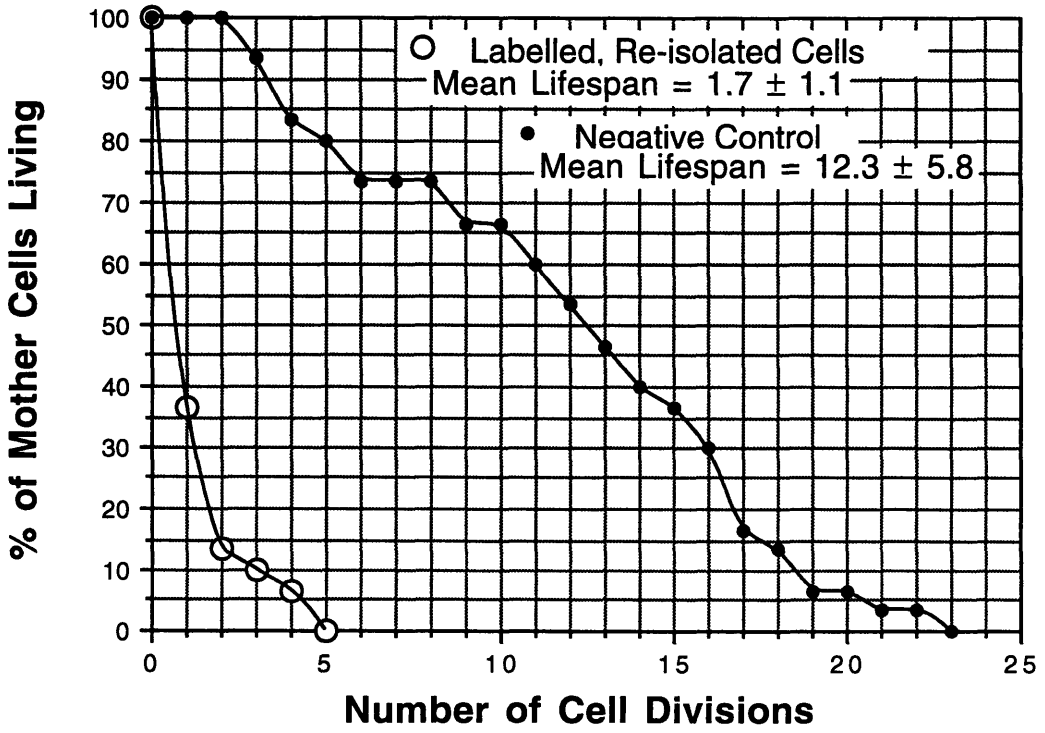


FIGURE 6: Labelled Cells Cultured for 20 Hours and Re-isolated Exhibit a Decreased Lifespan



number corresponds to 11.7 population doublings, so we expect the labelled cells to have already divided approximately 11 or 12 times prior to re-isolation and determination of life span. The life span data confirm this prediction -- the average life span for the re-isolated labeled cells was 1.7 cell divisions, while the negative control had a mean life span of 12.3 cell divisions (a difference of 10.6 cell divisions). The results of this experiment are shown in Figure 6.

Bud scars present on the cell surface are the only unambiguous indicators of age for an *S. cerevisiae* cell, so an examination of bud scars on sorted cells was carried out. A culture of *S. cerevisiae* was inoculated and allowed to grow up overnight. The exponentially growing culture was harvested the next morning, and a portion of the cells were stained with Calcafluor White and examined for bud scars under the fluorescence microscope. Theoretically, the fraction of an exponentially growing population having n bud scars should be $1/2^n$. In other words, 50% of the population have 0 bud scars, 25% have 1 bud scar, 12.5% have 2 bud scars, 6.25% have 3 bud scars, and so forth. The bud scar distribution observed for the sample of exponentially growing cells was very close to that predicted by theory. This is shown in Figure 7.

The remainder of the exponentially growing cells were fluorescently labeled by the biotinylation/avidinylation procedure and allowed to grow for 8 hours in rich media at 30 °C. Following this grow out, a portion of the culture was removed for analysis, while the remainder of the culture was allowed to continue growing. The portion withdrawn from the culture was sorted by FACS to give a 99% pure fluorescent population, which was then stained with Calcafluor White and examined for bud scars. The observed distribution shows that the sorted cells are of advanced age. Ignoring the cells with 0 bud scars, which were chiefly buds still attached to mother cells, one obtains a distribution which peaks at six bud scars. We have thus obtained a fairly pure population of cells with an average age of approximately six generations. This is shown in Figure 8.

FIGURE 7: Cells from Unlabelled, Exponentially-Growing Culture

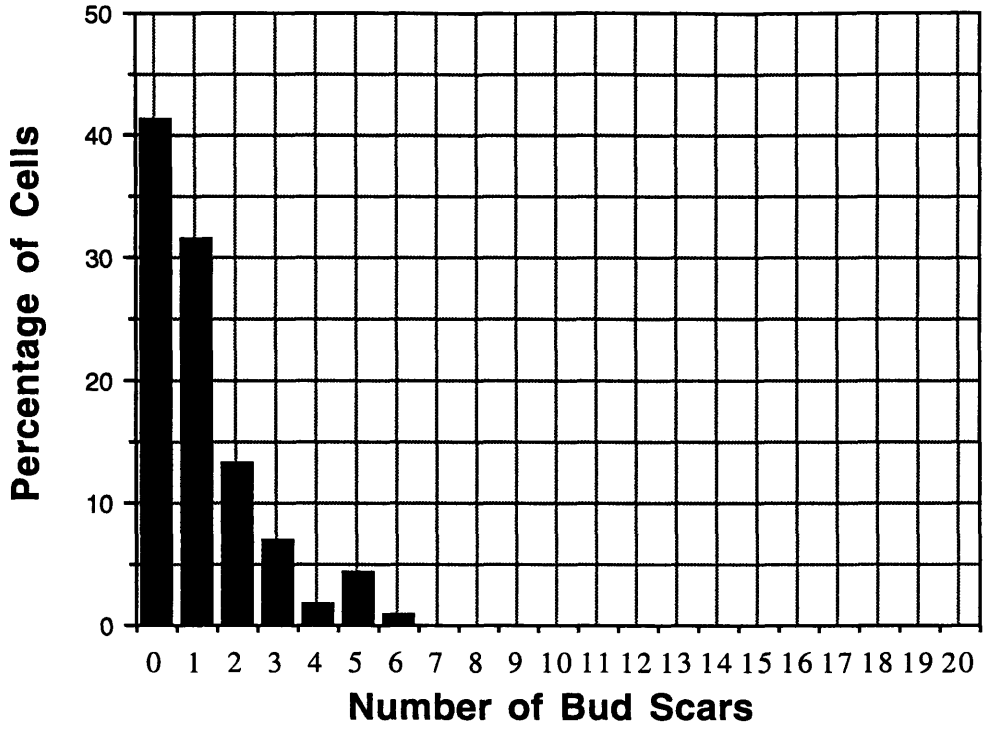
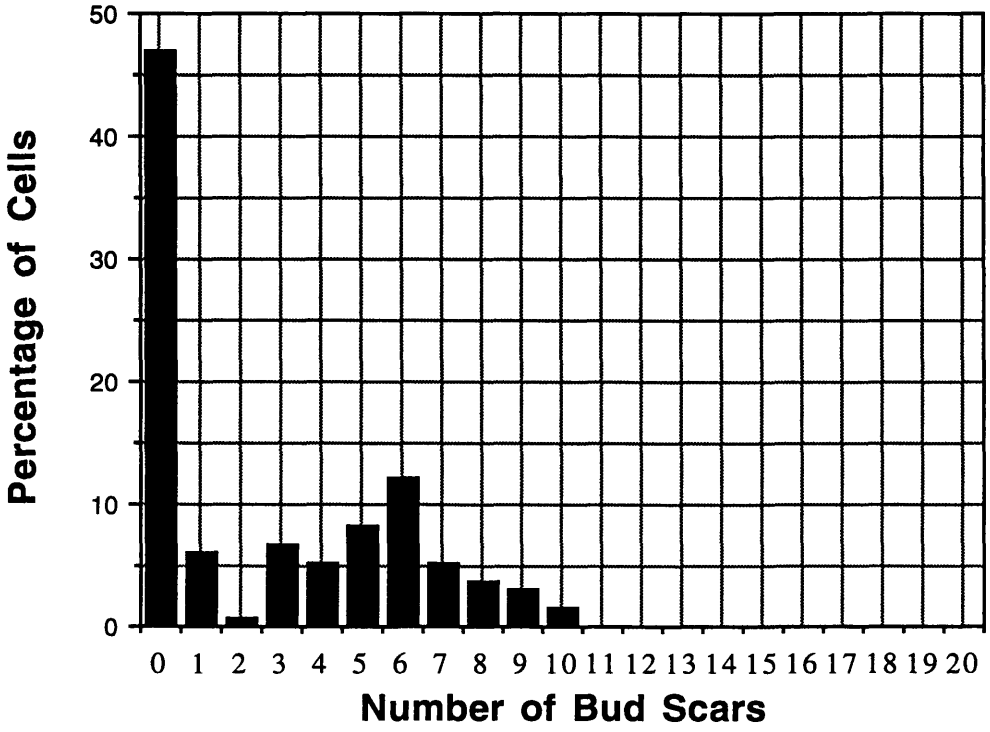


FIGURE 8: Labelled Cells Sorted From 8 Hour Culture



After a total of 18 hours, the cells still growing at 30 °C were divided into two aliquots. The first aliquot was sorted by FACS to give a 99% pure fluorescent population which was then stained with Calcafluor White and examined for bud scars. The second aliquot was simply stained with Calcafluor White and examined for bud scars. The sorted cells show a bud scar distribution centered around 11 bud scars, indicating that the cells grown for 18 hours are roughly 11 generations old on average. The unsorted cells show a distribution typical for an exponentially growing culture, as would be expected. These observations are shown in Figures 9 and 10.

TRIAL SORT FOR MUTAGENESIS: PURITY IS EXCELLENT

A possible application for FACS-based isolation of senescent *S. cerevisiae* is a screen for long-lived mutants, as follows. A population of virgin daughters obtained by rate zonal sedimentation[7] would be mutagenized and labeled by biotinylation/avidinylation. These cells would then be grown in rich media for a period of time such that virtually all cells retaining the longevity characteristics of the wild-type strain would have ceased division. The cell culture could then be sorted on a fluorescently-activated cell sorter (FACS) and the fluorescent population recovered at extremely high purity. Plating of the fluorescent fraction on solid media would allow any long-lived cells still possessing the capability for cell division to form colonies. Life spans of cells from each colony could then be determined using the micromanipulator and long-lived mutants identified.

To examine the time required for cells to reach mitotic arrest, and the purity obtainable in an actual mutagenesis, a trial sort was undertaken. A lightly-inoculated culture of BKY14C was allowed to grow in rich media for roughly 24 hours, at which point the exponentially-growing cells were harvested and labeled via the biotinylation/avidinylation protocol. Seven hundred labeled cells were plated on solid media, eventually giving rise to 442 colonies, an "efficiency of plating" (EOP) of 64%. The remainder of the labeled

[7]Sloat, B.F., Pringle, J.R. (1978) A Mutant of Yeast Defective in Cellular Morphogenesis. *Science* **200**:1171-1173

FIGURE 9: Labelled Cells Sorted From 18 Hour Culture

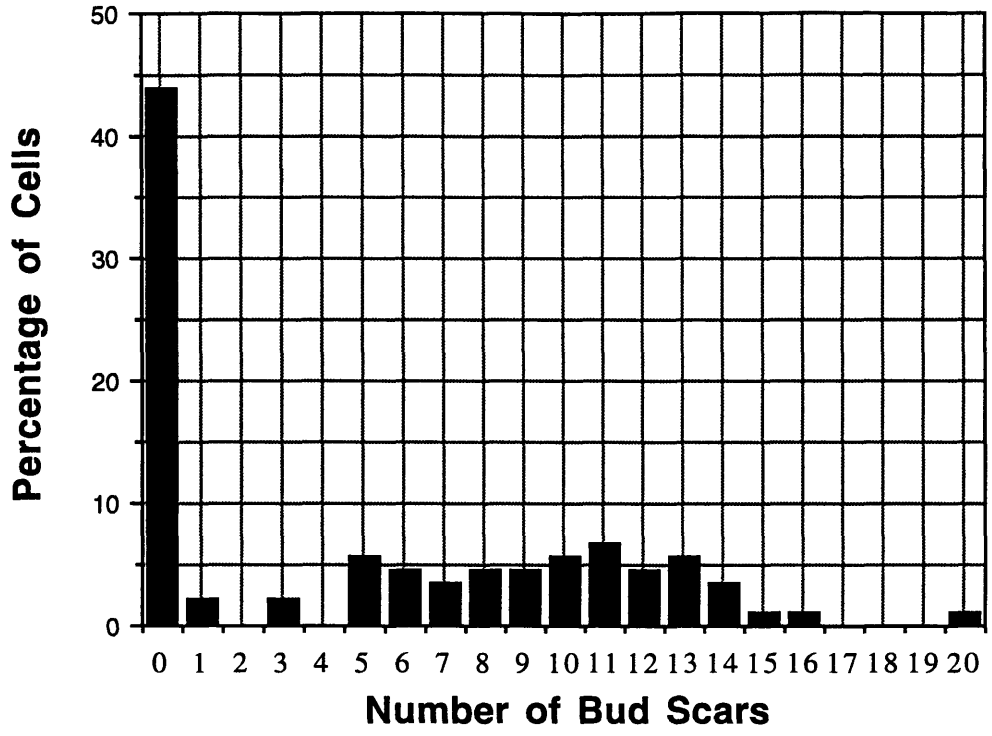
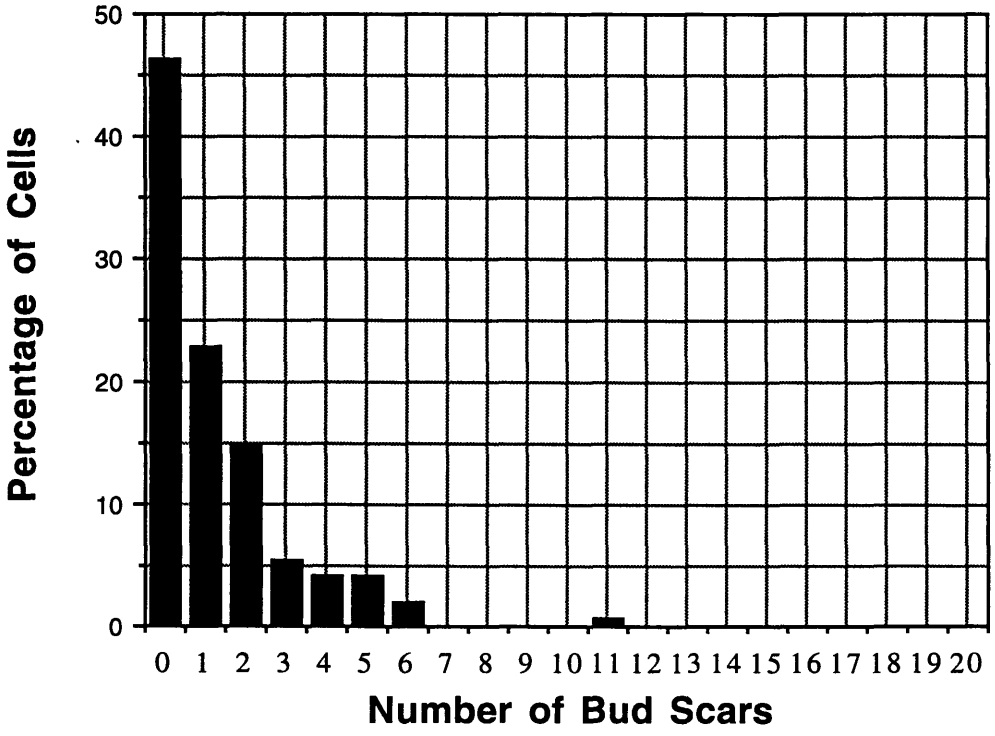


FIGURE 10: Cells from Unsorted 18 Hour Culture



cells were inoculated into rich liquid media and allowed to grow for 6 hours. Cells were then sorted on the FACS. An initial sort[8] enriched fluorescent cells from 19% to 63% of the total population, with 1.1 million fluorescent cells ("positives") sorted. A second sort enriched positives to 84% of the population, with 1.05 million positives sorted. At this point, fifty thousand cells were set aside and the remainder of the population was placed into rich media for further "grow-out". The fifty thousand cells were sorted two more times to achieve a purity of approximately ≥ 95%. Two hundred cells were then plated on solid media, eventually giving rise to 191 colonies, an EOP of 96%.

After eight hours of growth, the "fourteen hour" cell culture (founded by the fluorescent cells purified from the "six hour" culture) was sorted by FACS. An initial sort recorded 740,000 positives. A second sort enriched positives to 86% of the population, with 505,000 positives sorted. The purified cells were then placed into liquid media for another "grow out." No cells were plated for determination of EOP at this step.

After ten hours of growth, the resultant "twenty-four hour" culture (founded by fluorescent cells purified from the "fourteen hour" culture) was sorted by FACS. The first sort enriched positives from 1% of the population to 40%, with 340,000 positives sorted. A second sort enriched positives to 86% of the population, with 297,000 positives sorted. At this point 7,000 cells were set aside, and the remainder of the population was placed into rich media for further "grow out". The seven-thousand cells were sorted two more times to achieve a purity ≥ 95%. Seven-hundred cells were plated on solid media, eventually giving rise to 376 colonies, an EOP of 54%.

---

[8]The FACS may be configured to maximize purity (Normal-R mode) or to maximize recovery (Enrich mode). Figure 2 shows that it is possible to achieve a very high purity in a single sort using Normal-R mode and a relatively low flow rate. In preparatory cell sorts, the enrich mode was used to maximize recovery with a consequent decrease in the degree of purity obtainable in a single sort. Preparatory sorts were also generally run at very high flow rates (to minimize the time required for large sorts) and this also compromises the degree of purity obtainable in a single sort.

After ten hours of growth, the resultant "thirty-four hour" culture (founded by fluorescent cells purified from the "twenty-four hour" culture) was sorted by FACS. The first sort enriched positives from 1.3% of the population to 35%, with 317,000 positives sorted. An undetermined number of cells were lost due to the deflection plates not being turned on. A second sort enriched positives to roughly 85%, with 192,000 positives sorted. The purified cells were then placed into liquid media for another "grow out." No cells were plated for determination of EOP at this step.

After twelve hours of growth, the resultant "forty-six hour" culture (founded by fluorescent cells purified from the "thirty-four hour" culture) was sorted by FACS. The first sort enriched positives from 3% of the population to 46%, with 186,000 positives sorted. A second sort enriched positives to 95%, with 140,000 positives sorted. At this point 38,000 cells were set aside, and the remainder of the population was placed into rich media for further "grow out". The 38,000 cells were sorted five more times to achieve a purity $\geq$ 99.1%. 11,700 cells were plated on solid media, eventually giving rise to 379 colonies, an EOP of 3.2%.

After nineteen hours of growth, the resultant "sixty-five hour" culture (founded by fluorescent cells purified from the "forty-six hour" culture) was sorted by FACS. The first sort enriched positives from 1% of the population to 25%. A second sort enriched positives to 92%, with 80,000 positives sorted. Six more sorts yielded a purity of >99%. Four-thousand cells were plated onto solid media, eventually giving rise to 2 colonies, an EOP of 0.05%.

The sort can be summarized as follows. The fraction of small colonies, which either represent petites or simply indicate an age-related decrease in growth rate for the founding cell, is also noted. It should be noted here that previous experiments suggest that the percentage of petites do not increase with age[9].

[9]Unpublished results.

Total hours of growth time:	Number of cells isolated:	Efficiency of plating:	Fraction of small colonies (possibly petite)
0 hours	>1.1 million	64%	23%
6 hours	1.05 million	96%	32%
14 hours	505,000	n/a	n/a
24 hours	297,000	54%	49%
34 hours	192,000	n/a	n/a
46 hours	140,000	3.2%	56%
65 hours	70,000	0.05%	n/a

These results are interesting for a number of reasons:

First, the EOP data presents additional evidence that biotinylated-avidinylated cells undergo a relatively normal aging process. Cells which escape the rash of early deaths (EOP of 64% immediately after labeling) appear not to be adversely affected (EOP of 96% after 6 hours of growth). At the mean life span for a strain, it is expected that roughly half of the cell population will have entered mitotic arrest. Twenty-four hours of growth is roughly the time required for this strain to reach its mean life span, and at twenty-four hours only about 50% of the cells maintain the ability to form colonies. When determining life spans by micromanipulation for BKY14C, it is observed that very few cells are still dividing after 46 hours of growth, and the FACS sort is consistent with that observation.

Second, if we assume the worst and suppose that both colony-founding cells isolated at 65 hours were non-fluorescent young cells which slipped by the several FACS sorts, then we still have a purity of 99.95%, which should be more than sufficient to allow a screen for long-lived mutants to proceed with a very manageable number of false-positives.

CHAPTER THREE: MAGNETIC SORTING

BACKGROUND

It has been shown that fluorescent labeling coupled with FACS sorting is an effective means to isolate senescent cells. However, sorting large quantities of cells by FACS is a very time-consuming process. If positives are only 1% of a population, then the maximum effective sort rate of 10 million cells per hour yields only 100,000 positives per hour. Large scale isolations of 10^7 or more cells, as would be desirable for biochemical analysis, are impractical using the FACS method. For this reason, we sought a complementary method of isolation which would be practical at larger scales.

A magnetic cell sorter was obtained from Perseptive Diagnostics (Cambridge, MA) for evaluation. Whereas the FACS machine must sort each cell individually (albeit at a very high rate), the magnetic device sorts all cells simultaneously based on the presence or absence of a magnetic label. Essentially, a test-tube full of cells is placed beside a powerful magnet. Magnetically-labeled cells are quickly drawn to the side of the test tube, while unlabeled cells remain suspended or settle to the bottom. After 10 minutes, separation is complete and the supernatant solution can be withdrawn with a pipette, leaving the magnetically-labeled cells "pelleted" on the side of the test tube. Purity can be enhanced by placing fresh buffer in the test tube, vortexing, and allowing the separation to proceed again. The magnetic sorter has a capacity of roughly 10^8 cells, and thus a sort which requires 10 hours using the FACS can be accomplished in only 10 minutes with the magnetic sorter.

Streptavidin microbeads, consisting of a paramagnetic iron oxide core coated with streptavidin, were obtained from Perseptive Diagnostics for use as a magnetic label.

CRITERIA FOR EFFECTIVE SORTING

In order for magnetic sorting to be effective as a means of isolating

senescent *S. cerevisiae*, streptavidin beads must bind well to biotinylated cells, but must not cause significant toxicity in those cells. Furthermore, it must be possible to isolate magnetically-labeled cells at high purity. These criteria were tested prior to attempting an isolation of senescent cells.

MICROBEADS BIND BIOTINYLATED CELLS

Ten million cells of BKY14C were biotinylated and then incubated with streptavidin-iron oxide beads (100 beads per cell). The cells were suspended in 10 mL buffer, and 50 μL of this "initial suspension" were plated on rich media. The cell suspension was then placed in the magnetic sorter and allowed to stand for ten minutes. At that time the supernatant was withdrawn from the first test tube, placed in a clean test tube, and reinserted into the magnetic sorter. This was repeated a total of four times, during which the concentration of any non-magnetic cells should have remained unchanged. The final supernatant was then vortexed and 50 μL were plated on rich media. The results are as follows:

"initial suspension"	More than 5,000 colonies
"5th supernatant"	0 colonies

This experiment was repeated using 20 beads per cell with the following results:

"initial suspension"	1293 colonies
"5th supernatant"	0 colonies

Thus twenty beads per cell are sufficient to insure that essentially all cells are sufficiently labeled for effective sorting.

BEADS ARE NOT EXCESSIVELY TOXIC

Life spans were determined for 30 biotinylated/magnetically-labeled cells and 30 cells serving as a negative control. In addition, life spans were determined for 30 cells which were subjected to the entirety of

the biotinylation/magnetic-labeling protocol, but were not actually biotinylated or magnetically labeled. These data are shown in Figure 11.

As with fluorescent labeling, it appears that the stresses associated with buffer washes and centrifugations cause a number of early deaths, but the pattern of death due to aging is not significantly affected in magnetically-labeled cells.

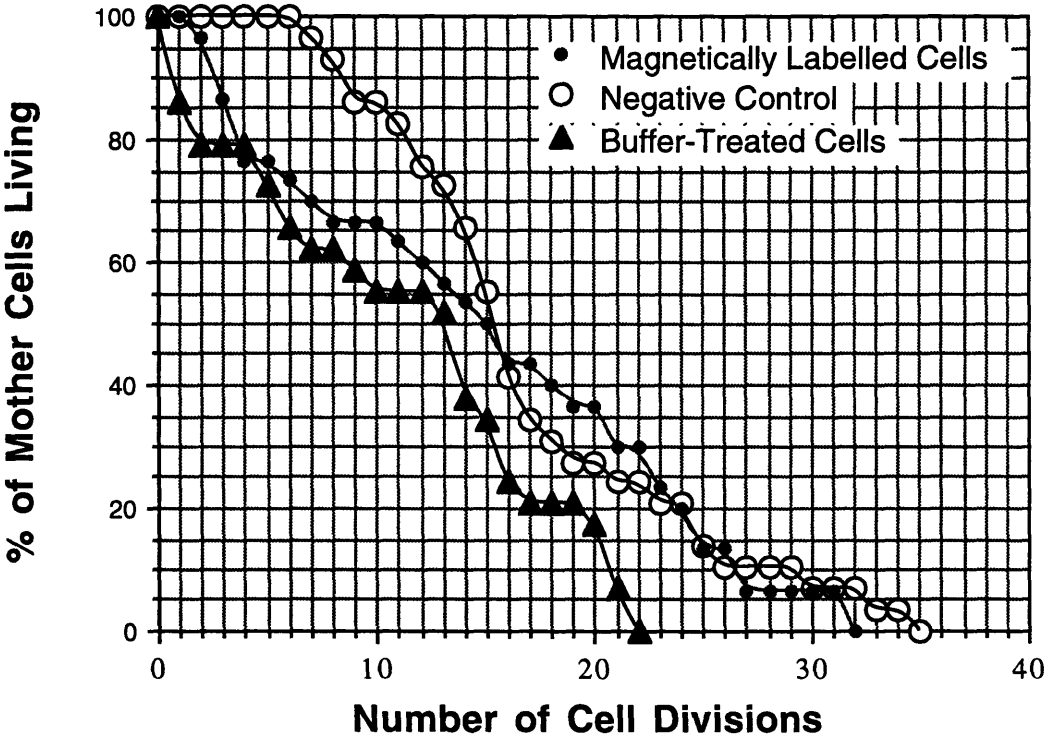
MAGNETIC SORTING YIELDS HIGH PURITY

Approximately 10 million cells of BKY14C were biotinylated and magnetically labeled by incubation with streptavidin beads (100 beads per cell). A large quantity of exponentially growing PSY142 cells were then added to the suspension. Aliquots of this mixture were plated onto both YPD plates (which support the growth of both BKY14C and PSY142) and adenine "drop-out" plates (which support the growth of PSY142 only). The cell suspension was then sorted six times with the magnetic sorter, each time taking the "magnetic pellet" of cells. The final pellet was resuspended and again plated on both types of plates. Results were as follows:

Name of cell suspension	Number of colonies on YPD plates	Number of colonies on "drop-out" plates	Apparent percentage of BKY14C
unsorted	133	102	23%
sixth sort	greater than 1000	0	greater than 99.9%

This experiment was repeated with cells labeled with 20 beads/cell. Purity was assessed after the first sort and after the fourth sort.

Figure 11: Effect of BioMag Labelling on Lifespan in Strain BKY5



Name of cell suspension	Number of colonies on YPD plates	Number of colonies on "drop-out" plates	Apparent percentage of BKY14C
unsorted	680	540	21%
first sort	178	24	87%
fourth sort	greater than 1000	0	greater than 99.9%

These data indicate that under optimal conditions, very high purity is possible with only a few sorts.

TRIAL SORTS

Initially we attempted to follow a protocol analogous to that used with fluorescent labeling, assuming that magnetic label would be retained by cells over the course of many generations of growth. Magnetically labeled cells were placed into rich media at 30 °C and allowed to grow for a period of time. A series of magnetic sorts was then carried out, and the purified magnetic fraction was stained with Calcafluor and examined for bud scars. Yield was found to be very low in this case and the small isolated population did not appear significantly advanced in age.

We then modified our procedure as follows. A population of biotinylated cells was allowed to grow for a number of hours at 30 °C. The entire culture was then spun down, resuspended in buffer, and incubated with paramagnetic streptavidin beads. The suspension was then sorted magnetically several times, and an aliquot of cells from the purified magnetic fraction was stained with Calcafluor and examined for bud scars. Yield was found to be very good and the isolated population appeared to be significantly advanced in age. The magnetic fraction was then placed into fresh media and allowed to grow again at 30 °C. After a period of time, the resulting culture was spun down and

sorted magnetically. Yield was found to be very low and the small isolated population did not appear significantly advanced in age.

From these two experiments it appears that the paramagnetic beads are not retained well by labeled cells over the course of several cell divisions. However, the FACS experiments show that the biotinylated components of the cell surface do persist over many generations of growth. To the extent that biotinylated sites are still available after an initial incubation with paramagnetic beads, or to the extent that biotinylated sites open up when beads are lost from the cell surface during the course of growth, a second incubation with paramagnetic beads should be effective in relabeling the biotinylated cells.

To examine this possibility, an exponentially growing culture of PSY142 was biotinylated via the standard protocol and 8.3 x 10^6 cells were inoculated into 500 mL of YPD and allowed to grow at 30 °C for 15 hours. At that time the cell density of the culture was determined to be 2,900 times the density of the inoculum, corresponding to 11-12 generations if one assumes that all cells divide at the same rate. The culture was spun down, resuspended in 10 mL 1x PBS, and incubated on ice with 50 beads/biotinylated cell for 2.5 hours, swirling every 15 minutes. Cells were sorted 9 times in 10 mL volumes of YPD. Nine mL of final sort were re-inoculated into 520 mL fresh YPD, and one mL was stained with Calcafluor to examine bud scars and count cells. The distribution of bud scars for the 2.0 x 10^7 cells isolated is shown in Figure 12. The distribution peaks around 12 bud scars, in good agreement with the value expected from the total increase in cell number.

After 15.5 hours, the culture inoculated with purified cells from the first sort was harvested and the cell density determined. The 1300-fold increase in cell density corresponds to 10-11 generations. Cells were spun down, washed with water, and resuspended in 10 mL 1x PBS on ice. Cell suspension was sonicated, and then incubated 3 hours with 125% of the amount of paramagnetic beads as were used in the first set of magnetic sorts. Cells were then sorted 9 times in 10

FIGURE 12: Labelled Cells Sorted From 15 Hour Culture (Magnetic Sort)

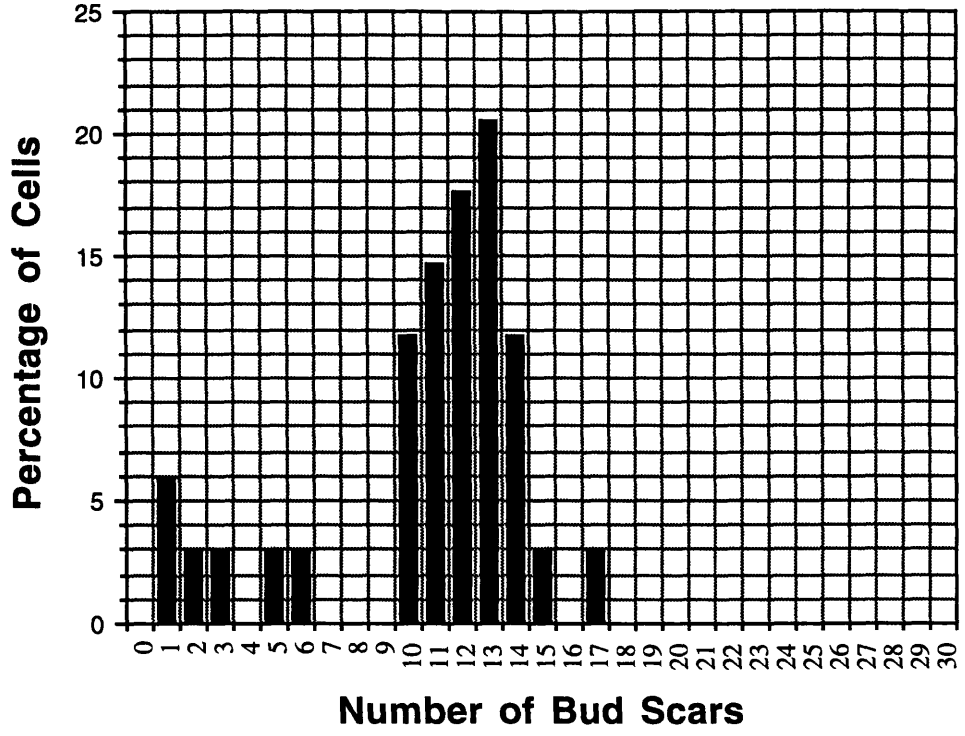
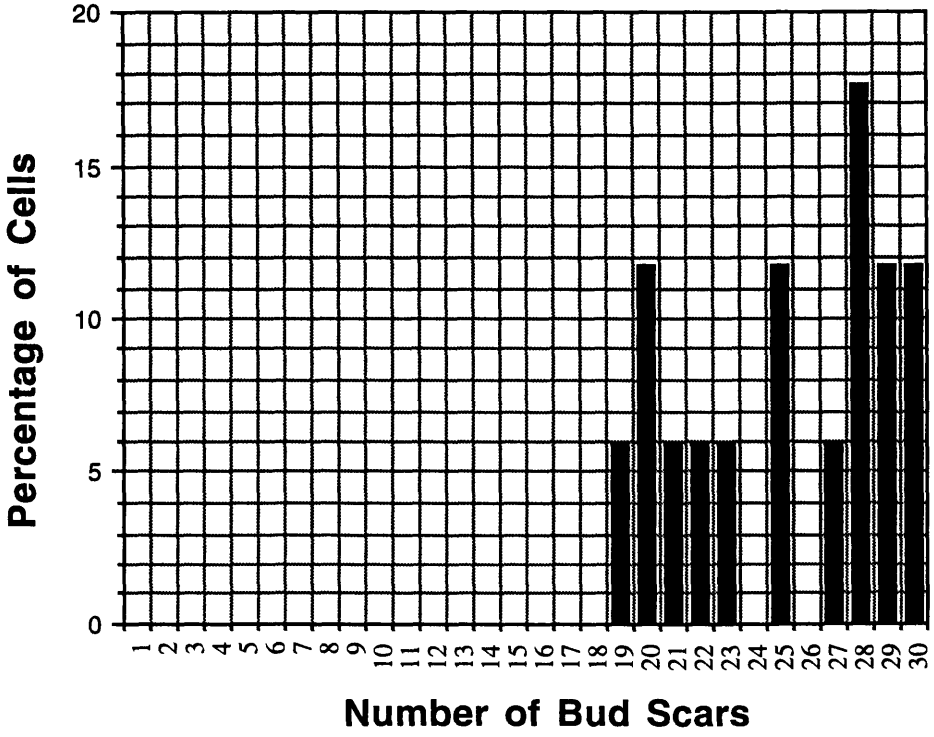


FIGURE 13: Labelled Cells Sorted From 30 Hour Culture (Magnetic Sort)



mL volumes of YPD. One tenth of the cells were stained and examined for bud scars. Total yield of sorted cells was 1.2×10^7, and the bud scar distribution was as shown in Figure 13.

Clearly the cells are quite pure and highly advanced in age. With so many bud scars on the cell surface it was difficult to be certain that bud scar counts were completely accurate. The microscope must be focused first on the front of the cell, and then on the back of the cell, with bud scars on the sides of the cell being particularly difficult to count accurately. The counts presented should be accurate to within about ±2 bud scars, however. Figure 14 shows the many bud scars present on the cell surface of isolated cells.

This sort has been repeated a number of times, verifying the reliability of the method. Yields and purity have varied to some degree, but the protocol stands as an effective way to isolate a large quantity of senescent cells.

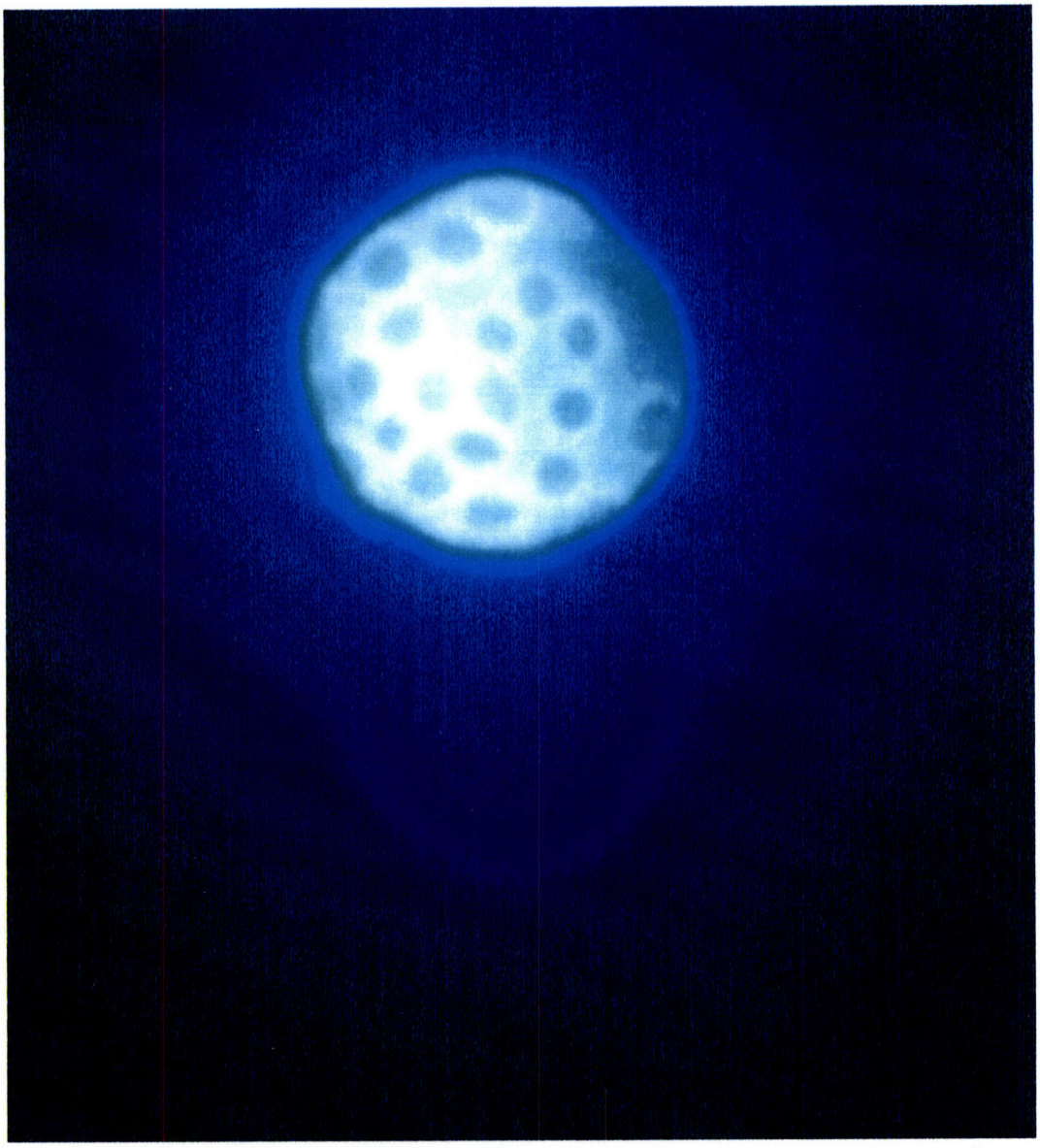
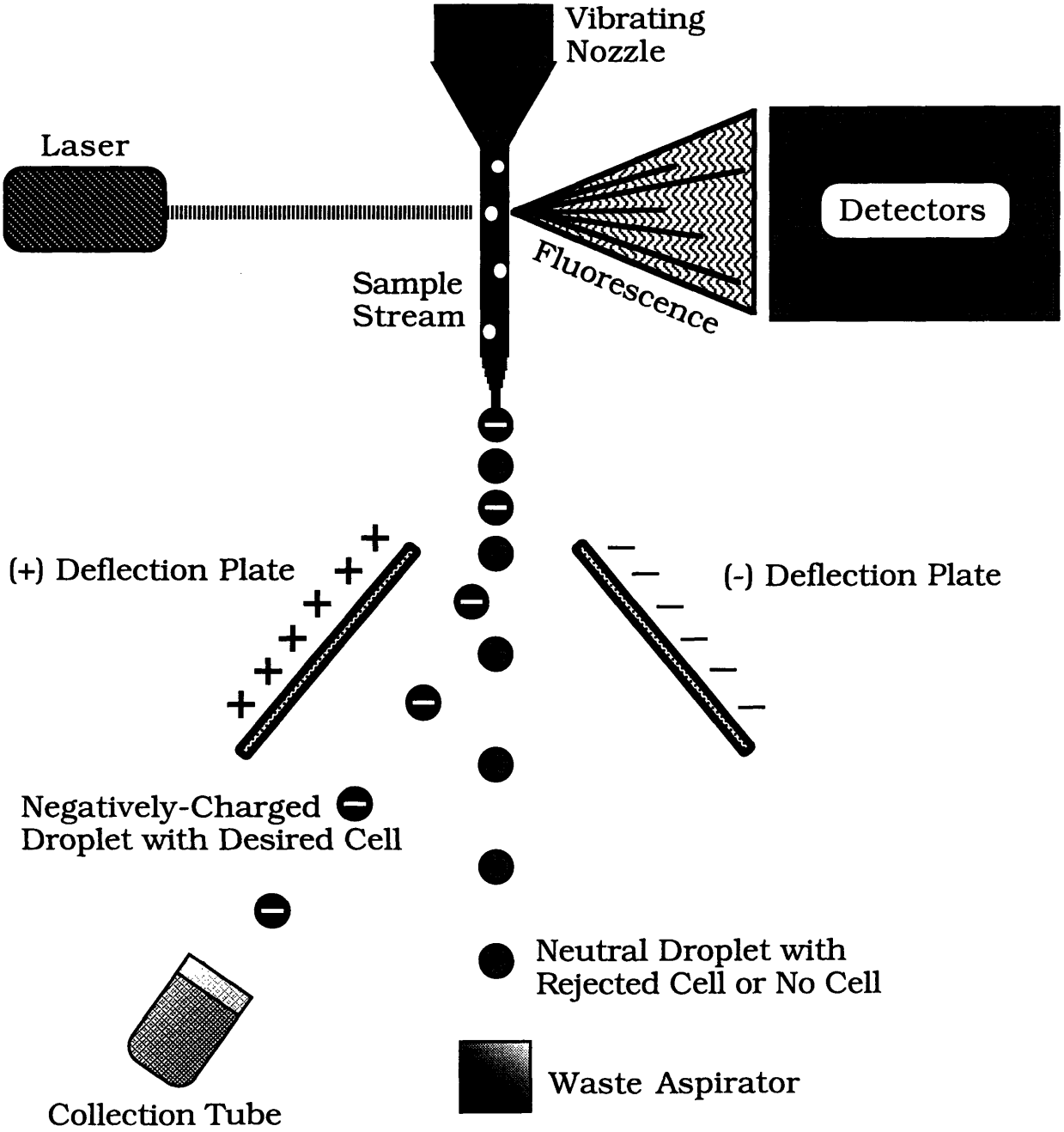
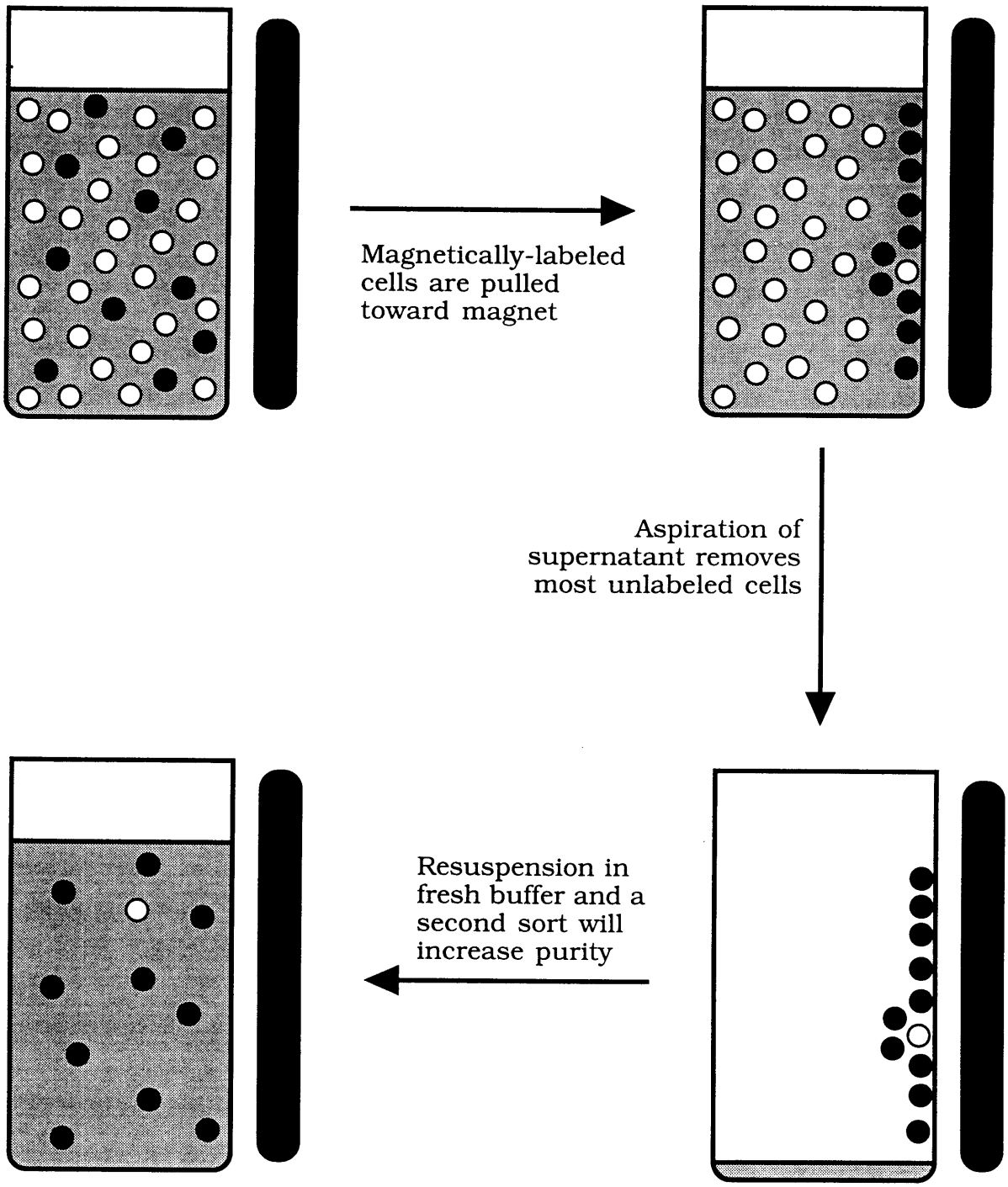


FIGURE 14: Bud scars present on the surface of a typical PSY142 cell isolated after 30 hours of growth in rich media. Additional bud scars would be made visible by focusing on the back surface of the cell.

APPENDIX 1: The fluorescently-activated cell sorter (FACS). Vibration of the nozzle at a tunable frequency causes the sample stream to break into droplets at a well-defined distance from the nozzle. Detectors monitor the magnitude of fluorescence produced by laser interrogation of cells passing by in the sample stream. When the fluorescence of a cell is within the desired range, a signal is sent from the detectors which causes the entire sample stream to be electrically charged at the precise moment the droplet containing the desired cell breaks away from the sample stream. Thus droplets containing desired cells carry a positive or negative static charge, while droplets containing undesired cells or no cells are electrically neutral. Droplets are then separated by passage through a large potential gradient arising from highly-charged "deflection plates".



APPENDIX 2: The magnetic cell sorter. A test tube containing both unlabeled and magnetically-labeled cells is placed in the cell sorter. A strong permanent magnet pulls labelled cells to the side of the test tube, while unlabeled cells remain suspended. Aspiration of the supernatant removes most unlabeled cells. The sort may be repeated to further purify the magnetically-labeled cells.



APPENDIX 3: Summary instructions for setting up the Becton-Dickinson FACS Vantage for cell sorting or analysis.

1. Computer power, then main power.
2. Printer power.
3. Fill sheath fluid.

    *Release pressure (pull up)
    *Be careful of tube on top of filter
    *Fill only to top weld
    *Put in long end of lid first

4. Empty waste fluid.
5. Turn on pressure switch.
6. Turn on stream lamps.
7. Turn to standby, rock between "standby" and "fill".

    *Check for air bubbles in nozzle with light.

8. Clean saline off nozzle with Q-tip.
9. Turn to "run", check for backdrip.

    *If no backdrip, clean out needle.

10. Hit green swith to turn on heat exchanger for laser cooling.

    *STEPS 10-11 MUST BE DONE IN PROPER ORDER!

11. Turn on "light switch" to pump cooling water through heat exchanger.
12. Turn laser on.
13. Turn on strobe light and drop drive.
14. Hit "Pulse/Sort" to see DDF (drop drive frequency).

    *Adjust DDF to 23,5oo

15. Turn on deflection plates (center first).
16. Turn on water jacket cooler, pump low.
17. Move camera to put viewing reference mark on laser intercept.
18. Move camera down 196 marks, and adjust phase and amplitude to get proper droplet breakoff.
19. Let run for long time to stabilize (> 0.5 hours).

APPENDIX 4: Summary instructions for Becton-Dickinson FACS Vantage alignment/ calibration and for FACS Vantage "shut down".

On computer:

1. "MasterPage" comes up automatically.
2. Click on "Lysis II".
3. Under LYS menu, select ACQ (acquisition).

    *Click OK for FACSVantage(SEM)

4. Under BD logo menu, go to "File Utilities".

    *Pull down on "MAST" at top of window that appears
    *Select #11

5. Double click on "0", click "OK".
6. Under BD logo menu, play Mousetracks (SUMT=SetUp Mousetracks).
7. Under Instrument Control menu, select "Get Instrument Settings".

    *Select "BEADSSL" (Beads Single Laser)

Alignment:

1. Press 1,3 then the "Horizontal/Vertical" button on the oscilloscope.
2. Put beads in tube, and switch flow control to "run".
3. Boost pressure until beads are visible.

    *Turn down sample differential => 50-100 beads/second

4. Adjust "Y-axis" and "Excitation Beam Focus" for highest signal.
5. Restrict iris to bring bead signal to center of oscilloscope.
6. Adjust "X-axis" to get tightest grouping and highest signal.
7. Adjust "Fluorescent Channel Height" to get highest signal.
8. Adjust "Fluorescent Channel Lens Focus" (VERY SENSITIVE) to get highest signal.
9. Open iris again.

Now adjust mirrors:

1. Press 1,2 Horizontal/Vertical.

*Move first mirror side to side to optimize signal.

2. Press 1,7 Horizontal/Vertical

*Move second mirro side to side to optimize signal.

3. Press 1,4 Horizontal/Vertical

*Move third mirror side to side to optimize signal.

4. Press 1,3 Horizontal/Vertical

*Close iris to where signal just begins to drop.

5. On computer, click "GO" and check CV's for each parameter.

*CV's should be ≤ 1 at half-height.

6. To select for proper drop delay, set up histogram region which excludes doublets, and load to computer as left sort window. Use "test mode" and "test sort" to check sort stream trajectories. Use counter mode to check recovery of beads using different drop delays. Choose drop delay which gives best recovery.

FACSVantage ShutDown

1. Press "ON/OFF" button on laser remote (hear "CLUNK").
2. Turn flow control to "RUN" position, and let drip for 10 minutes.
3. After 10 minutes, turn flow control to "FILL" position, and press "NOZZLE FLUSH" for 10 seconds. While continuing to press "NF", turn flow control to "OFF".
4. Turn pressure off. (Hear air leaving tank).
5. Turn off "light switch" to stop cooling flow to heat exchanger.
6. Turn off heat exchanger.
7. On computer, go to ACQ menu and choose ANALYSIS.
8. Under BD logo menu, choose quit.
9. Shut off main and computer power.
10. Turn off water bath.

APPENDIX 5: EXPERIMENTAL PROTOCOLS

BIOTINYLATION[1]

1. Scrape an *S. cerevisiae* colony from a plate and suspend in 1 mL of water. Withdraw 10 µL of the initial suspension and suspend in 1 mL of water (call this the "100x suspension"). Withdraw 10 µL of the 100x supension and suspend in 1 mL of water (call this the "1x suspension").

2. Prepare 4 sterile test tubes with 3 mL aliquots of YPD. Inoculate the test tubes with 100 µL of 100x suspension, 10 µL of 100x suspension, 100 µL of 1x suspension, and 10 µL of 1x suspension. Place at 30 °C on a roller.

3. After 16-24 hours growth, pick a tube that has an OD_{600} of 0.5 or less (by eye). Total number of cells[2] should be about 1-6 x 10^7. Split into two Eppendorf tubes and spin down on the microfuge for 8 to 10 seconds at maximum speed (start counting when you press the button and release the button when you reach 10). Dump the supernatant and add 0.5 mL ice cold 1x PBS to each tube, vortexing to resuspend. Combine the tubes and wash one more time with ice cold 1x PBS.

4. After dumping the supernatant from the second wash, draw 1 mL room temperature 10x PBS into a pipetman. Add roughly 1/3 of the volume to the pelleted cells and resuspend by vortexing. Uncap the tube containing the cell suspension and place it in a rack. Add the other 2/3 volume to 3.5 mg NHSLC biotin (Pierce) which has been pre-weighed in an Eppendorf tube. Dissolve the NHSLC biotin by vortexing and immediately add the cell suspension and vortex again to mix well. Tape the tube to a vortexer and shake at low speed for 15 minutes at room temperature.

5. Spin down the cells as before and wash with 1 mL ice cold 1x PBS. Repeat a total of eight times.[3]

<u>10x PBS</u>
19.9 g KH_2PO_4
2.84 g Na_2HPO_4
Add water to make 450 mL

---

[1]Procedure is a modified version of the methods of Goodloe-Holland and Luna, *Experimental Cell Research* **28**, 1987.
[2]Compared to Goodloe-Holland and Luna, this protocol uses a large excess of NHSLC biotin. When scaling this procedure up, it may not be necessary to scale up the amount of NHSLC biotin to the same degree.
[3]Fewer washes may be adequate. Also, washes could presumably be done with YPD. If viability is a problem, the effects of such modifications could be investigated.

Adjust pH to 8.0 with NaOH
Add water to give 500 mL final volume

AVIDIN-FLUORESCEIN LABELLING

1. Suspend up to 10^8 biotinylated cells in 1x PBS, and add 50 µL of fluorescein-conjugated avidin (Pierce, 5 mg/mL), vortexing briefly to mix.

2. Incubate for 10 minutes on ice, covered with foil to prevent possible bleaching.

3. Wash 2x with 1 mL aliquots of 1x PBS.

AVIDIN-PARAMAGNETIC BEAD LABELLING

1. Spin down biotinylated cells and their descendants on Sorvall (5 minutes at 3000 rpm). Wash once with water.

2. Resuspend cells in 10 mL ice-cold 1x PBS. Sonicate 30 seconds at 20% power (5s sonication, 5s on ice, 5s sonication, etc).

3. Add 50 streptavidin paramagnetic beads (Perseptive Diagnostics[4], Cambridge) per biotinylated cell and swirl to mix well.

4. Place cells on ice in 4 °C room. Swirl once every 15 minutes for 2 hours.

MAGNETIC SORTING

1. Place magnetically labelled cell suspension in test tube and place test tube in magnetic sorter in 4 °C room. Wait 15 minutes.

2. Aspirate supernatant with 10 mL pipette. Do not simply shove the pipette to the bottom of the test tube and then begin suction, as this will disturb the fluid level in the test tube and perhaps the magnetic pellet as well. Instead, place the tip of the pipette at the surface of the liquid and gradually lower the pipette as the liquid level drops. As the last bit of liquid is withdrawn a portion of the magnetic pellet tends to slough to the bottom of the tube; be careful not to aspirate any of the magnetic pellet.

---

[4]Perseptive Diagnostics was formerly known as Advanced Magnetics. In 1994 PD's "new and improved" cell sorting grade streptavidin beads were tried and found not to work at all. The older grade of streptavidin beads generally worked well, but may have been phased out of production.

3. Add 10 mL ice-cold YPD to test tube and vortex to mix. Place in magnetic sorter for ten minutes and repeat aspiration procedure.

4. Nine or ten sorts were usually performed when sorting cells of advanced age for final isolation.