Biomed Microdevices (2010) 12:1051–1059
DOI 10.1007/s10544-010-9459-5

Microfluidic sedimentation cytometer for milk quality and bovine mastitis monitoring

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Published online: 3 August 2010

Abstract We report a rapid, low-cost, portable microfluidic sedimentation cytometer (SeCy) for assessing the somatic cell count and fat content of milk in 15 min using a "sample-in, answer-out" approach. The system consists of 12 independent microfluidic devices, essentially flattened funnel structures, fabricated on the footprint of a single plastic compact disc (CD). Each funnel structure holds 150 μL of milk, has an inlet for milk filling and an outlet for air to escape, and ends in a narrow, closed-end microfluidic channel that facilitates packing of the cells into a column whose length is proportional to cell count. The closed-end channel provides accurate cell counts over the range 50,000–>3,000,000 cells per mL. The assay separates cells and fat globules based on their densities (by differential sedimentation), concentrating white cells in the closed-end channel near the outer rim of the CD for estimation of total "cell pellet" volume, while fat globules move toward the center of disc rotation, forming a fat "band" in the funnel. After adding milk to two or more microfluidic devices, the CD is loaded onto a custom-built reader unit that spins the disc for 15 min. Two low-cost microscopes in the reader image the centrifuged cell pellet and the fat band, providing a sufficiently accurate cell count to diagnose mastitis and measuring fat content as an indication of health and nutritional status.

Keywords Bovine mastitis · Milk · Cytometer · Centrifugal microfluidics · Sedimentation · Cell-counter · Somatic cells · Sample-to-answer device

1 Introduction

Bovine mastitis (BM) affects dairy cattle worldwide and causes annual loses of US$2 billion to the dairy industry in the US alone (Viguier et al. 2009). BM affects the composition of milk, reduces milk yield, and decreases milk quality and price (Hillerton et al. 2005). The costs of drug treatments, veterinary services, and labor, as well as milk buyers' penalties, are part of the economic losses due to mastitis (Huijps et al. 2008). Prompt diagnosis of infection at early stages can help improve milk production, prevent permanent harm to the cow, decrease the effective costs of BM, and reduce the widespread propagation of the infection to the rest of the herd and consequent culling, resulting in economic benefit to farmers and the general public (Hillerton et al. 2005; Huijps et al. 2008; Viguier et al. 2009).

BM is caused by several pathogenic organisms, most commonly bacteria, and results in the inflammation of the (milk-producing) mammary gland (Hillerton et al. 2005; Viguier et al. 2009). The gold standard in diagnosing BM is microbiological testing, which accurately identifies the afflicting pathogen in most cases; such tests are lengthy and costly, however, and therefore not feasible to implement as a routine screening test for mastitis. Other, simpler screening tests exist to determine the presence of an infection, such as measuring the electrical conductivity of

Electronic supplementary material The online version of this article (doi:10.1007/s10544-010-9459-5) contains supplementary material, which is available to authorized users.

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milk, the viscosity change on addition of detergent (*e.g.* California Mastitis Test), pH changes, or enzyme assays (e.g. NAGase, LDH, and esterase-catalysed enzymatic reactions), but these tests suffer from limited reliability (Pyorala 2003; Viguier et al. 2009).

Without identifying the causative pathogen, determining the number of somatic cells present in milk has become the gold-standard test to assess the potential presence of an infection (Pyorala 2003). Somatic cell counts are used to assess the quality and therefore the monetary value of the milk. In addition, somatic cell tests are an important component of udder health management for dairy producers (Schukken et al. 2003). For these health-management programs to be successful, somatic cell tests should be implemented on a frequent basis and on-site (Leslie et al. 2002).

Fat (cream) content is used mainly as a measure of milk's monetary value (Forcato et al. 2005), and is an indicator of nutritional status as well (Ashes et al. 1997), providing farmers with a potential decision point for nutritional supplementation. Milk with low fat content can indicate the existence of health problems with the animal (Forcato et al. 2005). It has also recently been observed that high fat: protein ratios are associated with increased risk of mastitis (Windig et al. 2005).

Current reliable methods to measure somatic cell counts (SCC) include direct microscopy, flow cytometry, and electronic particle detection (using a Coulter counter) (Moon et al. 2007). Flow cytometers are one of the most widely employed SCC test systems because of their high throughput and accuracy; their use has been standardized and recommended by international dairy organizations (Gonzalo et al. 2004). However, flow cytometers are expensive to purchase (>US$50,000) and to operate and maintain, precluding small farmers from adopting them for routine monitoring of herd health status. Typically, small farmers in developed countries send milk samples to government or centralized private laboratories to test the milk for somatic cell counts, along with other tests. This is less than ideal, as transportation of milk across long distances in non-refrigerated trucks, or delays in analyzing the samples, can affect the composition of milk, leading to inaccurate test results. Misplaced or mislabeled samples are also a recurrent problem (Berry et al. 2003). In developing countries, the main problem is the nonexistence of such laboratories. In both developed and developing countries, farmers would benefit greatly from an affordable, robust, easy-to-use "cow-side" test enabling regular monitoring of the herd's milk quality and incidence of mastitis. Several microfluidic systems have focused on miniaturization of the functionality of flow cytometers or electronic cell counters in hopes of reducing costs, automating manual tasks, and conferring portability, while also seeking to enable new discoveries and the processing of smaller sample volumes (Chung and Kim 2007; Ateya et al. 2008).

Although there have been successful demonstrations of miniaturized cytometers, providing similar sensitivity and quality of information to their benchtop counterparts, issues such as sample manipulation and automation are only beginning to be addressed (Huh et al. 2005; Chung and Kim 2007; Ateya et al. 2008). Current "micro-cytometers" suffer from: (a) elaborate microfabrication process sequences, some of which are not amenable to low-cost manufacturing (Huh et al. 2005; Chung and Kim 2007; Ateya et al. 2008); (b) a requirement for very precise flow control to create a stable sheath flow (Ateya et al. 2008); (c) clogging or fouling of microchannels (Ateya et al. 2008); (d) malfunctioning caused by air bubbles (Chung and Kim 2007); (e) need for multiple buffers and reagents for flow control and staining of cells (Huh et al. 2005; Chung and Kim 2007; Ateya et al. 2008); and (f) complexity of operation necessitating significant operator training (Moon et al. 2007). High-performance optics are commonplace in some commercial microfluidic flow cytometers, adding to cost and complexity (Moon et al. 2007; Ateya et al. 2008).

Recently, Toner and colleagues (Cheng et al. 2007a,b, 2009) reported innovative approaches for cell enumeration that addressed some of the shortcomings listed above: no reagents required, easy-to-use, low-cost fabrication, portability, and minimum instrumentation. They demonstrated the detection and count of CD4+ lymphocytes from whole blood using a combination of immunoaffinity and controlled-shear stress to selectively capture CD4+ cells (Cheng et al. 2007a,b, 2009) while preventing the capture of other types of leukocytes. They also demonstrated that captured CD4+ cells can be quantified using impedance spectroscopy by lysing the cells, which alters the bulk conductance of the solution (Cheng et al. 2007a,b). In another approach, Austin and colleagues demonstrated that cells can be efficiently sorted into different compartments—where presumably they could be counted—based on their sizes, using an array of microposts (Davis et al. 2006; Inglis et al. 2008). These innovative approaches demonstrate that miniaturization of bench-top instrumentation is not invariably the best strategy to address specific assays requiring cell counting and detection; rather, new approaches can exploit a range of bio/chemical/physical phenomena on the micro scale and at liquid/solid interfaces, leading to simpler, more economical, more practical solutions.

We report here a portable point-of-care (POC) microfluidic sedimentation cytometer (SeCy) to assess the numbers of somatic cells and the percentage of fat in milk; see Fig. 1. Rather than counting cells one by one, our approach is to centrifuge the somatic cells into a closed-end microfluidic channel, forming a packed pellet of cells

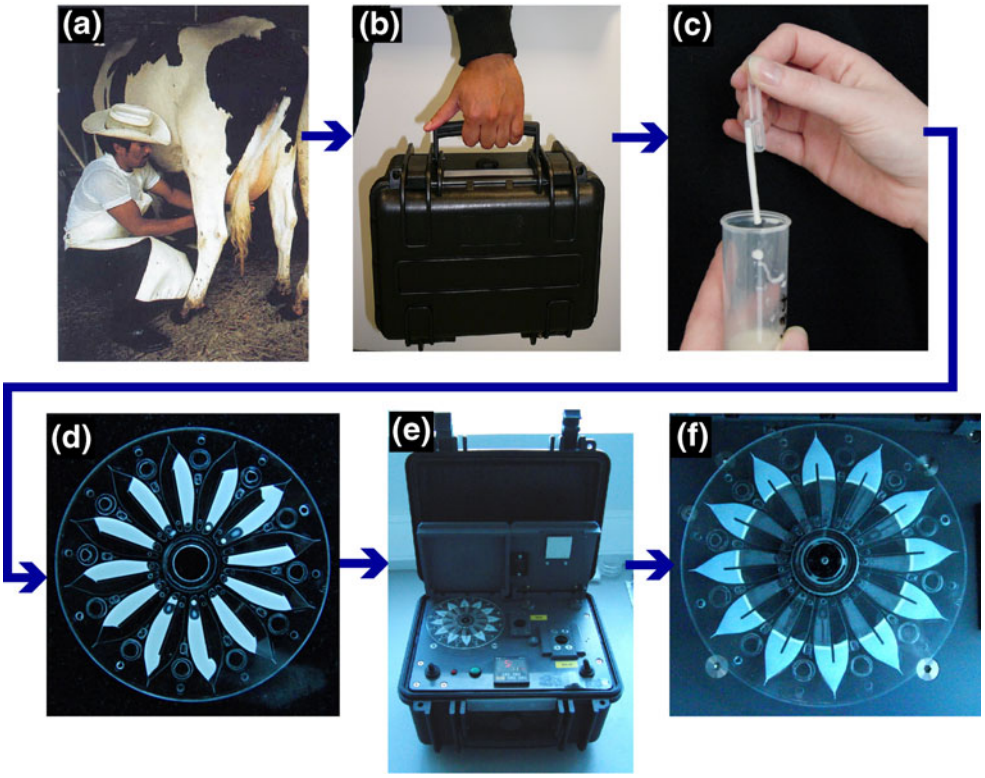

Fig. 1 General steps to operate point-of-care sedimentation cytometer. The process starts with a farmer milking a cow (**a**) and bringing the portable reader unit into the farm (**b**). The farmer then draws 150 μL of raw milk into a single-volume pipette (**c**). Next, up to 12 different milk samples can be dispensed into the microfluidic sedimentation cytometer unit (**d**). The disc is then loaded into the portable reader unit (**e**) which spins the disc for 15 min and analyses cell pellet size and cream band width (**f**)

whose volume is proportional to cell count. The device exploits the difference in cell and fat density from the rest of the milk components to separate them by centrifugation, forming the pellet and a fat band in opposite radial directions for convenient measurement.

The fluidic device has the same dimensions as a compact disc (CD) and allows for up to 12 tests to be performed at once; it is easy to use and handle, requiring no training other than learning to dispense 150 μL of raw milk into each of the 12 SeCy's using a single-volume pipette. Unlike other cell-counting techniques, this assay does not require any additional reagents or sample preparation. Completing the assay requires 15 min, at the end of which a reading of the cell count as well as the percentage of fat are made. Made of transparent polymer, the device is amenable to mass production, e.g. via injection molding. We also present a prototype portable reader unit to automate measurements, suitable for cow-side use; it includes two motors and two miniature microscopes (see Supporting Information for a description of the reader).

2 Device design

2.1 General concept of separation and analysis

Cells and fat globules can be separated from the rest of a sample of milk by centrifugal acceleration in a rotating CD platform because their densities and volumes differ substantially from the rest of the milk. The volumes of the cell pellet and the cream band formed after centrifugation can then be correlated with the number of cells and fat percentage of the milk, respectively. The cell pellet volume is more easily quantified by localization in a comparatively long and narrow closed-end channel, but there is sufficient fat to simply measure the width of the band of cream that forms.

A number of constraints were placed on the design of the SeCY disc, the most relevant fundamental parameters being the properties of milk, cells, and fat globules; however, we also considered the smallest volume of milk reasonably handled by a farmer with a pipette, the maximum number of individual SeCY's that could fit on the footprint of a CD, and a desire to operate the disc using a conventional CD player motor.

2.2 Number of cells

Assuming a normally functioning immune response, somatic cell numbers increase dramatically after a pathogen invades one of the mammary glands of the animal (Pyorala 2003). SCC thresholds are often used to predict infection and to determine if milk is suitable for human consumption: the legal maximum for somatic cells in milk Grade A is 400,000 cells/mL in the European Union, New Zealand, and Australia, whereas in Canada and the US it is 500,000 and 750,000 cells/mL, respectively (Hillerton et al. 2004; Nightingale et al. 2008). A threshold of 200,000 cells/mL

has been suggested as indicative of a healthy "quarter", i.e. as measured in milk collected from one of the four teats of the cow (Pyorala 2003). Flow cytometers can detect up to 10 million cells per mL. In designing this device, a target range of 0–3×10^6 cells/mL was selected.

2.3 Properties of milk, cells and fat globules

Milk is a complex matrix composed in its majority of water (87.1%), fat (in the form of spherical droplets, 2.2%–5.5% (Walstra et al. 1999)), lactose (4.6%), and proteins (3.3%) (Walstra et al. 1999). Present in lesser quantities are vitamins and minerals. Somatic cells in milk include mostly white blood cells (WBCs), and at lower percentages epithelial cells (0%–7% of the SCC total) (Lee et al. 1980). Table 1 shows the physical properties of cells, fat globules, and milk plasma (milk minus fat globules). The viscosity of milk plasma is 1.68 mPa•s (Walstra et al. 1999). Cells being denser than milk plasma and fat globules being less dense, a centrifugal force field moves the two particle types in opposite directions, cells away from the axis of rotation and fat towards it.

2.4 Device

Each SeCy has one inlet and one outlet separated by a ridge. The milk sample is introduced through the inlet and the gas is expelled through the outlet for reliable filling of the device. Attempts to use a SeCy containing a single inlet and no outlet were inconsistent because air was often trapped, preventing proper filling (Garcia-Cordero et al. 2008). See Supporting Information for a description of the indexing mechanism.

2.5 Sample volume and channel geometry and dimensions

The sample must be easily collected and handled by a farmer, preferably with a dropper or inexpensive pipette. Disposable pipettes are available with precise, pre-defined volumes ranging from 20 to 400 μL (Poly-Pipets, NJ, USA); the 150-μL version is well matched to the current SeCy unit design.

Table 1 Physical properties of somatic cells, fat globules, and milk plasma

Component	Diameter (μm)	Mass density (g/cm^3)
Leukocytes (WBC)	6–10	1.055–1.085 (Sethu et al. 2006)
Fat globules	1–8 (Walstra 1995)	0.916 (20°C) (Walstra et al. 1999)
Milk plasma	–	1.034 (20°C) (Walstra et al. 1999)

The funnel should have a small footprint to fit as many as possible on each CD. The dimensions of the closed-end microchannel are defined by milk sample volume, desired cell count resolution, and maximum number of cells to be measured. In this regard, the microchannel must be long enough to accommodate the maximum number of cells, narrow enough that the cell distribution is uniform across the channel width (easing readout of the pellet size), and shallow enough to give a pellet length conducive to a quantitative result. The resultant microchannel dimensions should be consistent with low-cost manufacture, preferably without requiring tight tolerances on tooling for reproducible assay results (injection molding yields highly reproducible part dimensions for a given polymer and set of process conditions, but sizing the mold tooling in advance to make an exact part size is somewhat more challenging).

Cells constitute less than 0.01% of milk volume, hence the device must contain sufficient milk to provide a readily countable number of cells. The selected milk sample volume is 150 μL, hence the closed-end microchannel must accommodate up to 450,000 cells (3×10^6 cells/mL), occupying approximately 235 nL (average cell radius of 5 μm; packing fraction is not considered for these calculations, as the cells are reasonably deformable (Donev et al. 2004).) The designed 200×200 μm closed-end microchannel cross section means that increments of 7,500 cells (50,000 cells/mL) correspond roughly to an increase in cell pellet length of 100 μm. The pellet length at the "healthy" detection level is therefore 400 μm.

Cell counting is greatly facilitated by well-chosen geometry, with a localization region where cells collect during centrifugation. Each SeCy comprises a funnel with a narrow closed-end channel as its tip, as shown in Fig. 2. The design process was a trade-off between guiding the cells into the closed-end channel with minimal chance of trapping along the walls at readily attainable rotational speeds, containing sufficient milk, creating a compact cell pellet that can be readily measured without high-resolution microscopy, and choosing an overall form factor consistent with a number of the structures fitting onto a single CD. The upper wall of the V-shaped part of the funnel decreases from a height of 900 μm–200 μm (where the closed-end channel begins) at an angle of 5.5°. The deeper region allows more milk sample to be contained in a given device area, while the shallower region creates a larger total area of compacted cells for quantitative measurement. Because the centrifugal acceleration can be separated into normal and tangential components at the sloping "floor" (Leatzow et al. 2001), the low 5.5° angle keeps the tangential component comparatively small, decreasing the likelihood that cells will become trapped along the sloping floor as they move into the closed-end channel. The V-shaped region of the funnel has a maximum angle of 37° relative to the disc radius,

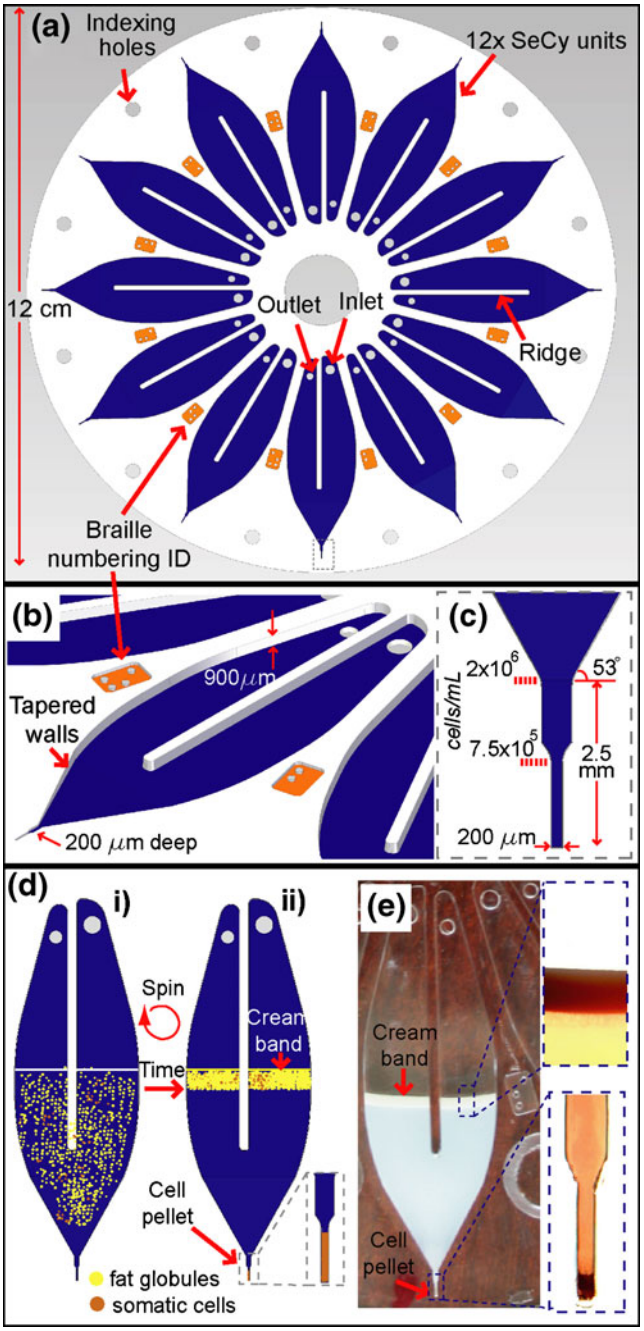

Fig. 2 Design and principle of the sedimentation cytometer disc. (**a**) Top view of the disc, which comprises 12 SeCy units, each being identified with a Braille code. Indexing holes in the disc are used by the reader to facilitate correct and reproducible positioning of the disc relative to the microscopes. (**b**) A 3D view of a SeCy unit shows the tapered walls, which reduce gradually the height of the structure from 0.9 mm to 0.2 mm. (**c**) Magnification of the end of the closed-end channel. (**d**) Schematic of the SeCy unit with milk (*i*) shows that after spinning the disc for 15 min, the fat globules and cells separate into two distinct regions (*ii*): the cream band forms near the upper part of the funnel while a cell pellet forms in the closed-end channel, its length proportional to cell count. (**e**) Images of a SeCy device spun for 15 min, captured with a conventional pocket digital camera. Insets show the cream band and cell pellet as viewed with the microscope

possibly larger than ideal for minimizing cell trapping along this region of the side walls, but the sample volume, height of the disc, and radius of the disc constrained this dimensional choice, which proved to be acceptable.

3 Experimental section

3.1 Disc fabrication

Once a design was proven to work using micromilling and lamination techniques (Supporting Information includes detailed description of fabrication), the design was adapted to injection mold tooling and discs were mass-fabricated in 1.4 mm thick cyclic olefin copolymer (COC) (5013-S04, Topas Advanced Polymers GmbH, Germany) by Microfluidic ChipShop (Jena, Germany) using injection molding. All channel features are included in the single molded layer. A flat 150-μm-thick COC (8007, Topas Advanced Polymers GmbH, Germany) cover layer was bonded to the 1.4-mm-thick disc with proprietary technology (Microfluidic ChipShop).

Unbonded discs and closed-end microchannels in bonded devices were characterized with a scanning electron microscope, SEM (Evo LS 15, Zeiss, Germany). Four SeCy units from a single disc located at 90° to one another were analyzed. Closed-end channels on bonded discs were manually cut with a knife for SEM characterization.

3.2 Milk collection

Milk samples were obtained from the Irish Dairy Research Center, Teagasc (Fermoy, Co. Cork, Ireland). The samples came from local Teagasc dairy research herds and were analyzed using a milk analyzer, MilkoScan FT 6000 (FOSS, Denmark), which includes a flow cytometer to count somatic cells. Milk samples then were stored at 4°C and transported to our facilities for analysis.

3.3 Spinning station and optical setup

Raw milk volumes of 150 μL from individual samples were dispensed through the inlet of each SeCy unit using a pipette. Milk flowed into the left side of the SeCy unit as shown in Fig. 1. No cross-contamination was observed between units when loading each device. Up to 12 samples can be processed by the SeCy disc.

Discs were mounted on the spindle of a brushless DC motor with an integrated optical encoder (Series 4490, Faulhaber, Switzerland). The motor was controlled by a host computer using WinMotion (v2.02, Faulhaber).

Images of the channel tips were recorded with a low-cost microscope (MIC-D, Olympus, USA). Images in bright light provided enough contrast to distinguish the cell pellet

and fat layer from the rest of the milk. Cell pellet images were converted to a black-and-white image with manual adjustment of the threshold using GNU Image Manipulation Program (GIMP) (v2.6.6, Free Software Foundation). Next, the pellet area was processed and measured with custom software (MATLAB, Mathworks, USA). GIMP tools were also used to measure the fat band size. Image processing results were compared to cell counts from the FOSS MilkoScan system.

4 Results and discussion

4.1 Characterization

The tip of the closed-end channels had a height of 191± 9 μm and a width of 215±10 μm, a 2.6% increase relative to the designed area for a 200-μm square (see SEM images in Supporting Information). This deviation is small relative to the standard deviations determined by experimental measurement (see Cell Counts Results section) and therefore is not of concern.

4.2 Operational angular speeds

Air pockets that became trapped in the closed-end microchannels set the minimum angular speed of operation. Removal of all air pockets and filling of closed-end channels with cells was possible at speeds of 4000 rpm and above, which is equivalent to a pressure of 183 kPa at the tip of the channel (see Supporting Information for discussion on calculations).

4.3 Fat globule sedimentation time

Calculation of the minimum time to form the cell pellet and cream layer, including the general case of the time for a single cell and a single fat globule to travel the full length of the liquid in the rotating SeCy unit, is presented in the Supporting Information. To determine the time it takes to form the band of cream (fat), experiments at different angular speeds were performed using raw milk with 4.5% w/w of fat. Figure 3 shows the time evolution of the width of the cream band size at 2500, 4000, and 5500 rpm. The time for a fat globule with a mean diameter (Fox and McSweeney 2003) of 3.5 μm to travel from the tip of the SeCy unit, r_1=57 mm, to the top of the liquid, r_2=35 mm, was estimated using

$$t = \frac{9\eta}{2\omega^2 R^2\left(\rho_p - \rho_m\right)} \frac{1}{(1-C)^P} \ln\left(\frac{r_2}{r_1}\right) \quad (1)$$

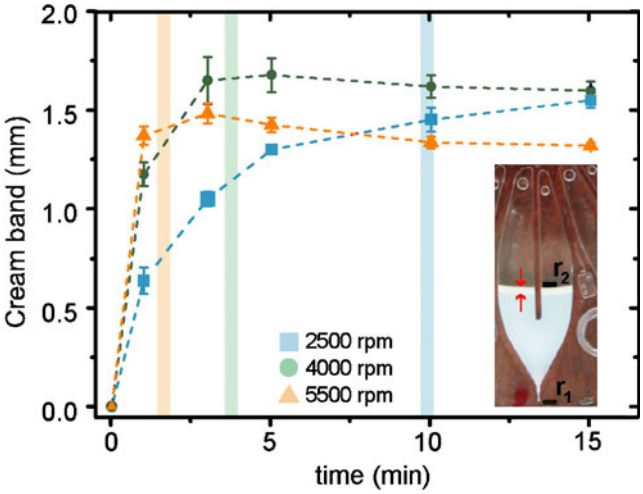

Fig. 3 Evolution of the cream band width over time at three different angular speeds (2500, 4000, and 5500 rpm). Vertical stripes indicate the theoretical time required for a fat globule to travel from r_1 to r_2, calculated using Eq. 1. The error bars represent the standard deviations from three independent measurements

See Supporting Information for the derivation of this equation. Calculated times were 9.6, 3.8, and 2 min for angular speeds of 2500, 4000, and 5500 rpm, respectively, and are represented as stripes in the same Figure. At 2500 rpm, it takes almost 15 min before the cream band attains a constant area (unchanged by additional spinning). A perceptible band is formed in ~3 min at 4000 rpm and remains constant for the next 12.5 min. After 15 min, the 2500-rpm band converged to the same width as the 4000-rpm band.

At 5500 rpm, the cream band is rapidly formed in 2 min, increases slightly in width to a maximum in the next minute, then shrinks in size over the next 7 min, remaining almost constant for a subsequent 5 min. From the calculated theoretical values, the width of the cream layer was expected to remain constant after 2 min as was the case for the 4000-rpm band. The reduction in cream band width was unexpected, but careful observations on the disc revealed a significant protruding "bump" on the bottom part of the SeCy unit, i.e. protrusion of the 150-μm bonded polymer cover layer. This bulge caused the height of the SeCy unit to increase, and thus reduced the width of the cream band while accommodating the same cream volume.

Calculated times from Eq. 1 agree well with experimental findings and should serve as a rough guide in estimating the minimum amount time to form the cream band.

4.4 Somatic cell sedimentation time

Experiments at different speeds and times determined the appropriate conditions to sediment the cells. Figure 4 shows experiments carried out with raw milk with a cell count of

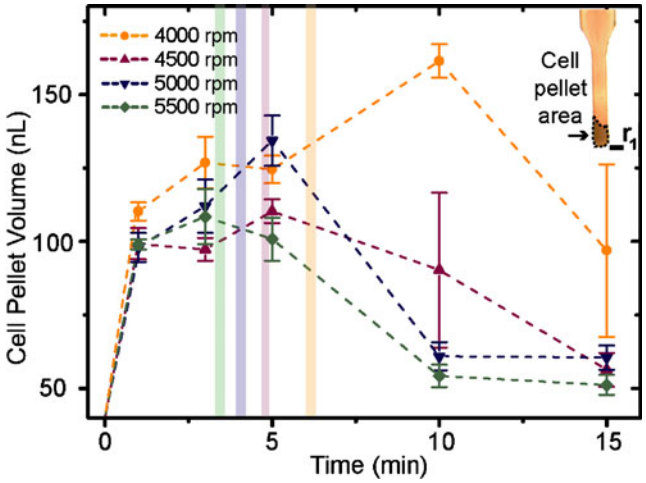

Fig. 4 Evolution of the cell pellet volume with time at four different angular speeds (4000, 4500, 5000, and 5500 rpm). Vertical stripes indicate the theoretical time for a cell to travel from the upper layer of the liquid to the tip of the SeCy unit, calculated using Eq. 1. Image processing software recognizes and measures the area occupied by the cells in the channel and computes the volume. The error bars represent the standard deviation from three independent measurements

4.74×10^5 cells/mL (as determined with the MilkoScan system) and a high fat content of 4.94%. A reproducible cell pellet is formed between 10 and 15 min at 5000 and 5500 rpm. The data show that the cell pellet is more compacted at 5500 rpm than at 5000 rpm. Error bars are smaller at faster speeds (≥5000 rpm) than when slower speeds are used.

In analogy to the fat globules, the time was calculated for a cell with a mean diameter of 6 μm to travel from r_1 to r_2. Calculated times of 6.2, 4.9, 4, and 3.3 min for angular speeds of 4000, 4500, 5000, and 5500 rpm, respectively, are represented as vertical stripes in Fig. 4. These times are not consistent with our experimental findings: there is a difference of 7 min between expected and calculated values at 5500 rpm, and at 4000 rpm this difference is 9 min and the cells did not seem to form a constant pellet. Thus, Eq. 1 does not predict cell sedimentation times nearly as well as the times for fat band formation.

Figure 4 shows a sharp rate of increase in the cell pellet volume vs. spin time for the first few minutes, followed by an almost two-fold decrease in subsequent minutes. Although it was anticipated that the cell pellet would compress over time in the closed-end channel, a two- or more-fold packing density increase was unexpected, even for a packing fraction approaching 100% (Donev et al. 2004). In some cases, small debris or clusters of fat globules were observed slowly migrating center-wards from the region of the closed-end channel; the debris or clusters likely are initially trapped in the channel and slowly move center-wards as they detach from the cells already trapped in the closed-end channel. The cell pellet volume ultimately remains stable at times exceeding 10–15 min for high angular speeds (>5000 rpm).

The disagreement between calculated and experimental values for sedimentation times of somatic cell can be attributed to multiple factors, fat globules or debris (from the raw milk samples themselves) trapped in the channel being one. The fat globules tend to aggregate in clusters and could interact with cells, perturbing their flow path and reducing their velocity. The large difference in concentration between fat globules and somatic cells, ~4% versus 0.01%, makes this factor worthy of serious consideration. As shown by Fig. 3, after 1 min at 5500 rpm, a dense cream band has formed that could block cell sedimentation. It is also likely that some of the cells roll along the wall(s) as they move toward the closed-end channel, decreasing the average settling velocity. Leatzow et al. found that small differences in the angles of tapered walls can cause as much as a 6-fold difference in the separation time (Leatzow et al. 2001).

During experiments, we noted bulging of the 150-μm layer bonded to the device at all speeds greater than 4000 rpm, more noticeable still at 5500 rpm. Upon stopping the disc, the bulge slowly returns to an almost normal position. This bulging may have a detrimental effect on the capture efficiency of the SeCys and will be further studied in the future.

Despite this fairly complicated behavior and the potential non-ideal effects, the correlation between cell pellet volume and somatic cell count is still close to 90% (see below).

4.5 Cell counts and fat percentage

After developing and characterizing the protocol to sediment the cells and fat globules, we tested a number of samples with different cell counts and fat percentages. All experiments were carried out for 15 min at an angular speed of 5500 rpm using an angular acceleration of 150 rpm/sec to reach that speed.

Figure 5 shows results from various samples, comparing the width of the cream band with readings from the MilkoScan system. The SeCy compares favorably to this bench-top instrument, measuring samples with fat percentages ranging from 2% to 6.6%.

Figure 6 compares the results of the SeCy to the flow cytometer readings. The SeCy detects cells in the range from 5×10^4 to 5×10^6 cells/mL with good correlation to the commercial system (R^2=0.88); the estimated lower limit of detection is 5×10^4 cells/mL. The data gap between 2×10^6 and 5×10^6 cells/mL is due to a lack of samples in that range. High-cell-count samples are uncommon in well-maintained herds exhibiting low incidence of mastitis; during the timeframe of these experiments, we could obtain only 3 samples above 1×10^6 cells/mL.

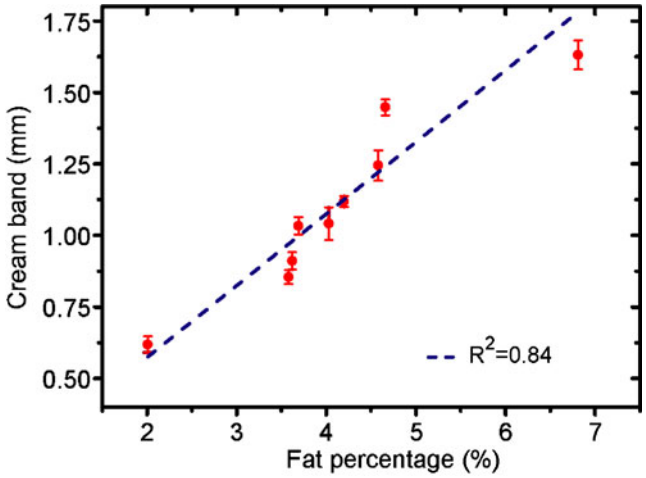

Fig. 5 Width of cream band compared to measurement of the same sample using the MilkoScan system. The error bars represent the standard deviations of three independent measurements

The cell pellet volumes obtained with the SeCy devices are very close to the expected values. Below 500,000 cells/mL, the SeCy system provides a better correlation (R^2=0.92) than at much higher cell counts (>500,000 cells/mL), where it is possible that some somatic cells become trapped in the dense cream band that forms very rapidly at 5500 rpm. In addition, at the onset of an infection and for cell counts higher than 200,000 cells, most of the somatic cells are neutrophils, which engulf not only bacteria, but micelles and fat globules as well (Harmon 1994; Kehrli and Shuster 1994), thus changing the physical properties (density) of some of those cells and precluding them from reaching sedimenting in the closed-end channel.

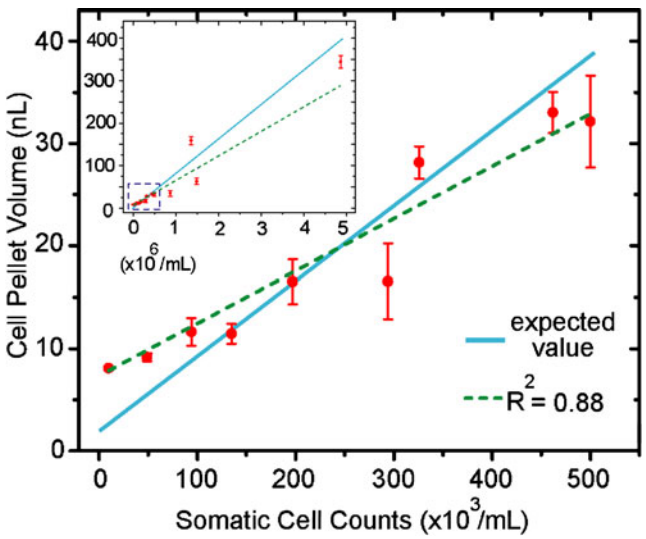

Fig. 6 Cell pellet volumes obtained with the SeCY system compared to readings from the same sample using the MilkoScan system. The "expected value" line represents the theoretical limit of 100% cell-capture efficiency in the closed-end channel. Inset graph shows the comparison of up to 5×10^6 somatic cells. The error bars represent the standard deviations of three independent measurements

A few parameters can be optimized to improve cell capture efficiency at high cell counts: for example, reducing the speed of rotation in the first minutes slows initial formation of the cream layer and may increase the chances of all cells sedimenting into the closed-end channel. The effect of reducing or increasing the milk sample volume might also be investigated. Future work could also involve simulations to understand the effect of the geometry (wall angles, aspect ratios) on the efficiency of sedimentation of the cells, and characterization of other phenomena such as the Coriolis effect (Fromer and Lerche 2002).

5 Conclusions

We demonstrated the design, fabrication, and characterization of a low-cost, portable microfluidic system for assessing the number of somatic cells and fat content of whole, unprocessed milk in 15 min. This system is one of the few demonstrations of a microfluidic concept providing a "sample-in, answer-out" capability. Unlike other cell-counting techniques, this assay requires no additional reagents. It is easy to operate and handle: the user only needs to dispense 150 μL into each SeCy unit and insert the disc into a portable reader.

Our CD-based device design was demonstrated to be manufacturable in large quantities using injection molding of COC thermoplastic resin and a thin polymer cover layer, in analogy to conventional CD manufacture. The CD includes 12 independent sedimentation cytometer units, each resembling a flattened funnel with sloped walls to facilitate the cell collection and featuring a narrow closed-end channel where cells sediment and form a column whose length is proportional to cell count.

We investigated the formation of the cream layer over a range of angular speeds and compared them with theoretical values, and performed similar experiments for cell pellets. Results from our device correlate well with those from a bench-top flow cytometer designed specifically for milk analysis for both somatic cell counts and fat percentage measurements. Our SeCy's measure fat content in the range 2.0–6.5% and cell counts from 5×10^4 to 5×10^6 cells/mL. The SeCy has captures efficiencies above 80%.

A 'point-of-cow' reader system was developed to automate the measurement of milk samples in a format suitable for use on farms.

Acknowledgements This work was supported by Science Foundation Ireland under Grant No. 05/CE3/B754. We are grateful to Jim Flynn and Brendan Kavanagh in Teagasc, MoorePark for providing and analyzing milk samples in the MilkoScan system. We also acknowledge Lorcan Kent for help with SEM images and Caroline Viguier for help during initial experiments. We thank Prof. Luke P. Lee for helpful

discussions and Ivan Dimov for helping with image analysis. We also thank Dr. Holger Becker of Microfluidic ChipShop for helpful discussions in adapting our CD design to the injection molding process.

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SUPPLEMENTARY INFORMATION

Microfluidic sedimentation cytometer for milk quality and bovine mastitis monitoring

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Contents

Device Design – Indexing mechanism

An indexing system based on Braille code, embossed in the surface of the disc at a consistent radial distance, is used to identify each SeCy unit on a given CD. Numbers in Braille consist of a cell of six dots arranged in a matrix of 2 columns by 3 rows. The Braille code (numbers from 1 to 12) will enable a future reader to identify the funnel being scanned using pattern-recognition software.

Indexing holes facilitate correct and reproducible positioning of the disc in the reader system: pins in the reader lock the disc in place and prevent movement to ensure that each image is collected in the same region of each SeCy unit.

Disc fabrication

Initial CD-based assay prototypes were fabricated using a CAT3D micromilling machine (Datron, UK). Cyclo olefin polymer (COP, Zeonor 1060R, Zeon Chemicals, Japan) CDs were obtained from Åmic (Sweden) for these prototypes. The discs had a 1.2 mm thickness, with inner hole and outer diameters of 15 mm and 120 mm, respectively. The design was laid out in a CAD program (Excalibur 5.0, Progressive Software Corporation, USA), then converted into micromilling machine code using the same software. Surface profiles of the fabricated devices were obtained using an optical profiling system (Wyco NT1100, VEECO, USA). Sheets of 250-μm-thick poly(methyl methacrylate), PMMA, (Goodfellow, UK) were laminated onto a sheet of 80-μm-thick double-sided pressure-sensitive adhesive, PSA (Adhesives Research Ltd, Limerick, Ireland). The sheets were then cut to the size of a CD using a laser cutting system (Optec Micromaster, Belgium). After removal of the liner, the PSA was laminated onto the milled plastic CD.

The low throughput of this fabrication technique (three hours to fabricate a single disc; two more to characterize the dimensions), the challenges of alignment and reproducibility for small features on opposite extremes of the discs, and a need to understand a path towards commercialization, compelled the development of a high-volume manufacturing approach.

Device Characterization

The tip of the closed-end channels had a height of 191 ± 9 μm and a width of 215 ± 10 μm, a 2.6% increase relative to the designed area for a 200-μm square (see Fig. 7).

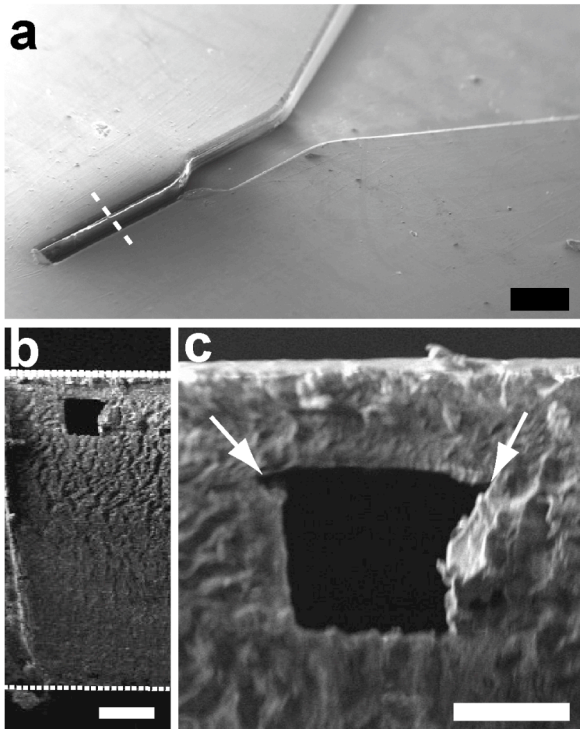


Fig. 7 SEM images of the SeCy device showing images of the bottom part of the funnel (a); dotted line shows the approximate locations where the cross sectional images were obtained in (b) and (c). Cross sectional view of disc, highlighting disc borders with dotted lines (b), and cross sectional view of closed-end channel. Arrows in (c) point to defects during release of molded disc from mold insert and subsequent bonding. Scale bars are 500 μm, 200 μm, and 100 μm in images (a), (b), and (c), respectively.

Device: Operational angular speeds

The pressure can be calculated from (Madou *et al.* 2006):

$$P = \rho\omega^2\overline{r}\Delta r \qquad \text{(S1)}$$

where ω is the angular velocity, ρ the density of milk, and $\overline{r}$ and Δr the average radial distance from the CD center and length of the milk sample in the SeCy unit, respectively. The milk sample in the SeCy unit had an inner radius of 3.5 cm and the tip of the SeCy unit is 5.7 cm from the center of the CD. This inner radius distance, determined by the milk sample volume dispensed into the SeCy unit, is constant.

Previous SeCy designs demonstrated that filling of wider closed-end channels was possible at angular speeds of 3000 rpm (Garcia-Cordero *et al.* 2008). The dimensions of the channel affect the filling and can be optimized according to the application.

The maximum angular speed was set by pressures at which delamination of the sealed CD occurs. Fluid leakage started to occur above 6000 rpm, equivalent

to a pressure of 413 kPa. Thus, the proper functioning for cell counting of the SeCy ranges between 4000 and 6000 rpm.

Theory: sedimentation time

We performed a theoretical analysis to estimate the minimum amount of time to form the cell pellet and cream layer; the general case of the time for a single cell and a single fat globule to travel the full length of the liquid in the rotating SeCy unit is considered. Cells and fat globules are assumed to be rigid spheres, not subject to deformation for this analysis, and the same local concentration of particles is assumed throughout the trajectory. We also assumed milk plasma (whey) as the liquid medium where particles are suspended and the liquid has Newtonian properties.

Four forces act on a spherical particle of radius R that is rotating in the SeCy unit at an angular speed ω: centrifugal, $F_C = \omega^2 r V_p \rho_p$; buoyant, $F_b = -\omega^2 r V_p \rho_m$; viscous drag, $F_d = -\zeta u_\infty$; and Brownian, F_f; (Sharma *et al.* 2009) where r is the radial distance from the center of rotation to the particle, $V_p = 4/3\pi R^3$ and ρ_p are the volume and density of the particle, respectively, ρ_m is the density of the medium, u_∞ is the velocity of the particle, and u_C is the (terminal) settling velocity of the particle corrected by a factor that considers the volume fraction of particles present in the solution.

The viscous friction coefficient, ζ, is related to the size of the particle as $\zeta = 6\pi\eta R$, where η is the viscosity of the medium. Because the mean diameter of somatic cells is about 10 μm, Brownian force is not included in these calculations. A cell reaches its terminal velocity in a uniform centrifugal field in a matter of microseconds (Corry and Meiselman 1978), so it is safe to assume that the total force acting on it is $F_T = F_C + F_b + F_d = 0$ or $\omega^2 r V_p(\rho_p - \rho_m) - \zeta u_\infty = 0$. Thus, the settling velocity can be found from the following equation (known as the Stokes equation (Sharma *et al.* 2009)):

$$u_\infty = \frac{dr}{dt} = \frac{\omega^2 r V_p \left(\rho_p - \rho_m\right)}{\xi} \equiv \frac{2}{9}\frac{\omega^2 r R^2 \left(\rho_p - \rho_m\right)}{\eta} \quad \text{(S2)}$$

This formula applies only to low-Reynolds-number conditions, $Re < 1$, for a single particle (Russel *et al.* 1992). During sedimentation, hydrodynamic interactions with other cells or fat globules can decrease the settling velocity (Kynch 1952; Maude and Whitmore 1958; Corry and Meiselman 1978; Sharma *et al.* 2009). To correct for the particle concentration in solution, Eq. S2 must be adjusted by a correction factor,

$$u_C = u_\infty \left(1 - C\right)^p \quad \text{(S3)}$$

also known as Richard-Zaki correlation, where C is the solid volume fraction and p is an empirical constant equal to 5.5 for hard spheres and low Re (Martin *et al.* 1995). The correction factor does not consider wall effects. Eqs. S2 and S3 have been studied for cubic and cylindrical containers (Burger and Wendland 2001; Kuusela *et al.* 2004), hence the geometry of our device may require an alternate formulation. To simplify the analysis we do not include wall effects, particularly rolling of cells to the upper taper walls as they sediment, which could significantly affect settling velocity (Leatzow *et al.* 2001). Thus, we assume that the majority of the cells remain in the bulk of the solution for most of the sedimentation process.

The total solids volume fraction in milk is 0.21, 0.05 from the fat content plus 0.16 from other components (casein micelles, lactose, and whey protein) (Fox and McSweeney 2003). Although sedimentation processes are dynamic and give rise to the propagation of kinematic waves that change the volume fraction as a function of distance, Eq. S3 remains a good approximation of the settling velocity (Lerche 2002). A more thorough study of sedimentation processes in a centrifugal field can be found elsewhere (Burger and Concha 2001; Bürger *et al.* 2004).

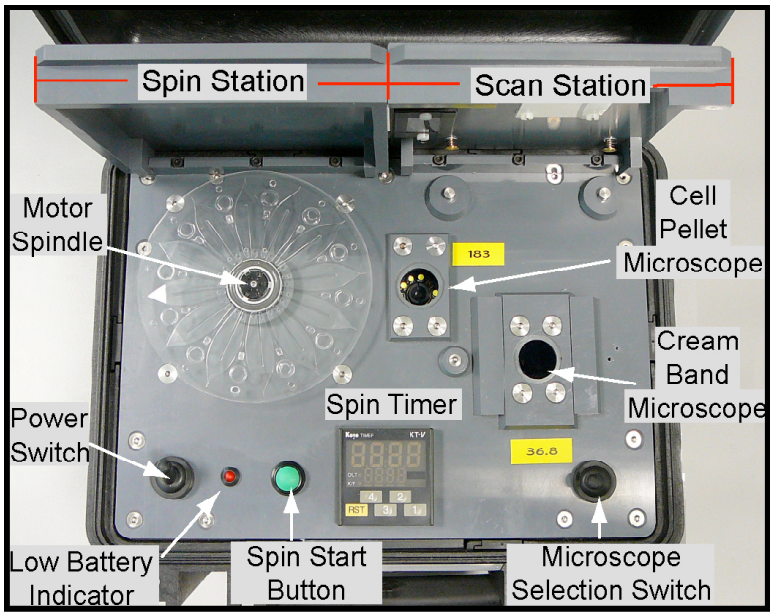
To calculate the time it takes for a particle to travel from a radial distance r_1 to r_2, Eqs. S2 and S3 are integrated to give

$$t = \frac{9\eta}{2\omega^2 R^2 \left(\rho_p - \rho_m\right)} \frac{1}{\left(1 - C\right)^p} \ln\left(\frac{r_2}{r_1}\right) \quad \text{(S4)}$$

Reader

We developed a portable 'point-of-cow' (POC) reader system in parallel to the manufacturing and testing of the SeCy chip; it is housed in a 'farm-appropriate,' rugged box. The reader, built by AOS Technology Ltd. (Leicestershire, UK), incorporates electronics, two microscopes, and a motor to spin the disc. The reader weighs 4 kg and measures 30 cm wide x 22 cm deep x 20 cm tall and includes spin and scan stations. The spin station rotates the disc at a fixed angular speed for a controllable time. The spin station also features a lid with interlock mechanisms that only allows the disc to rotate when the lid is closed as shown in Fig. 8.

Fig. 8 'Point-of-cow' sedimentation cytometer reader system. The reader consists of spin and scan stations to facilitate and automate the cell pellet and cream band measurements.



The scan station contains two USB digital microscopes with an integrated camera (Dino-Lite AM413t, Absolute Data Services Limited, UK), one to measure the cell pellet area and another to measure the cream band width. The former microscope is fixed at a magnification of 183X, the latter at 36.8X. Due to spatial constraints, the microscopes are located at a 90° angle to one another, viewing different SeCy units. Selecting between the two microscopes is accomplished with a toggle switch. The reader is connected to a portable computer to acquire images. Electrical power is supplied from a rechargeable lithium ion battery. USB and electrical connectors are located in the back panel of the box.

To operate the POC-reader, the user inserts the disc into the spin station, closes the lid, and presses the spin button. The disc rotates for a fixed time. After the disc stops, the disc is manually transferred to the reading station where the two microscopes capture images (Dino-Capture software) of the cell pellet and the fat band. Images can then be analyzed with a portable computer. A future version of the POC reader will integrate a microcomputer and display in the same box.

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