Sales Book 1: Cell Line Development

Purpose

To help identify how the WOLF Cell Sorter and N1 Single Cell Dispenser can help support Cell Line Development. This can include cloning cells for Biopharma protein production, cloning of stem cells, or the generation of reporter lines. This book contains an introductory Whitepaper, several Application and Technical Notes and a Sales Presentation. Each are available on the NanoCellect Distributor Portal, as well.

Table of Contents

This book contains an overarching Whitepaper, several Application Notes and a Sales Presentation. Each are available on the NanoCellect Distributor Portal, as well.

1. **Whitepaper 001:** Cell Line Development 2
2. **Application Note 003:** Single-Cell Cloning for Cell Line Development 10
3. **Application Note 004:** Better Cell Sorting and Antibody Discovery from Single Bovine B-Cells 14
4. **Application Note 005:** Gentle Sorting Accelerates Generation of Genome-Edited iPSCs 16
5. **Application Note 009:** Isolate Antibody-Secreting CHO Cells for Single-Cell Cloning with the WOLF 18
6. **Application Note 010:** Induced Pluripotent Stem Cell Preparation for 10x Genomics Applications 20
7. **Application Note 017:** WOLF Exceeds Limiting Dilution Cell Cloning Efficiency 23
8. **Application Note 018:** Maximizing Protein Production with WOLF Bulk Sorting 25
9. **Application Note 022:** Increase HEK293T Single-Cell Clonal Outgrowth Under Standard Conditions 26
10. **Technical Note 005:** Fast Plating Mode Increases Single-Cell Cloning Throughput 28
11. **Sales Presentation:** Cell Line Development 29

© 2020 NanoCellect Biomedical, Inc. All rights reserved.

9525 Towne Centre Dr., Suite 150, San Diego, CA 92121
(877) 745-7678 info@nanocellect.com

WOLF Cell Sorter Whitepaper 1: Cell Line Development

William Alaynick, Ph.D.
NanoCellect Biomedical, Inc., San Diego CA

Cell Line Development

Cell line development, or CLD, is a form of cell culture that is most often used in industrial biopharmaceutical applications in an effort to produce proteins as drugs. These protein drugs, or biologics, are typically hormones, such as insulin or human growth hormone (hGH), soluble receptors, such as etanarcept (Enbrel®), or monoclonal antibodies, such as adalimumab (Humira®). Collectively biologics produce $450 billion in annual sales and are expected to grow.

In order to produce biologics, mammalian cells adapted for robust protein production and high growth rates have been established as immortalized cell lines. The two most common are Chinese Hamster Ovary (CHO) cells and Human Embryonic Kidney (HEK) cells. Each of these have several subtypes that suit various culture methods, such as adherent or suspension. And each requires specialized culture media to have their best performance.

Because biologics are administered almost exclusively by injection or infusion, they must be produced in a cGMP environment and be scrupulously clean. This places restrictions on the types of cell culture media that can be used. While sera, such as fetal bovine serum (FBS) can be used for research, animal products are typically eliminated from cell culture that will be used to produce drugs for human use. Animal products have the potential to carry viruses or prions, and therefore must be avoided. Specific recombinant growth factors and animal product free serum replacements may be used, however, to help achieve good outgrowth.

The steps for CLD include,

1. Introducing nucleic acid that encodes the biologic into the cells. This can be done by transient transfection, stable transduction, viral transduction, and other methods.
2. Selecting a single cell that expresses that protein and expanding that into a monoclonal culture. This can be performed by limiting dilution, cell sorting or cell printing.
3. Validating that the colony is monoclonal. This is typically performed with a plate imager.
4. Quantifying the production of protein by that clone (titer).
5. Scaling up that clone to several liters.
6. Scaling the culture to hundreds or thousands of liters.
7. Collecting the desired biologic from the culture.
8. Purifying the biologic to pharmaceutical quality.

Broadly speaking, CLD is divided into *upstream* and *downstream* sections. Upstream describes the steps required to reach scale up. While Downstream encompasses the steps involved in collecting and purifying the biologic. NanoCellect serves the key step of the Upstream process: plate sorting, the selecting a specific cell and placing that single cell into the well of a 96- or 384-well plate.

Plate Sorting

Plate Sorting is the process of using fluorescence activated cell sorting (also known as FACS) to identify a cell based on one or more optical parameters, sorting that cell, and directing it to a specific well of a 96- or 384-well plate. Even without fluorescent labeling (label-free), cells can be selected based on their size (forward scatter) or internal complexity (back scatter), as well as some level or endogenous or background fluorescence. Live cells can be discriminated from dead or dying cells and debris to help improve the quality of unlabeled cells placed in wells. Furthermore, this method is more efficient than limiting dilution because up to 95% of wells have a single cell—while limiting dilution can only reach about 20%. This improves the efficiency of subsequent steps of incubation and plate imaging while expanding these single cells into colonies.

Additional measures can be made by using dyes to identify live or dead cells. To identify live cells, fluoresceine diacetate (FDA) can be used to find cell that still have esterase activity that cleave the FDA and result in fluoresceine with greater fluorescence. To identify dead cells, we can use membrane-impermeant dyes that only enter dead or dying cells with

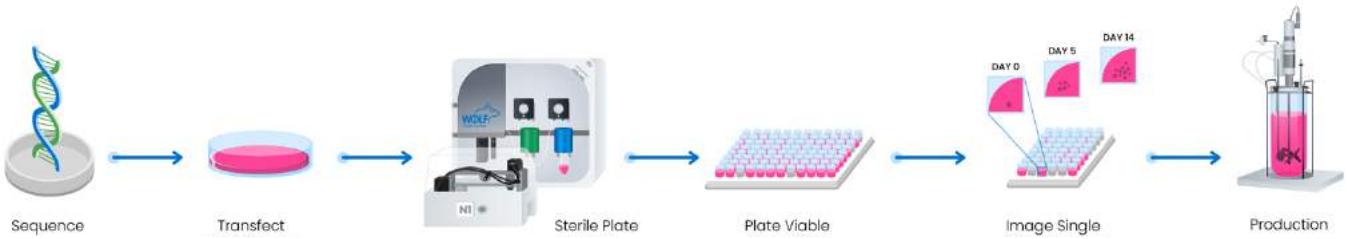


Figure 1. Cell Line Development Workflow Overview. In order to produce a desired protein, genetically reprogrammed cells are sorted into 96- or 384-well plates for monoclonal outgrowth, validation of monoclonality, measurement of protein production (titer), and scale-up for production.

© 2020 NanoCellect Biomedical, Inc. All rights reserved.

9525 Towne Centre Dr., Suite 150, San Diego, CA 92121
(877) 745-7678 info@nanocellect.com

porous or broken membranes. These dyes then intercalate in DNA to become more fluorescent. Examples include propidium iodide (PI) and 7-AAD.

Additional reagents can be used to label antibody production, such as a fluorescently labeled Protein A, or a fluorescently labeled antigen to which the antibodies were raised, or a fluorescent antibody against the protein being produced. Collectively these reagents can increase the likelihood of selecting a living cell that is producing the protein of interest. This further increases the efficiency of the subsequent incubation and plate imaging. Care must be taken, however, the be sure the reagents used will conform to the regulatory requirements of the organizations, e.g. cGMP, etc. In other words, cGMP reagents may be required.

Challenges with Droplet Sorting and Key Benefits of the WOLF Cell Sorter

While there are several products that can perform plate sorting, they can be divided into two broad categories. First there are droplet-based cell sorters. These are very fast and have lots of optical parameters (e.g. colors), but they suffer from four key drawbacks.

Complexity. Droplet sorters are difficult to operate and maintain, requiring dedicated, expert operators, lab space and expensive service contracts. They have complex fluidics carts and pumps that are common source of failure.

Sterility. These systems are nearly impossible to truly sterilize. They can be rendered aseptic with harsh chemicals, but they are very difficult to truly sterilize by autoclaving, gamma-irradiation or gas sterilization. Only the Sony FX500 has truly sterile fluidics—yet it is still an open, droplet system.

Biohazards. Because these systems use droplets, there is a potential to spread biohazards to the operator, especially when working with sample of human origin or in infectious disease research. This can require a dedicated room and cumbersome containment hoods and venting to abate aerosols.

Cell injury. Droplet sorters require relatively high pressures (20-80 psi), force cells through a small aperture (nozzle) resulting in shear stress, and then have a rapid decompression to ambient pressure (decompression shock). Collectively, these stresses can injure or kill cells to substantially reduce the number of viable cells in the cell culture plates. Initially promising cell dispensing can lead to disappointing outgrowth as cells die in the subsequent days.

The second class of instruments used for plating cells includes microfluidic cell sorters, also known as mechanical sorters. These systems have fewer optical parameters (5 maximum). These have the following advantages

Simplicity/ease-of-use. These systems can be operated by a non-expert on a bench on in a tissue culture hood. They are lower cost and have lower service contract prices.

Sterility. Some of these systems are truly end-to-end sterile (WOLF, NanoCellect), while others have sterile components, yet rely on non-sterile fluid reservoirs.

Contained Biohazards. Because these systems do not use droplets to sort, they do not create aerosols. Furthermore, because some have end-to-end disposable fluidics (WOLF, NanoCellect), potentially hazardous samples are simply disposed.

High Viability. Because of microfluidics, there is more laminar flow and less shear stress. In addition, no nozzles are needs, nor are any droplet formed. Importantly, the pressure of microfluidic sorters in on the order of 2 psi or less.

Figure 2: Cell Line Development Researchers

AbVision | Acceleron | AGC
Aridis Pharmaceuticals | Agilent
ATUM | AveXis / Novartis | AZTherapies
Baylor | Beacon Discovery | Momenta
Biogen | BioMilQ | Bolt Biotherapeutics
Case Western Reserve | CRISPR Therapeutics
Dewpoint | Eikon Therapeutics | Eli Lilly & Co
GNF / Novartis | Gyeongsang University
HebeCell | HHMI Harvard Stem Cell Institute
University of Oxford | Janssen / Johnson&Johnson
JOINN | Loma Linda University | Merck
Medimmune / AstraZeneca | Tocagen | UC Davis
Mt. Sinai School of Medicine | Multi-Bio
New York Stem Cell Foundation | NGM
Oklahoma State University | Oncorus
Parvus | Penn State College of Medicine
Rice University | Sorrento Therapeutics
St John's University | Takeda
USDA | Ultragenyx | Vanderbilt University
Viacyte | Zhuhai Livzon Pharmaceutical

© 2020 NanoCellect Biomedical, Inc. All rights reserved.

9525 Towne Centre Dr., Suite 150, San Diego, CA 92121
(877) 745-7678 info@nanocellect.com

Advantages of the WOLF Cell Sorter

In order to understand the advantages of the WOLF, one must appreciate the advantages in, 1) simplicity/ease-of-use, 2) sterility and biosafety, 3) gentleness and viability, 4) the strength of NanoCellect's Technical Support and Service, and lastly, 5) a lower price and cost of ownership. Relative to existing products on the market, if we examine the WOLF simply by numbers (speed, colors, lasers) we may overlook key benefits of the WOLF Cell Sorter that emphasize cell qualities of viability, safety, sterility, and access.

Speed: Rather than focus on the cell sorting speed as an isolated parameter, it can be helpful to ask, "How many cells do you need"? Or, "How many cells can you process per day?" In other words, what is the limiting step in the workflow—it could be a limited amount of incubator space or numbers of plates that can be analyzed by their cell imager per day. We often find that throughput requirements are based on a certain level of expected loss based on previous methods, such as droplet sorter results or limiting dilution; or an overly conservative safety tolerance for the downstream steps. For example, their previous low efficiency in plating has required that 8-10 plates to get 200 clones. With higher efficiency, 1-2 plates with high-density, high-viability and high-outgrowth might be enough.

When considering the WOLF within a workflow, it is also helpful to ask, "What is needed or sufficient?", rather than what is a customer's first guess or an old protocol. We often find that smaller numbers of high-quality cells can work in many applications. And this avoids being drawn into the 'spec wars'. Droplet sorters can sometimes win on initial plating speed, but they may be simply providing a lot of dead or dying cells with a lower overall efficiency in the workflow.

Colors: With the advent of genetic labels and a huge catalog of antibodies and fluorescent markers available, many experiments can be achieved with 1-3 colors. There will always be certain applications or workflows that require multiple lasers and more colors. Walking through the actual experimental requirements—not all imaginable experiments—can help focus the customer on what is sufficient or required. It is critical to establish a commitment from the customer that the WOLF's optical parameters can work—and this commitment must come from all stakeholders in the purchase: buyers, users, and evaluators.

Sterile vs Aseptic. Sterile conditions are achieved with heat (autoclaving), gamma radiation, or gas (hydrogen peroxide, ethylene oxide). Sterilization is used to prepare medical grade products, such as needles, sutures, and implantable devices. This is in contrast with aseptic treatment, that avoids contamination, but does not definitively remove existing microbes. With one exception (the Sony FX500), sorters can only clean the system with chemicals and dilution. This can be insufficient to remove all microbes, especially those that can produce spore-like forms. Some systems have replaceable components, such as a replaceable cuvette and sample line (Sony). In contrast, the WOLF has an end-to-end sterile, disposable cartridge and fluidic path. And it compact enough to fit into a standard tissue culture hood, BSL3 hood, or anerobic chamber. In addition, replacing the WOLF's cartridge and fluidics between samples definitively eliminates cross contamination.

Biosafety: The processing of biological samples on droplet-based machines can create aerosols that pose a biohazard threat. Even if the threat is minimal, there are formal regulatory practices that require stringent control of aerosols with abatements systems and containment hoods. When processing samples of human origin or with known pathogens, the stringency of biohazard precautions is elevated. Elaborate cleaning protocols increase these burdens. In contrast, the WOLF operates at less than 2 psi and therefore lacks the pressure necessary to form aerosols. Furthermore, the system's fluidics are entirely isolated from the WOLF hardware to eliminate contamination. And because the fluidics are disposable, there is no possibility of cross-contamination between samples. The cleanup is to simply remove and dispose of the fluidics in less than 1 minute. The WOLFs small size allows users to move the system into and out of containment hoods, including BSL3, without assistance from an engineer.

Other Cell Line Development Tools

Four approaches can be used to place single cells into the wells of 96- and 384-well plates.

Limiting Dilution

By diluting cells that then distributing them to wells, one can use the principle of the Poisson distribution to have, on average, one or fewer cells per well.

Positives: This method is low-tech, has no capital equipment costs, and is gentle.

Negatives: This system is low efficiency, resulting in approximately 20% of the wells having a single cell. This low efficiency means that for a typical 200 colony campaign, more plates are needed to reach 200 single-cell colonies. This requires more space in the incubator and more time to perform such as are they alive, and if are they expressing protein of interest—measures that further increase the efficiency of CLD workflows. Using the WOLF can improve the efficiency and productivity of employees and the facilities by 2-5 times.

Droplet Sorting

Droplet sorters have the ability to detect many colors and operate at high speeds—although to preserve viability, sorting speeds must be limited to less than 10,000 cells per second to minimize shear stress. Competitors include BD (FACSAria, FACSFusion, FACSMelody, FACSJazz), Sony (SH800S, FX500, MA900), Beckman-Coulter (Astrios).

Positives: Droplet sorters have the highest speeds and greatest number of colors for plate sorting. They can often complete a 96-well plate in just a several seconds; and have the ability to process large numbers of cells to select for rarer cells. They can also dispense cells in a very small volume (100 nL) to avoid dilution—although this is not a concern for cell culture where cells go into 100 µL of media or more.

Negatives: Droplet sorters are complex to operate and maintain—especially the fluidics carts that are a common point of failure. The complexity of operating a droplet sorter is

© 2020 NanoCellect Biomedical, Inc. All rights reserved.

9525 Towne Centre Dr., Suite 150, San Diego, CA 92121
(877) 745-7678 info@nanocellect.com

Droplet Plate Sorting vs WOLF in Cell Line Development

	SH800S Sony	FX500 Sony	MA900 Sony	FACSAriaIII BD	FACSMelody BD	Beckman-Coulter Astrios	WOLF NanoCellect
Events/sec (eps) Sorts/sec (cps)	100,000 12,000	100,000 12,000	100,000 12,000	100,000 10,000	34,000 10,000	100,000 10,000	5,000 300
Colors	Up to 6	Up to 6	Up to 12	Up to 28	Up to 11	Up to 30	3
Sorting	2-way	2-way	4-way	4-way	2-way	4-way	2-way
Instrument Price Cartridge Price	$140-200k $60	$150-200k $60	$150-220k $60	$225-$1M No cartridge	$150k-$250k No cartridge	$225-$1M No cartridge	$98.5k + $20-30k N1 $195 cartridges
Viability	+++	+++	+++	++	++	+++	++++
Scatter (large angle)	FSC (180° BSC)	FSC (180° BSC)	FSC (180° BSC)	FSC (90° SSC)	FSC (90° SSC)	FSC (90° SSC)	FSC (180° BSC)
Fits in hood	No, custom	No, custom	No, custom	No, custom	No, custom	No, custom	Yes
Portable (kg)	No, 98 kg	No, 100 kg	No, 100 kg	No, >100 kg	No, 41 kg + 36 kg	No, >100 kg	Yes, 20 kg
Sort Mechanism, Pressure Aerosol	• Cuvette sorting • ~70-20 psi • Aerosols	• Cuvette sorting • ~70-20 psi • Aerosols	• Cuvette sorting • ~70-20 psi • Aerosols	• Cuvette sorting • ~70-20 psi • Aerosols	• Cuvette sorting • ~70-20 psi • Aerosols	• Jet-in-air Sorting • ~70-20 psi • Aerosols	• Microfluidic Sorting • 1.5 psi • No Aerosols
Selling point (claim, feature)	• Ease of use • Microfluidic sorter (false)	• Disposable sterile fluidics • Microfluidic sorter (false)	• One button start (aseptic cleaning, QC, and sort). • Microfluidic sorter (false)	• High sensitivity • High speed • High parameters	• Same optics as Aria • Ease-of-use software • Form factor.	• High sensitivity • High speed • High parameters	• Ease of use • 1.5 psi • High viability • Sterile, disposable fluidics • Low biohazards
Negatives	• Non-Sterile fluidics • Droplet shear stress • Open droplet sorting • Not microfluidic sort, only microfluidic analysis • Biohazard aerosols • Fluidics cart service	• Droplet shear stress • Open droplet fluidics • Biohazard aerosols • Fluidics cart service	• Non-Sterile fluidics • Droplet shear stress • Open droplet fluidics • Biohazard aerosols • Fluidics cart service	• Non-Sterile • Highest shear stress • Open droplet fluidics • Biohazard aerosols • Complex operation • Fluidics cart service • High cost of ownership	• Non-Sterile • Highest shear stress • Open droplet fluidics • Biohazard aerosols • Complex operation • Fluidics cart service • High cost of ownership	• Non-Sterile • Droplet shear stress • Open droplet fluidics • Biohazard aerosols • Complex operation • Fluidics cart service • High cost of ownership	• Lower speeds • 5 parameters, max

enhanced when we think of setting of the droplet stream to reach the bottom of each well. This can be challenging as the droplets are electrostatically charged and can stick to the dry wall of the plate, rather than landing in the media at the bottom. This can be especially true in the outermost wells, the so-called 'edge effect'. With high speed comes the cost of high shear-stress and decompression shock. As a result of these mechanical forces, cells can reach the well, only fail to grow or to die in the coming hours or days.

plate imaging and more plates to analyze for titers. This method lacks the ability to measure the qualities of the cells,

Microfluidic Sorting

There three systems that use fluorescence activated cell sorting and microfluidic sorting to place cells into wells of 96- or 384-well plates. These systems operate at low pressures, place cells into wells, and have varying degrees of sterility.

Namocell. Namocell has a compact (10kg) system that can perform 1-way sorting into 96- or 384-well plates using a total of 4 optical parameters collected from a 488 nm laser. It uses 1 scatter (FSC) and three colors in the case of the first-generation system, simply called the Namocell. Their second-generation system, the Hana, uses 2 scatter (FSC and 180º BSC) and two fluorescent colors. In both cases, the flow cytometry software is less capable than the WOLF and only allows for threshold and rectangular gates, not polygon gating. Care must be taken in evaluating their published specifications as they tout an extremely high processing rate *and* a very high single-cell plating speed. However, there are two modes, an enrichment mode, and a purity mode. So, it can be fast *or* pure, but not both.

The system requires loading a sample onto their sterile, disposable chip. The outboard fluidics, however, for sheath and cleaning are not sterile. Overall, the Namocell is a less refined product than the WOLF. The build and software are not as robust or polished. For instance, key steps require power-cycling the instrument to reset it, rather than using the software.

On-Chip. This Japanese company has a system that can be outfitted with a 488 nm laser and two of the following lasers, 405 nm, 561 nm, 637 nm, and detect up to 6 colors. The sorting mechanism is called "flow shift" and can sort up to 1000 cells per second—although plating requires another system and is much slower. There are two chip types, 80 x 80 micron and 150 x150 micron and both are available in sterile and non-sterile packs of 10. There is a single cell dispenser that does not sort cells, it uses a pipette to draw up a small volume of sample and uses microscopy to check for zero, one or greater than one cells in the 7 to 200 micron size range. If only one cell is detected, it will be placed into a well of the 96- or 384-well plate. If not, it will repeat the process. The plating takes 60 minutes for a 96-well plate and 200 minutes for a 384-well plate. They report >90% single-cell accuracy. Two discreet instruments are required, therefore, to perform plate sorting. The sales and support of On-Chip is much more limited outside of Japan. There is one sales and support person in North America, for instance.

Positives: Microfluidic or mechanical sorters have several advantages. First, these have higher cell viability and RNA integrity because of the lower mechanical strain on cells than droplet sorters. By operating as lower pressures, they avoid large changes in pressure. Lower pressure results in lower cell

© 2020 NanoCellect Biomedical, Inc. All rights reserved.

9525 Towne Centre Dr., Suite 150, San Diego, CA 92121
(877) 745-7678 info@nanocellect.com

velocities and changes in velocity, as well. Flow within and 150x150 micron and both are available in sterile and non-sterile packs of 10. There is a single cell dispenser that does

microfluidic structures is typically laminar flow with more consistent velocities and turbulence to reduce tumbling and shear forces. Second, biosafety of the cells and operators is improved. Because of the low operating pressures, microfluidic sorters cannot form aerosols, protecting the operators from potential biohazard exposures and giving microfluidic sorters and excellent safety profile, especially for work with human samples or when live virus is used to modify cells, such as in vaccine production. Third, because these systems use disposable cartridges or chips, in the event of a clog, the system can be exchanged and restarted without help from a service engineer. In the case of the WOLF Cell Sorter, the use of peristaltic pumps allows for the exchange of the entire fluidic path, making disposal of potential biohazards and avoidance of microbial growth easy.

Negatives: With the above benefits come the following challenges. First, because of the lower pressures and speeds and the slower mechanical sorting mechanisms, there are fewer cells per second that can be sorted—although this is not a concern in Cell Line Development where sorting 1 cell per second is adequate. Second, these systems have constant flow of samples and sheath resulting in more dilution. Here again, this is not of concern in Cell Line Development because the cells are dispensed into a hundred or more microliters of media. A minor concern or difference is that all sorters that use cartridges or chips use a different form of large-angle scatter, 180º back-scatter, and cannot collect 90º side-scatter because the cartridge is in the 90º path of the scattered light. These two forms of scatter are practically equivalent, especially in Cell Line Development. The Namocell and OnChip sorters use pressure pumps and therefore retain water with salts in the system between runs. This can result in failure of mechanical parts and has the potential for microbial growth. The pressure pumps cannot be sterilized, but only rendered aseptic by cleaning with harsh chemicals. The WOLF Cell Sorter is the only microfluidic sorter capable of plate sorting that has sterile, rather than aseptic, end-to-end fluidics.

Cell Printers

Cell printers have been specialized to serve cloning work, such as Cell Line Development, and this specialization comes at the expense of measuring optical parameters used in flow cytometry. These systems use photomicroscopy to detect 1 cell or >1 cell at the interrogation point, and then attempts to place a single cell into the well of a 96- or 384-well plate. These are usually bright field images, however newer systems have added a single fluorescent color to allow for the identification of fluorescent probes.

Namocell. This can be considered a hybrid product with aspects that are like a sorter and aspects that are like a cell printer. It is addressed above in the *Microfluidic Sorter* section.

Cytena/Cellink. The first generation Cytena, now called the scp, uses bright field microscopy to analyze a cell between 5 and 35 microns at the detection zone with a 10X objective prior to dispensing. If no cell is seen, it continues dispensing. If a single cell is seen, the cell is dispensed through a 45 x 45 micron nozzle into the well of the plate in 150 pL of volume, with about 95% accuracy. If more than one cell is seen, it uses an aspirator to discard that event. The process is then repeated. The cartridge can hold 5 to 80 uL of media with cells at a concentration of 10^4 to 10^7 cells per mL, with an optimal range of 10^5-10^6 . This system was developed by the German Freiberg University spin-out, Cytena GmbH, founded in 2014, and eventually acquired by the Swedish publicly traded company, Cellink, in 2019. Cytena was distributed outside of Germany by Molecular Devices, a division of Danaher, from

Cell Printers vs WOLF for Cell Line Development

	Namo/Hana Namocell	VIPS Solentim	CloneSelect SCP	f.sight	CellenOne Scienion (Cellenion)	On-Chip SPiS	WOLF NanoCellect
Plating Speed	1 minute	10 minutes	10 minutes	10 minutes	4 minutes	60 minutes	2.5 minutes
Colors	3/2	0	0	1	0	0	3
Sorting	1-way	1-way	1-way	1-way	1-way	No	2-way
Price of instrument / price of cartridge	$80k $65	$120k $100	$160k $200	$160k $200	$150k	$100k $200	$118.5k $175
Viability	++++	++++	++++	++++	++++	++++	++++
Scatter	FSC/FSC BSC	No	No	No	No	No	FSC, BSC
Fits in hood	Yes	Yes	Yes	Yes	No	No	Yes
Portable (kg)	Yes, 10 kg	No, 70 kg	No, 30 kg	No, 30 kg	No, 205 kg	No, 100 kg	Yes, 20 kg
Selling point (claim, feature)	<2psi, small, easy, automated, chip-based (closed)	Suspension and adherent cells. Automated limiting dilutions, bright field imaging	Disposable chip, label-free sorting, high viability, Small volumes, automated limiting dilutions, bright field imaging	Disposable chip, label-free sorting, high viability, Small volumes, automated limiting dilutions, bright field imaging.	Low volume, high viability	Disposable chip, label-free sorting, high viability	Gentle 1.5psi, cell integrity (karyotype), disposable chip and tubing, ease of use
Weaknesses	Single cell speed is slow. Limited sample voumes.	No fluorescence or cytometry	No fluorescence or cytometry	No cytometry	Large footprint, expensive	No fluorescence or cytometry. Slow, at 1 hour per 96-well plate.	

© 2020 NanoCellect Biomedical, Inc. All rights reserved.

9525 Towne Centre Dr., Suite 150, San Diego, CA 92121
(877) 745-7678 info@nanocellect.com

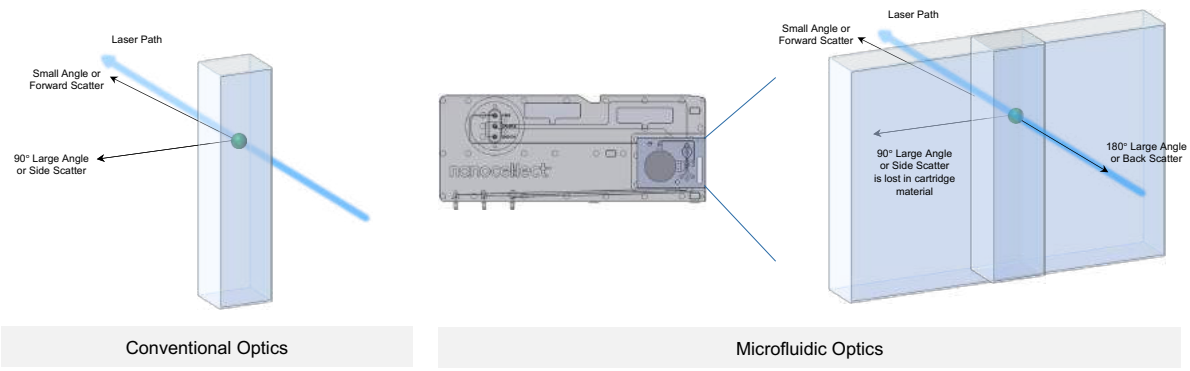


Figure 3. Differences in Large Angle Scatter in Cell Sorters. Conventional Optics. A traditional cuvette flow cell used in droplet sorting measures small-angle forward scatter and 90° large-angle side scatter. **Microfluidic Optics.** The WOLF, and other sorters that use cartridges or chips, collect small-angle forward scatter like a traditional cuvette flow cell. However, large-angle scatter is collected as back scatter, BSC, at 180° instead of SSC at 90° to accommodate the microfluidic cartridge design. For more information see: Technical Note 2020-06, Identifying Leukocyte Populations: WOLF Back Scatter Versus Traditional Cytometer Side Scatter

creating some confusion by consumers regarding the manufacturer.

Cytena/Cellink's next generation systems have a new form factor for this technology, some with new features, but sharing, with one exception, the same chips, nozzle sizes, and dispensing volumes. The c.sight™ is the most similar to the Cytena with bright field imaging and plating of cells into 96- and 384-well plates to serve CLD and single-cell genomics. The up.sight™ is similar to the c-sight™ with the addition of an inverted microscope plate imager to follow outgrowth and verify monoclonality. A fluorescence-capable version of the c.sight™ is called the f-sight™, and can be configured with a 488 nm laser or a 531 nm laser to identify fluorescent labels, typically fluorescent proteins such as GFP or RFP, mCherry, etc. It collects brightfield and fluorescent images. Lastly, the b.sight™ is intended for microbial work and uses brightfield imaging with a 20X objective to image cells in the ~1 to 10 micron range. The chip as a 20 x 20 micron nozzle and dispenses 35 pL per cell.

On-Chip. On-Chip manufactures a cell printer does not sort cells, in addition to their cell sorter that does not plate cells. The SPiS uses a pipette to draw up a small volume of sample and uses microscopy to check for zero, one or greater than one cells in the 7 to 200 micron size range. If only one cell is detected, it will be placed into a well of the 96- or 384-well plate. If not, it will repeat the process. The plating takes 60 minutes for a 96-well plate and 200 minutes for a 384-well plate. They report >90% single-cell accuracy.

Solentim. The VIPS (Verified In Situ Plate Seeding) uses a low-pressure system to dispense one droplet with one cell into a dry well. This is verified by inverted scope microscopy and automated cell detection to confirm a single cell in a given well. If a single cell is confirmed, the well is filled media and the process repeated at the next well. It takes about 10 minutes to complete a 96-well plate. The plates can be analyzed by the system's plate reader over subsequent days. The system is small enough to fit into a biosafety hood.

Scienion/Cellenion (Cellink Companies). The CellenONE uses a capillary pipette to analyze and dispense single cells. The pipette is placed in front of a microscope objective to visualize zero, one, or more than one cell. If a single cell is detected, a three-axis stage moves the pipette to a given well and dispenses a droplet that falls into the well. If more than one cell is seen, the sample goes to a sample recovery tube. It takes approximately 4 minutes per 96-well plate; and can process 384- and 1536-well plates, as well—presumably at approximately 16 and 48 minutes, respectively.

© 2020 NanoCellect Biomedical, Inc. All rights reserved.

9525 Towne Centre Dr., Suite 150, San Diego, CA 92121
(877) 745-7678 info@nanocellect.com

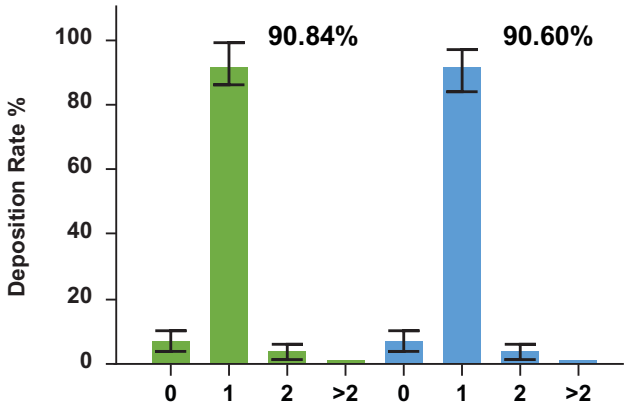


Figure 4. The WOLF Cell Sorter and N1 Single-Cell Dispenser achieves over 90% single cell deposition. Deposition of GFP-positive CHO cells was performed with the WOLF and N1. In both 96- and 384-well plates, the deposition rate was over 90% as assayed by a plate imager.

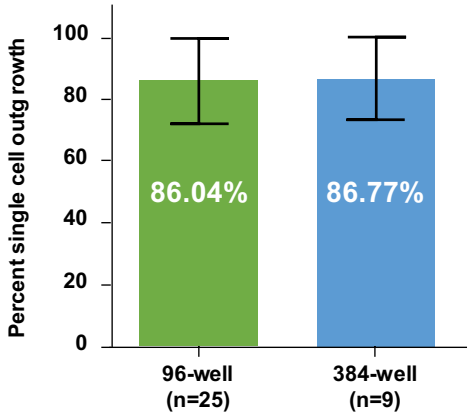


Figure 5. High Monoclonal Outgrowth with the WOLF Cell Sorter and N1 Single-Cell Dispenser. Monoclonal outgrowth was seen in over 86% of the wells that had a single cell deposited. Colony outgrowth was assayed on Day 14 with a plate imager.

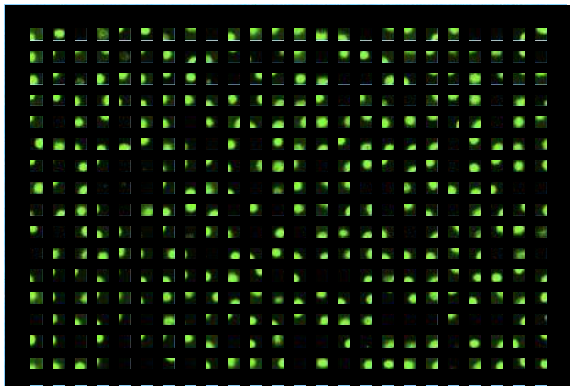


Figure 6. High Efficiency Outgrowth. Over 86% of the cells deposited into a 384-well plate had monoclonal outgrowth.

© 2020 NanoCellect Biomedical, Inc. All rights reserved.

9525 Towne Centre Dr., Suite 150, San Diego, CA 92121
(877) 745-7678 info@nanocellect.com

Optics	
Excitation	488 nm; 30 mW diode laser (rated at 45,000 hr life)
Laser Profile	25 x 75 µm
Scatter Detection	Forward (0 Degrees, +/-15) Back (180 Degrees, +/-15)
Emission Detection	3 Colors, user exchangeable bandpass filters Standard set installed: • 525 ± 25 nm (FITC, GFP) • 585 ± 20 nm (PE) • 665 Longpass (PI, PE-CY5, mCherry, tdTomato)
Optical Alignment	Fixed alignment, no maintenance required

Performance	
Scatter Resolution	Resolves lymphocytes, monocytes & granulocytes
Fluorescence Resolution	8 peak separation with the Spherotech Rainbow calibration beads in the 3 fluorescence channels
Analysis Speed	>5,000 events/second
Sorting	1 and 2 way
Back-to-back Sorting Speed	300 events per second
Plating Speed	8 minutes for 96-well and 32 for 384-well
Absolute Counts	Yes
Volumetric Analysis	Yes
Warm-up Time	Less than 1 minute
Sorting Output	1.5 mL and 5.0 mL, 96- and 384-well plates
Sorting Purity	Up to 99% purity

Fluidics	
Sample Input	1.5 and 5.0 mL tubes
Sheath Input	50 mL conical tube
Sheath Fluid	PBS, water, cell culture media, etc.
Sheath Fluid Usage	7.5 mL/hour
Sample Flow Rate	24 µL/minute
Sheath Flow Rate	160 µL/minute
Dead Volume	50 µL
Tubing Inner Diameter	200 to 500 µm
Flow Cell	200 x 75 µm
Sample Pressure	< 2 psi
Aerosols	None
Sterility	Sterilized with ethylene oxide (EtO)

Physical Specifications	
WOLF Dimensions	15.5W x 14.4H x 15D in (39.34 x 36.6 x 38.1 cm)
WOLF Weight	44 lbs / 20 kg
WOLF Electrical	AC Input: 100-240V, 50-60Hz, 3-1.5A
N1 Dimensions	8.42W x 6.5H x 8.34D in (21.4 x 16.5 x 20.9)
N1 Weight	5.5 lbs / 2.5 kg
N1 Electrical	DC Input: 24V 1A

© 2020 NanoCellect Biomedical, Inc. All rights reserved.

9525 Towne Centre Dr., Suite 150, San Diego, CA 92121
(877) 745-7678 info@nanocellect.com

nanocellect Biomedical, Inc.

Application Note

Single-Cell Cloning for Cell Line Development

Aly Krasny, Huailu Chen, Walker Daniels, Emily Bozek, Beth Leary, William Alaynic, and José M. Morachis, Ph.D.

Introduction

Generation of stable monoclonal cell lines is required for a wide variety of applications, including the development of biologics, such as therapeutic antibodies, or to create CRISPR-engineered cells for disease-in-a-dish models. While the demand for engineered cell lines has increased, production of monoclonal lines is still limited by the current technology for single-cell sorting and dispensing. Here we contrast the limitations of traditional technologies with newer technologies, such as microfluidic sorters and dispensers (WOLF Cell Sorter, NanoCellect, Inc.) that can analyze, sort and dispense single cells into 96- or 384-well plates with high viability and outgrowth. In addition, we highlight that both robust immortalized cell lines (e.g. CHO) and more sensitive cells can be used to generate monoclonal cell lines in a sterile format with little cellular stress.

Technologies for single-cell cloning need a boost

Traditional methods like laser capture microdissection (LCM) and colony picking by micromanipulator enable isolation of single cells, however, their workflows are not compatible with living cells and/or are very low throughput. Using a classical technique, like limiting dilution, has been a go-to solution with minimal upfront resource requirements and gentle handling of cells. However, limiting dilution is inefficient and cannot positively select protein-producing cells or avoid dead cells, resulting in redundant downstream imaging and titer work to isolate monoclonal lines.

Fluorescence Activated Cell Sorting (FACS) methods allow analysis and sorting of specific cells with higher throughput than LCM, micromanipulation, or limiting dilution, with the ability to analyze multiple cell parameters (fluorescence and scatter) and directly sort into 96- or 384-well plates in minutes. Yet the traditional droplet-based cell sorting mechanism and fluidics system exposes cells to substantial shear stress and large, rapid changes in pressure (decompression shock) that can kill or injure cells.

This reduces outgrowth of cell colonies—especially in delicate, engineered lines or stem cells. Sample-to-sample contamination is also a major concern with traditional FACS because fluidic lines are not disposable and must be cleaned chemically. To address stress and sterility, cell printer technologies can plate single cells into plates, but these lack the analytical advantages of flow cytometry that allow the selection of living, single-cells that express a protein of interest to further increase plating efficiency.

The WOLF tackles traditional limitations

The WOLF Cell Sorter addresses challenges in sterility, viability and plating efficiency by combining microfluidic cell analysis and sorting with single-cell plating in the cell line development workflow. NanoCellect's technology detects and sorts unlabeled or fluorescently labeled cells within a disposable microfluidic system, and then distributes the cells one-at-a-time to 96- or 384-well plates using the N1 Single Cell Dispenser module. A peristaltic pump gently pushes cells through microfluidic channels with low pressure (<2 PSI) and low shear stress. Robust laser-based excitation with sensitive PMT detection are used to identify cells that are gently sorted with a precise piezo actuator.

Figure 1. Single-cell cloning workflow: The mixture of cells is collected in a microfuge or FACS tube in buffer of choice and loaded onto the WOLF Cell Sorter with ~50mL of the sheath fluid of choice. Target cells are selected (based on up to 5 optical flow cytometry parameters) and then dispensed into 96-well plates, pre-filled with media. Cells are incubated in culture and imaged or assayed to confirm genetic and monoclonal status.

© 2020 NanoCellect Biomedical, Inc. All rights reserved.
APN-003 2020-02
9525 Towne Centre Dr., Suite 150, San Diego, CA 92121
(877) 745-7678 info@nanocellect.com

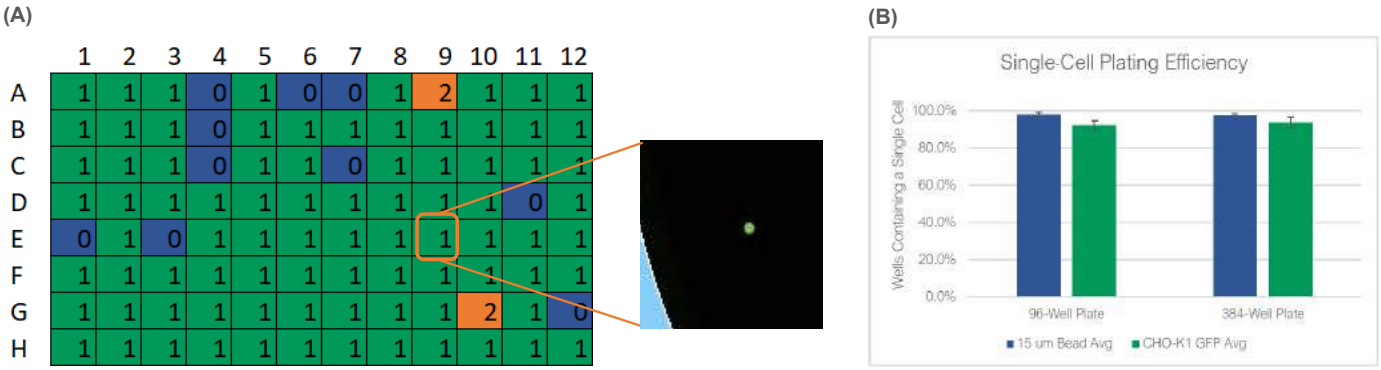


Figure 2. High efficiency single-cell sorting into 96- and 384-well plates with the WOLF Cell Sorter: (A) Heatmap view using the Synentec NyOne plate imager a with representative bead image on a 96-well microtiter culture plate. The number in each well indicates the number of control beads or cells counted. **(B)** Percent of wells in a plate with single control beads or single CHO-K1 GFP cells dispensed was 98.1 ± 1.2% (beads) and 92.5% ± 2.3% (CHO cells) in 96-well plates. Similar efficiencies were achieved in 384-well plates with 97.8% ± 0.9% (beads) and 93.8% ± 2.8% (CHO).

Microfluidic cartridges and tubing sets are provided in sterile, disposable kits to ensure a clean environment for cell analysis, sorting, and dispensing. Because of the microfluidic design, the WOLF uses only about 1% the amount of sheath fluid required by traditional cell sorters. This makes it feasible to use more expensive cell culture medium as sheath, if desired, to maintain cell health. Sorted cells are then dispensed via the N1 to a multi-well plate according to a user-defined layout. The tubing set connects magnetically to the N1 Single Cell Dispenser assuring end-to-end sterility of the fluid path. As each cell is analyzed and sorted within the WOLF's cartridge, it immediately flows through to the N1 unit and is gently dispensed in 7 µL droplets that are 'touched-off' into each well. By integrating gentle cell sorting and single-cell dispensing in a single system, cells have minimal handling and stress. This maximizes monoclonal viability, outgrowth, and titer in cell line development, as demonstrated by the following data.

Results

High single-cell dispensing efficiency and outgrowth

To test the WOLF sorting efficiency and accuracy, we sorted and dispensed 15 µm calibration beads (Dragon Green, Bangs Labs) and Chinese Hamster Ovary (CHO) cells, a cell line commonly used in the production of biologics. A stable GFP-expressing line of CHO cells was purchased (CHO-K1, ATCC), cultured and sorted on the WOLF by selecting high GFP expression and single-cells (doublet-discrimination). The WOLF sorted single, GFP+ cells into 96-well plates containing 200 µL/well culture media and into 384-well plates containing 50 µL/well culture media. Control plates were also tested with calibration beads. Fluorescent and brightfield images were taken and analyzed on Day 0 with a plate imager (NyOne, Synentec) with 4X magnification to measure singlet plating efficiency. Plates were then incubated at 37°C, 5% CO_2 and imaged on day 14 to evaluate outgrowth. Three technical replicates were completed for 96-well (N=9) and 384-well (N=3) plates.

The WOLF Cell Sorter dispensed beads with a singlet fidelity of 98.1% on 96-well plates and 97.8% on 384-well plates. Similarly, dispensing CHO cells resulted in 92.5% and 93.8% single cells into 96- or 384-well plates, respectively (Figure 2). These results are comparable with traditional FACS technology, yet does so with lower mechanical stress. High shear stress commonly reduces colony outgrowth to below 30% with FACS. However, with the WOLF low-pressure microfluidics, >87% of wells had monoclonal outgrowth after 14 days in both 96- and 384-well plates (Figure 3).

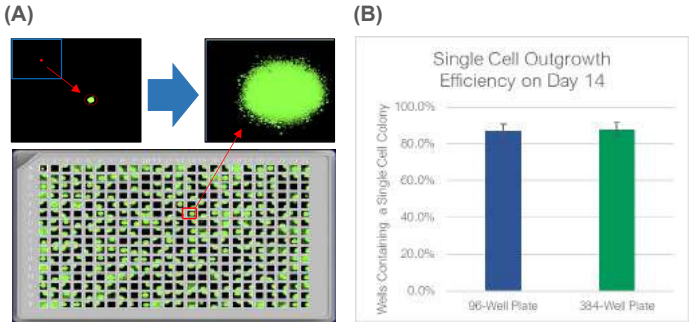


Figure 3. Monoclonal CHO cell outgrowth after analysis, sorting, and plating: (A) Plates were imaged for fluorescence on day 14 and counted for colony presence. Counts were compared to Day 0 heatmap counts to determine monoclonality (derived from a single plated cell). **(B)** After 14 days, the average number of wells on a 96-well plate containing a single-cell colony was 87.4 ± 3.3% (N=9) and on a 384-well plate was 88.1 ± 3.7% (N=3).

© 2020 NanoCellect Biomedical, Inc. All rights reserved.

9525 Towne Centre Dr., Suite 150, San Diego, CA 92121
(877) 745-7678 info@nanocellect.com

Sensitive cells can be sorted to create >250% more colonies

Cells engineered to produce large amounts of exogenous proteins can be very sensitive to manipulation and stress from traditional cell sorters. We compared the WOLF to a traditional droplet-sorter in the lab of a Top-10 biopharma collaborator. Their existing workflow used a FACSAria Fusion (Becton Dickinson) to analyze, sort, and plate a proprietary and highly-sensitive engineered Jurkat cell line with poor outgrowth. The WOLF Cell Sorter was evaluated in a side-by-side comparison to the FACSAria Fusion in analyzing, sorting, and plating cells into 96-well plates, based on a specific fluorescent marker. Both plates were incubated for 2.5 weeks with one media addition midway through outgrowth. Cells from each of the wells were counted via flow cytometry based on a viability marker to evaluate colony growth efficiency.

Compared to the FACSAria Fusion, the WOLF sorter plated 20% more single cells (Figure 4). However, more importantly, after incubating 2.5 weeks, the WOLF-sorted plates contained 2.6-fold more colonies (100 cells or more). The gentle sorting mechanism of the WOLF Cell Sorter greatly improved the viability of these sensitive cells, compared to traditional FACS, resulting in better monoclonal outgrowth.

Generating monoclonal, high-titer colonies

Inefficient generation of cell lines results in increased plating and subsequent downstream screening, such as quantification of protein production. In collaboration with an industry-leading developer of antibody therapeutics, we evaluated the ability of the WOLF to rapidly identify very high-titer antibody cell lines. To do so, we challenged the WOLF by sorting our collaborator's most difficult-to-grow proprietary cells stained with a FITC-conjugated line-specific antibody.

Engineered cells were bulk-sorted into a mini-pool of ~15,000 cells and evaluated for polyclonal antibody production in a 14-Day Fed-Batch Assay (Figure 5A, next page). This simple bulk sort increased polyclonal productivity by more than 3-fold. Additionally, we attempted to improve monoclonal production. Sorted single cells were dispensed with the N1 into 96-well plates and incubated for 20 days with intermittent imaging to establish monoclonality. This yielded substantially more monoclonal lines than previously achieved by the collaborator. Once monoclonal lines reached 50% confluence, cells were transferred to a 24-well plate, put on a shaker, and evaluated for antibody titer on Day 3 (Figure 5B). Six of the high-producing colonies were evaluated in an 11-Day Batch-Fed

(A) FACSAria Fusion Sorter

	1	2	3	4	5	6	7	8	9	10	11	12
A	61	2	0	0	0	0	0	5	1	0	2	328
B	1	0	1	2	3	0	0	0	73	1	3	1
C	5	0	30	0	0	2	0	60	58184	169	6	3
D	5	0	20239	8	2	2214	27	5223	515	1	2	3
E	1	1	3921	87	350	2	0	6	40732	17001	0	1
F	4	1	9	0	0	1	0	2	142	3	0	1
G	1	0	1	0	1	0	0	0	1	2	0	0
H	8	1	4	0	0	0	1	3	2	0	1	6

(B) WOLF Sorter

	1	2	3	4	5	6	7	8	9	10	11	12
A	36	1	30	0	0	1302	118	1	1	13	0	4
B	6	27	7458	2	1	54862	293	1	5	3195	41	4
C	7	7	66	445	44321	889	23	1	1	9	146	0
D	6	11	2190	45	1	3614	17519	15	1477	20	25898	8
E	5	1	0	0	184	29857	206	1	373	120	121	1
F	161	4577	4	39	0	98	6	2	37598	725	0	3
G	17	102	0	0	0	0	3	30	532	37739	0	0
H	10	166	0	0	2	1	0	2	153	553	1	4

Figure 4. WOLF improved monoclonal outgrowth by >250% over the FACSAria Fusion: Proprietary engineered Jurkat cells were analyzed, sorted and single-cell plated with the FACSAria Fusion **(A)** or the WOLF Cell Sorter. **(B)** Wells were analyzed via flow cytometry after incubating for 2.5 weeks.

© 2020 NanoCellect Biomedical, Inc. All rights reserved.

9525 Towne Centre Dr., Suite 150, San Diego, CA 92121
(877) 745-7678 info@nanocellect.com

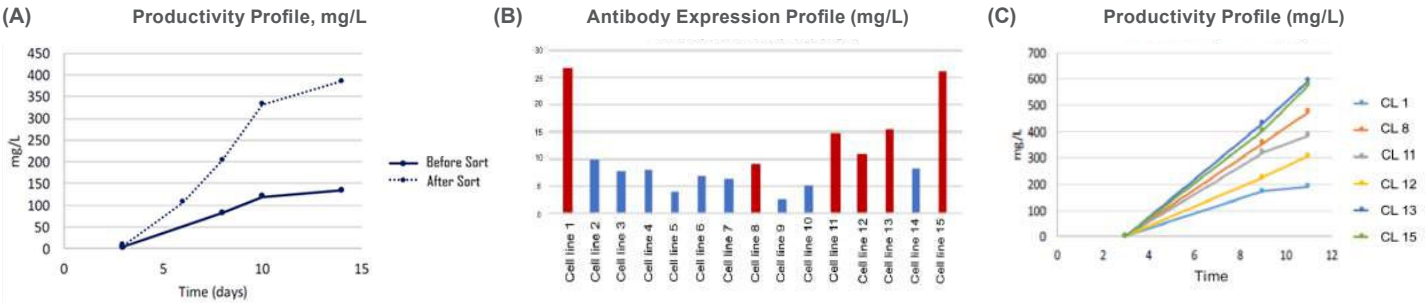


Figure 5. Antibody productivity using the WOLF cell sorter for industrial antibody development: (A) Unsorted cells produced 130 mg/L of antibody in a 14-day batch-fed assay. This increased to almost 400mg/L after bulk sorting by selecting based on high GFP expression. **(B)** Single cells were also dispensed into 96-well plates and incubated for 20 days. Of the monoclonal colonies that emerged in the plate, 15 were evaluated via mini-antibody titer. **(C)** Six of those colonies (>9 mg/L) were selected to evaluate in an 11-day batch-fed assay and produced up to 6x more antibody than unsorted cells.

Assay for scaled-up antibody production. Two clones (1 and 15) demonstrated production up to 6-fold higher than unsorted cells (Figure 5C). These improvements in cell outgrowth and protein production save time and resources and are being applied by the collaborator to additional lines to improve titers and turnaround times.

Conclusion

Current technologies struggle to combine analysis, sorting, and single-cell plating in an efficient, streamlined workflow for cell line development. Methods such as manual picking can effectively isolate cells of interest but are time consuming and low throughput. While technically easy and gentle, methods such as limiting dilution lack selection and therefore suffer from low efficiency. The WOLF combines multiparameter selection with gentle single-cell plating into a workflow that with just 15 minutes of start-up, can efficiently generate monoclonal lines with high outgrowth and titers. The WOLF dispenses 96-well plates in 8 minutes with 92.5% efficiency and 87% outgrowth. Viability is highlighted by the ability of the WOLF to produce >2.5-fold more colonies when compared to traditional FACS technology. In summary, the microfluidic WOLF can improve cell line development workflows by improving sterility, viability, outgrowth, and titers.

For more information, visit nanocellect.com or email info@nanocellect.com

© 2020 NanoCellect Biomedical, Inc. All rights reserved.

9525 Towne Centre Dr., Suite 150, San Diego, CA 92121
(877) 745-7678 info@nanocellect.com

nanocellect Biomedical, Inc.

Application Note

Better Cell Sorting and Antibody Discovery from Single Bovine B-Cells

Adapted from the Smider Lab results presented at Antibody Engineering & Therapeutics 2018[1]

Introduction

A distinct feature of bovine antibodies, ultralong CDR H3 regions, have not been observed in any other species and have the capacity to access epitopes on structurally complex antigens. As such, bovine immunoglobulins have great potential for development as clinical treatments and research tools targeting a broad variety of antigens. However, unlike other species, techniques for studying bovine immunoglobulin genes at the single-cell level have not been comprehensively developed. The Smider group at the Scripps Research and the Applied Biomedical Science Institute established a new method for amplification of bovine immunoglobulin VH and VL genes at the single-cell level utilizing NanoCellect's novel cell sorting technology. They employed the WOLF Cell Sorter and N1 Single Cell Dispenser to sort individual B cells into wells for cDNA production and single cell (sc) PCR amplification, followed by nested PCR. Due to high cell integrity, approximately 53% of the wells yielded sequences for both heavy and light chains.

Low-pressure sorting improves RNA integrity

Conventional single-cell sorting into 96-well plates for scPCR has used complex instrumentation optimized for speed rather than cell viability. Results from individual cells collected on a traditional droplet-based cell sorter (BD FACSAria™ II) and NanoCellect WOLF Cell Sorter were compared. For PCR amplification of the bovine IgG genes, primers were designed and ratios of each V_H and V_L primer, along with other conditions, were optimized for production and amplification of cDNA using a QIAGEN™ OneStep RT-PCR Kit.

Figure 1. Schematic of heavy and light chain gene amplification from a single bovine B cell: After immunization of a cow with an antigen of interest, blood was collected, PBMCs isolated, and sorted for IgG+ B cells. QIAGEN OneStep RT-PCR Kit, with primers that anneal to mRNA for bovine V_H and V_L leader peptides and constant regions, was used to produce V_H and V_L cDNA via specific reverse transcription. V_H and V_L genes were subsequently amplified with HotStarTaq PCR, then further amplified with nested primers containing restriction sites for cloning. Nested primer PCR products were cloned into expression vectors and sequenced.

© 2020 NanoCellect Biomedical, Inc. All rights reserved.

9525 Towne Centre Dr., Suite 150, San Diego, CA 92121
(877) 745-7678 info@nanocellect.com

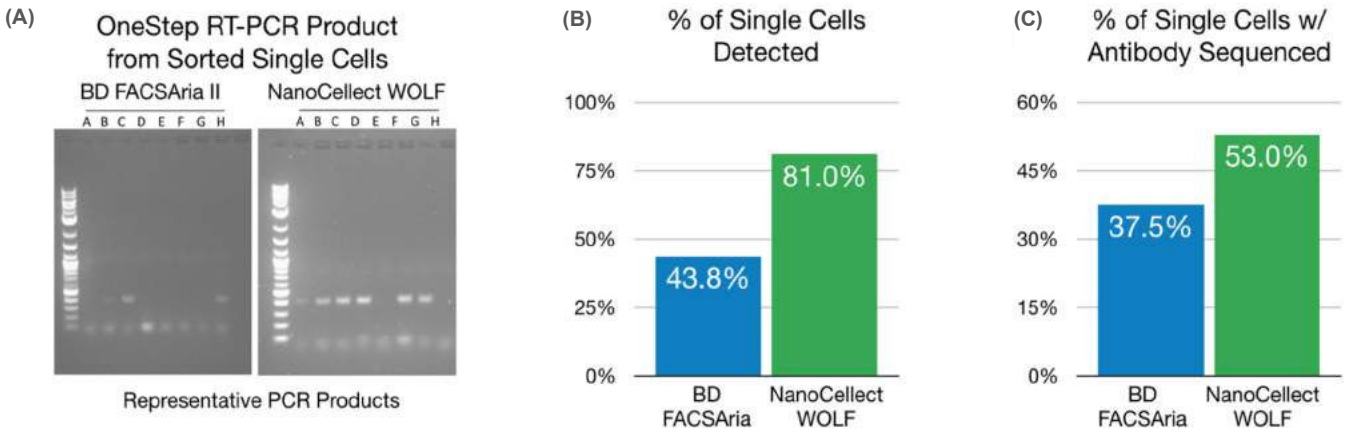


Figure 2. The cell sorter impacts scPCR success: (A) Results obtained from cells sorted by BD FACSAria II versus NanoCellect WOLF Cell Sorter were compared for detection of scPCR gel product. (**B)** Analyzed and combined results (n = 16 for Aria and n = 21 for WOLF) of scPCR product detected. (**C)** Proportion of total number of cells sorted that yielded full antibody sequences (GeneWiz). These results show that percent of cells yielding visible scPCR products and full antibody sequences are higher when cells are sorted by the NanoCellect WOLF Cell Sorter than by BD FACSAria II.

Results and conclusion

This is the first protocol to comprehensively optimize amplification of antibody genes from single bovine B cells. Sorting with the WOLF Cell Sorter resulted in nearly 200% more single cells with detectable OneStep RT-PCR product and 41% more single cells were sequenced for both heavy and light chains, compared to traditional FACS. Therefore, the best condition for scPCR to amplify bovine VH and VL chain genes from sorted B cells is highly dependent on the type of cell-sorting instrument used and the optimization of PCR amplification conditions. This new technique's production of monoclonal antibodies with heavy and light chain pairs expressed from individual bovine B cells will be useful for rapid identification of recombinant bovine antibodies. Little is known about bovine antibody gene repertoires and this method will enable the evaluation of bovine heavy and light chain genetic repertoires at the single-cell level.

For more information, visit nanocellect.com or email info@nanocellect.com

References

1. *Cloning bovine immunoglobulin VH and VL genes from single B cells.* Gabrielle F. Warner, Jeremy K. Haakenson, Vaughn V. Smider; Scripps Research and the Applied Biomedical Science Institute. *Antibody Engineering & Therapeutics* 2018

© 2020 NanoCellect Biomedical, Inc. All rights reserved.

nanocellect Biomedical, Inc.

Application Note

Gentle Sorting Accelerates Generation of Genome-Edited iPSCs

Jean J. Kim,[1] Anel LaGrone,[1] Aly Krasny,[2] Nick Sullivan,[2] Will Alaynick,[2] and José Morachis, Ph.D.[2]
1. Baylor College of Medicine, Houston, TX 2. NanoCellect Biomedical, Inc., San Diego, CA

Introduction

CRISPR-edited iPSCs for *in vitro* disease models

Induced pluripotent stem cell (iPSC) generation, from patients or healthy controls, combined with rapid CRISPR genome editing has greatly accelerated the generation of *in vitro* "disease-in-a-dish" model systems for studying human disease mechanisms. However, substantial limitations still exist in the workflow. In the derivation of iPSCs from patient cells, specific selection criteria could be used to isolate pluripotent cells from those that have not been reprogrammed. The differentiation process could be improved by using specific markers to enrich cells of interest. Lastly, selecting and plating single cells that have undergone CRISPR editing would improve the efficiency of the workflow. At each of these steps, low-pressure sorting can be used for selection while avoiding undue stress on these sensitive cells.

Reconsidering flow sorting to optimize workflow

Fluorescence activated cell sorting (FACS) is a common method of purifying cells from a heterogenous mixture, however it has been avoided for delicate iPSCs due to harsh sorting conditions. The shear stress, high pressures (up to 70 psi), and decompression shock of traditional droplet sorters have been associated with poor post-sort viability. Microfluidic mechanical sorters, such as NanoCellect's WOLF Cell Sorter have reduced shear stress due to microfluidic laminar flow, low pressure (<2 psi), and a droplet / aerosol-free mechanism that eliminates decompression shock to improve viability.

The WOLF detects and sorts unlabeled or fluorescently labeled cells within a sterile disposable microfluidic system. Cells can be sorted into standard microcentrifuge or 5 mL tubes for bulk sorting or sorted one-at-a-time into 96- or 384-well plates using the N1 Single Cell Dispenser module. By integrating sterile, gentle cell sorting, and single-cell dispensing in one system, the WOLF offers a viable option to greatly improve the monoclonal selection and outgrowth of genome-edited iPSCs.

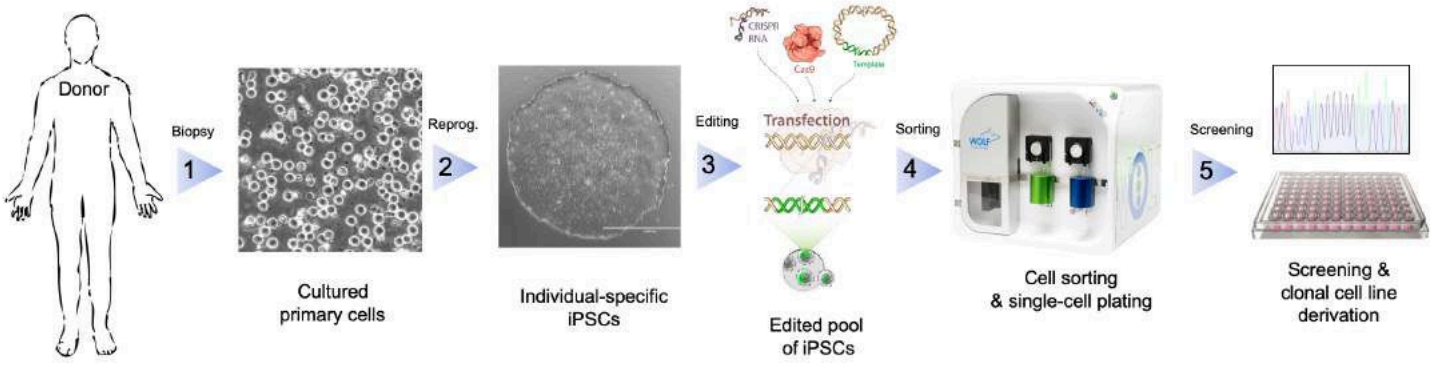


Figure 1. Improved iPSC and CRISPR workflows with microfluidic cell sorting and plating. Generation of reprogrammed pluripotent stem cells, generation of differentiated cells, and selection of CRISPR-edited colonies are all inefficient processes that can benefit from cell selection. Here we show that the microfluidic sorter, WOLF, can improve the workflow of monoclonal line generation.

© 2020 NanoCellect Biomedical, Inc. All rights reserved.

9525 Towne Centre Dr., Suite 150, San Diego, CA 92121
(877) 745-7678 info@nanocellect.com

Results

Monoclonal sorting and plating accelerate colony selection

The WOLF yields a single-cell plating efficiency of up to 93% which is significantly more efficient than non-sorting methods, such as limiting dilution. To evaluate the WOLF's iPSC plating performance, a genome-edited iPSC line was cultured with mTeSR1 (STEMCELL Technologies) and StemFlex (Thermo Fisher Scientific) on hESC-qualified Matrigel plates (Corning). Cell-cluster passaging was performed using ReLeSR (STEMCELL Technologies) and single-cell dissociation was performed using Accutase. Unlabeled cells were sorted based on forward- and back-scatter properties to isolate singlets (Figure 2A), which were then dispensed into Matrigel-coated 96-well plates. Single iPSC clones were grown in StemFlex and were supplemented with 10μM ROCK inhibitor for the first 24 hours.

The WOLF shows improved monoclonal outgrowth

The microfluidic WOLF Cell Sorter and the conventional BD FACSAria II were each used to generate a 96-well plate of monoclonal cells to evaluate plating efficiency and outgrowth. The single-cell clones were allowed to grow and imaged to quantify colony growth. Though single cells were accurately dispensed into both, the cells in the Aria plate were no longer viable one week after plating. In contrast, the WOLF yielded outgrowth of 14 single cell colonies at the same timepoint (Figure 2B).

Sorting allows for selection of only viable, transfected cells

In addition to selecting and dispensing cells based on scatter properties, the WOLF Cell Sorter allows you to sort and dispense based on fluorescence detection, such as GFP expression and fluorescent viability stains. Fluorescent labels can be used with conventional plasmid transfections (Figure 3, top row), as well as with those utilizing the much more efficient CRISPR-Cas9 system. In addition, dead cells can be excluded with dyes, such as 7-AAD, that label cells with compromised cell membranes (Figure 3, bottom row). To minimize doublets, which is critical for single cell cloning, the software can be used to gate and sort only singlets.

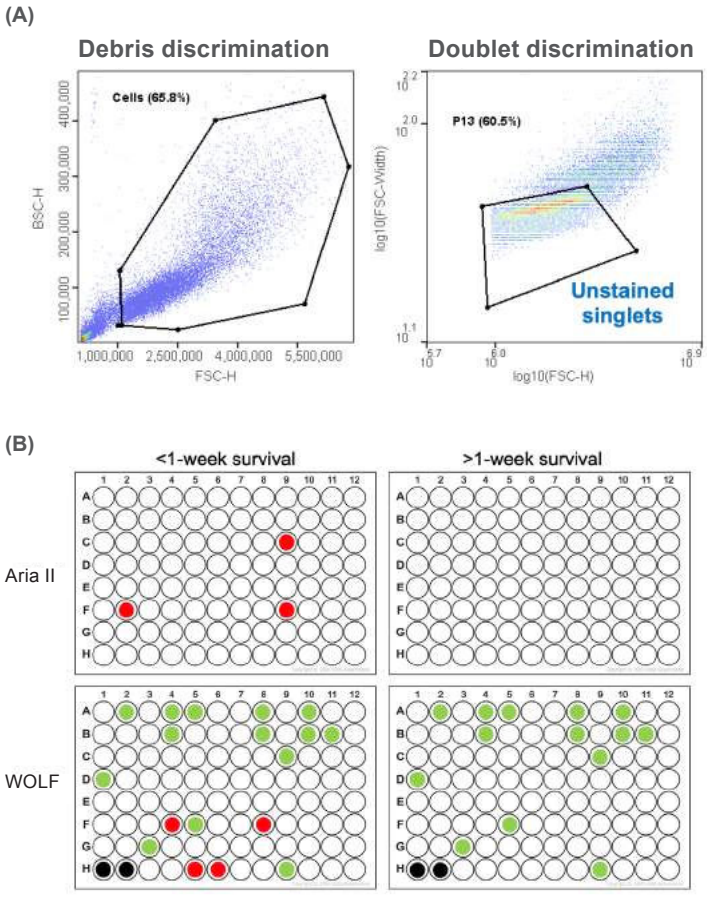


Figure 2. Viability and 1-week survival of cells sorted on the microfluidic or conventional sorter: (A) Edited iPSCs were sorted for viable singlets based on forward- and back-scatter properties. **(B)** Microfluidic sorting resulted in 14.9% of post-sorted wells containing monoclonal colonies, whereas none of the cells plated with the conventional sorter, BD FACSAria II, survived longer than a week. Red wells survived for less than one week, green wells survived beyond 1 week, and black-labeled wells were controls.

© 2020 NanoCellect Biomedical, Inc. All rights reserved.

9525 Towne Centre Dr., Suite 150, San Diego, CA 92121
(877) 745-7678 info@nanocellect.com

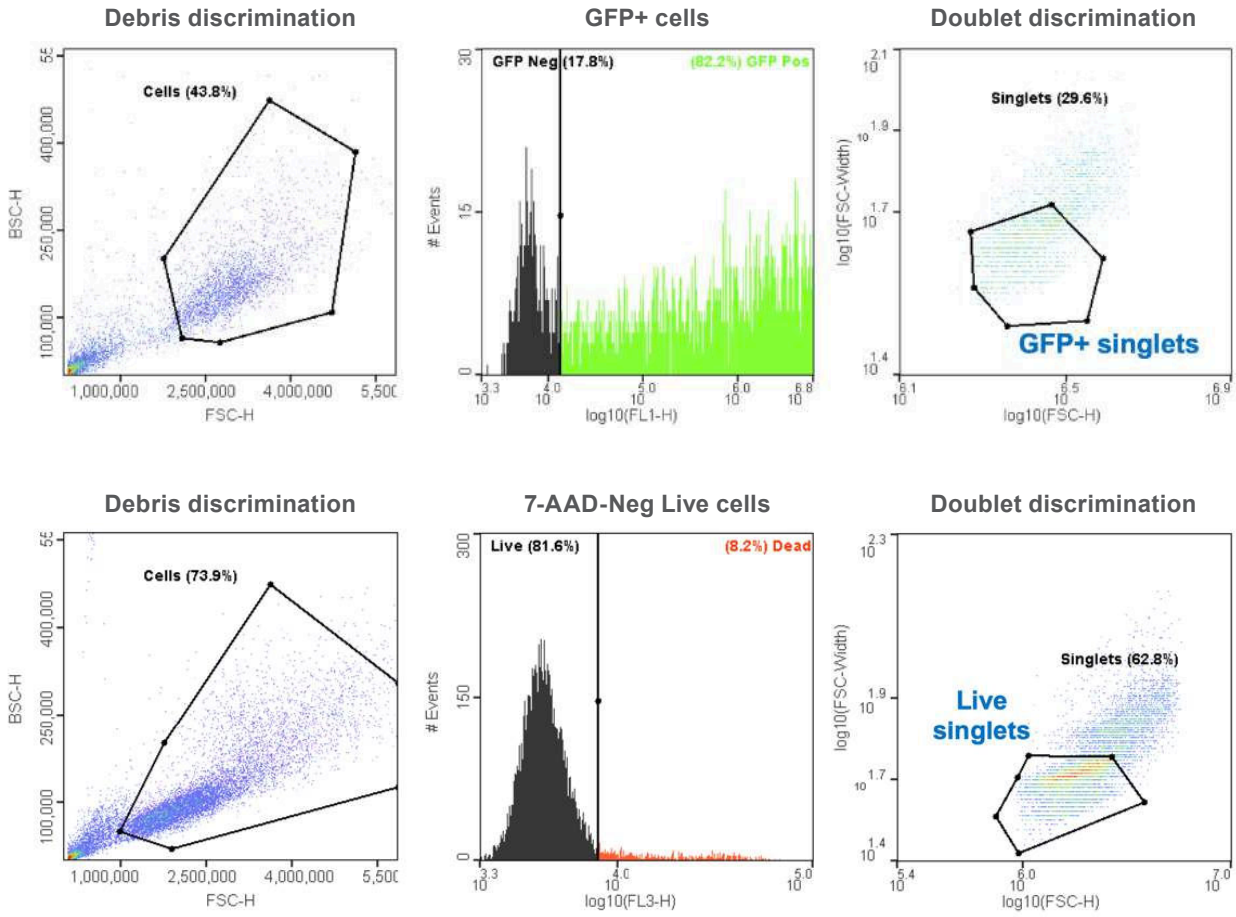


Figure 3. Cells selected based on transfection and viability status: Top: Cells were nucleofected with pMAX-GFP plasmid. GFP-positive singlet cells were sorted based on 500-550 nm emission. **Bottom:** Non-transfected viable single cells were selected on the basis of 7-AAD (>665nm emission) exclusion and forward- and back-scatter, respectively.

Conclusion

Significant advances have demonstrated the scientific value of *in vitro* patient-cell-derived disease models. The increased use of these models requires improvements that reduce labor and increase efficiency in selection and cloning. Utilizing traditional FACS instruments to sort and isolate edited single cells has proven to yield very few or a complete lack of viable cells for outgrowth, rendering it an unusable option for delicate cell types. However, the WOLF Cell Sorter uses microfluidic sorting to overcome these challenges, resulting in reliable cloning for iPSCs. This high viability, disposable microfluidic technology reestablishes cell sorting as a viable option to advance the generation of engineering sensitive model cell lines.

For more information, visit nanocellect.com or email info@nanocellect.com

© 2020 NanoCellect Biomedical, Inc. All rights reserved.

9525 Towne Centre Dr., Suite 150, San Diego, CA 92121
(877) 745-7678 info@nanocellect.com

nanocellect Biomedical, Inc.

Application Note

Isolate Antibody-Secreting CHO Cells for Single-Cell Cloning with the WOLF

Aly Krasny,[1] Alena Bartakova,[1] and JOINNBIO Cell Line Development Team[2]
1. NanoCellect Biomedical, Inc., San Diego, CA, 2. JOINN Biologics US Inc. Richmond, CA

Introduction

The therapeutic antibody discovery and development process can last over 10 years before reaching the commercial manufacturing stage. Therefore, it is critical to make every element of this process efficient. Transitioning from the discovery phase to pre-clinical characterization and manufacturing requires the development of a stable, high-producing monoclonal cell line. Isolating a single, antibody-producing clone can be time-consuming with traditional techniques such as limiting dilution, which can leave 41%-77% of wells empty and only 25%-43% of remaining wells yielding on-target single-cell colonies[1]. Cell printers can improve upon the low dispensing efficiency however, they still lack the ability to select for live, protein-producing target cells.

Fluorescence-activated cell sorters, such as the WOLF, allow for the selection of single cells based on scatter and fluorescent tags that indicate protein or specific antibody secretion and viability. The N1 Single Cell Dispenser can then deposit single cells into 96- or 384-well plates with higher than 90% efficiency2. The low pressure of the system (< 2 psi) ensures gentle sorting and does not disturb the secretory activity of the cells. With a small, 2-cubic-foot form factor, the WOLF can easily fit into a biosafety cabinet to provide a sterile workspace. The disposable cartridge and fluidics are also sterile and ensure a workflow completely free of cell contamination.

This sterile workflow and the ability to select for high-producing cells are what make this a powerful tool to improve the efficiency of stable cell line generation in CDMOs (Contract Development and Manufacturing Organizations), like JOINN Biologics. JOINN Biologics provides a complete range of services, from early custom development to clinical manufacturing. Their JOINN Laboratories group focuses on the early development phase of biological therapeutics, including manufacturing cell line development. Their commitment to state-of-the-art technology platforms, coupled with CHO-based host cell systems, includes utilizing the WOLF to sort cells that are actively producing the recombinant antibody-of-interest, as demonstrated below.

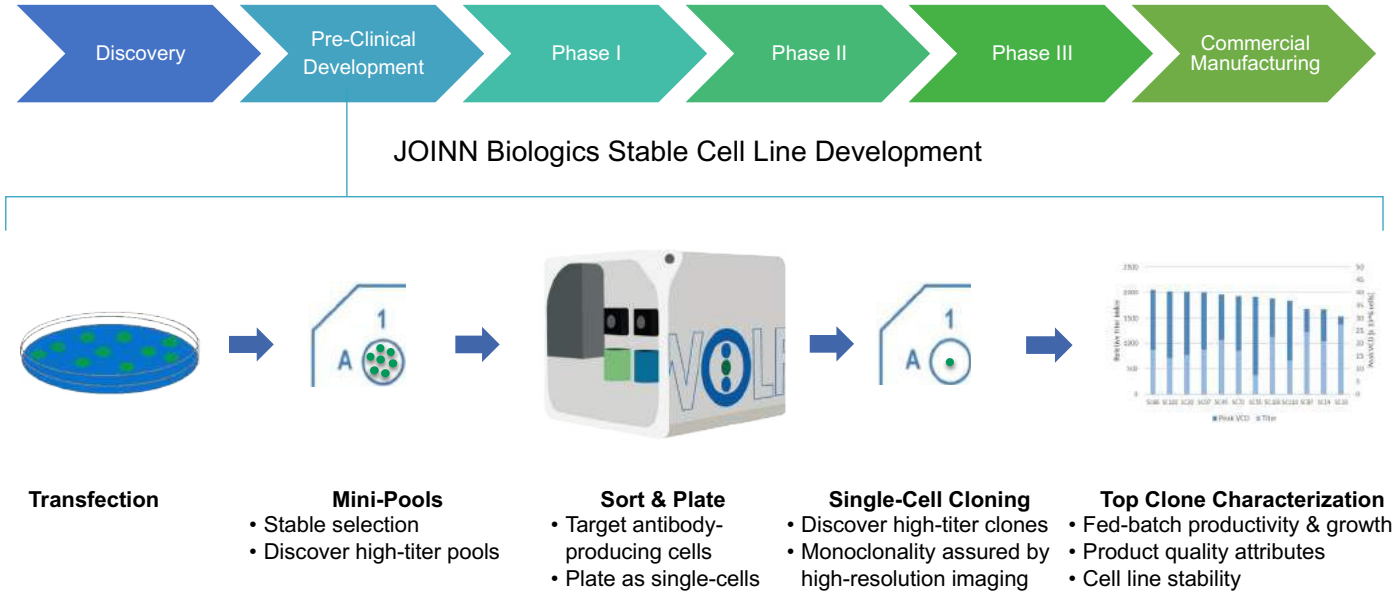


Figure 1. The WOLF replaces limiting dilution to improve JOINN Biologics' protein and antibody production workflow: CHO cells are transfected to produce the desired antibody and tested as mini pools for high titer levels. Instead of plating via limiting dilution, the highest producing pools are sterilely sorted based on antibody secretion, plated as single cells, and evaluated for antibody titer. The highest titer single-cell clones are then characterized for a variety of attributes.

© 2020 NanoCellect Biomedical, Inc. All rights reserved.
APN-009 2020-04
9525 Towne Centre Dr., Suite 150, San Diego, CA 92121
(877) 745-7678 info@nanocellect.com

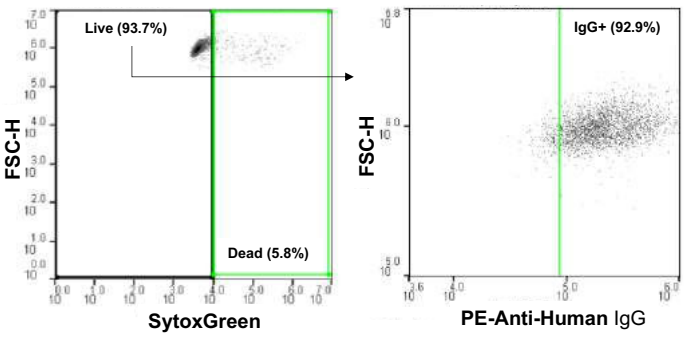


Figure 2. Antibody-secreting cells were detected and sorted on the WOLF: Dead cells were eliminated from the 100% transfected population via SytoxGreen positive staining and 92.9% of the Live-gated cells were PE-Anti-Human IgG+.

Method

To simulate varying levels of transfection efficiency and evaluate the WOLF's accuracy in detecting antibody-secreting cells, varying amounts of stably transfected CHO cells were mixed with wild-type. The transfected cells, which produce a recombinant human IgG antibody, were titrated at total concentrations of 100%, 50%, 20%, and 10%. All centrifugations, incubations, and media steps were kept at 4oC to minimize changes in secretory activity. Cells were centrifuged at 350 x g for 5 minutes, washed once, and resuspended at 1x105 cells/mL in media. Cells were incubated in the dark with PE-anti-human IgG for 20 minutes and washed twice with media. SytoxGreen was added at 2 drops/mL and incubated in the dark for 15 minutes.

Cells negative for SytoxGreen and positive for PE-anti-human IgG were sorted at 4°C, using ice-cold media as sheath (Figure 2), and dispensed into 96-well plates with room temperature media. One plate was sorted and dispensed for each concentration and analyzed for outgrowth with the CloneSelect™ Imager. Wells with outgrowth were analyzed for Protein A binding with the Octet Red96e.

Results

The WOLF was able to detect IgG+ cells for each concentration mix (Figure 3A, left) and yielded similar levels of single-cell antibody-producing colonies, despite the varying initial concentrations. On average, 70% of the cell outgrowth on each plate was from a single antibody-producing cell (Figure 3A, right). This on-target outgrowth is significantly higher than the average 43% (2.6% CV) seen in previous studies for a 0.5 cell/well limiting dilution with a very robust CHO cell line. The CV for outgrowth of single-cell producing clones was 7.2% across all four plates and the Octet Red96e confirmed titers of 15 to 80mg/L for each of the single-cell clones (Figure 3B).

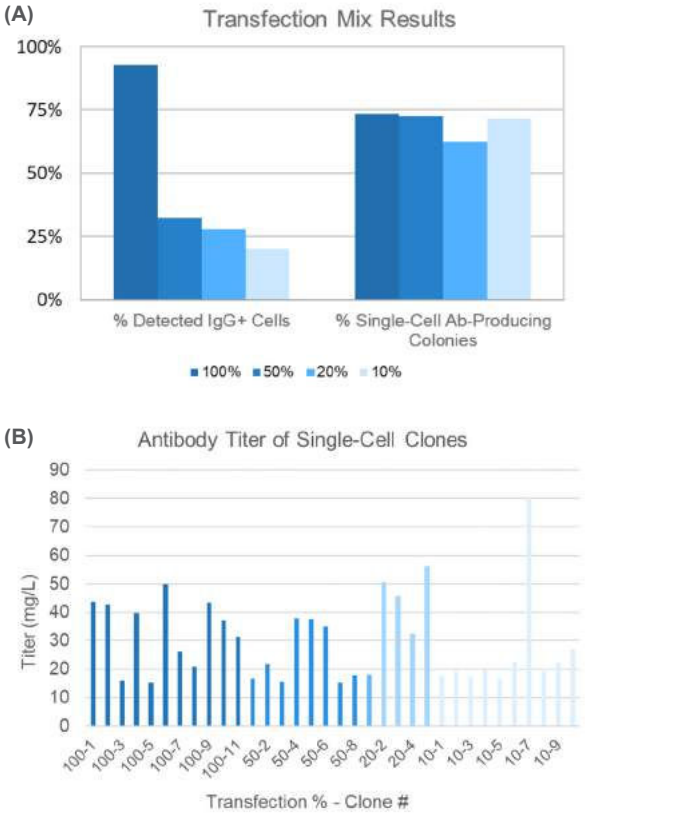


Figure 3. Single-cell antibody-producing clones are consistently isolated despite initial target population: (A) For each plate, single-cell antibody-producing clones made up 73%, 73%, 63%, and 71% of the wells with cell outgrowth. **(B)** Single cells sorted from the varying transfection mixes yielded high-producing clones.

Conclusion

The WOLF accurately detects and sorts antibody-secreting cells at target populations as low at 10% with maximum monoclonal outgrowth and titer levels. This methodology can expedite development of high-producing, clonal stable cell lines from novel transfections in the antibody production process. The WOLF's fluorescence-activated cell sorting capabilities, gentle pressure, and sterile fluidics make it a powerful tool to accelerate therapeutic antibody workflows.

For more information, visit nanocellect.com or email info@nanocellect.com

References

1. Krasny, A. & Morachis, J. M. (2019). WOLF Exceeds Limiting Dilution Cell Cloning Efficiency. Retrieved from https://nanocellect.com/scientific-content/
2. Krasny, A., Chen, H., Daniells, W., Bozek, E., Leary, B., Alaynick, W., & Morachis, J. M. (2019). Single-Cell Cloning for Cell Line Development. Retrieved from https://nanocellect.com/scientific-content/

© 2020 NanoCellect Biomedical, Inc. All rights reserved.

nanocellect Biomedical, Inc.

Application Note

Induced Pluripotent Stem Cell Preparation for 10x Genomics Applications

Nicole Jagnandan, Ph.D.

Introduction

Induced Pluripotent Stem Cells (iPSCs) are adult cells, typically collected from the skin or blood, that are genetically reprogrammed to have embryonic properties. iPSCs have made a dramatic impact in biological research where they are used to study tissue and organ development, drug discovery, and a wide range of diseases. Notably, iPSCs can be derived from affected patients, such as Alzheimer's, to produce disease-in-a-dish models. Furthermore, single cell genomics, such as single-cell RNA sequencing (scRNA-seq), has become a valuable tool to study the diversity and heterogeneity of cells contributing to a phenotype. Transcriptional profiling of pluripotent cells is of extreme importance to scientists because it facilitates the discovery of disease development at the molecular and cellular level. iPSCs are fragile cells and mechanical stress can obscure or skew the scRNA-seq results by activating genes related to cell stress or differentiation, rather than the process of interest. Therefore, it is important to minimize mechanical stress when handling cells. Furthermore, the quality of cDNA libraries is improved by reducing dead cells and debris prior to cDNA generation and library preparation. Fluorescence activated cell sorting is a common technique used by researchers to enrich specific cell populations while avoiding dead cells and debris. However, conventional cell sorting methods that use a high-pressure droplet-based sorting cause mechanical shear stress on cells; inducing gene expression changes and/or RNA degradation.

The WOLF Cell Sorter is a gentle, low-pressure (<2 psi) microfluidic cell sorter. Using a low-pressure cell sorter reduces the shear stress on cells to enable healthier cells and higher RNA integrity post-sort. The WOLF also uses a disposable microfluidic cartridge so that everything the sample and sheath fluid touches is sterile, disposable, and free of sample-to-sample contamination. To demonstrate how this gentle, sterile cell sorter affects single-cell RNA transcriptomics in a 10x Genomics workflow, we compared sample preparation in unsorted iPSCs to cells sorted on either the NanoCellect WOLF or BD FACSAria™ II.

Method

Reprogrammed iPSCs from human skin fibroblasts (Coriell Institute, GM23338) were divided into three samples: 1) unsorted control; 2) cells sorted on a conventional high-pressure droplet sorter, the BD FACSAria II; and, 3) cells sorted on the low-pressure microfluidic WOLF Cell Sorter. Prior to sorting, propidium iodide (PI, ThermoFisher #R37169), was used to identify dead cells. On the BD FACSAria II, iPSCs were sorted by the Sanford Burnham Prebys Flow Core using a 100 µm nozzle and pressure reduced to 20 psi. On the WOLF, PBS+ 0.05% BSA was used as the sheath. WOLF sort gates were set to exclude debris using BCS-H/FSC-H, and to exclude doublets with FSC-H/FSC-W. Live cells were gated as the PI-negative population. BD FACSAria II sort gates were set to exclude debris using SSC-A/FSC-A, and to exclude doublets with FSC-H/FSC-W and SSC-W/SSC-A. Live cells were also identified as the PI-negative population. Sort plots are shown in Figure 1.

50,000 cells were sorted on both the BD FACSAria II and NanoCellect WOLF into a FACS tube precoated with 0.5 mL

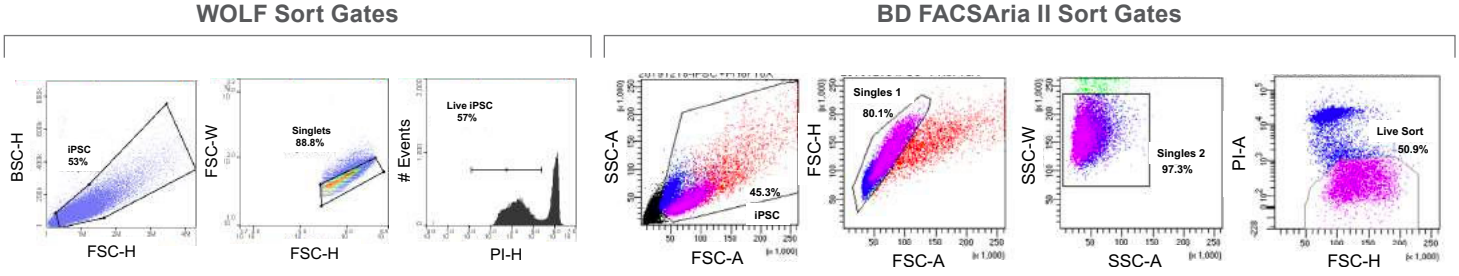


Figure 1. Sort Gates on the WOLF and BD FACSAria II: 50,000 live iPSCs were sorted on the NanoCellect WOLF and BD FACSAria II using scatter to sort single cells from debris and doublets. A viability dye, propidium iodide (PI), was used to identify dead cells.

© 2020 NanoCellect Biomedical, Inc. All rights reserved.
APN-010 2020-04
9525 Towne Centre Dr., Suite 150, San Diego, CA 92121
(877) 745-7678 info@nanocellect.com

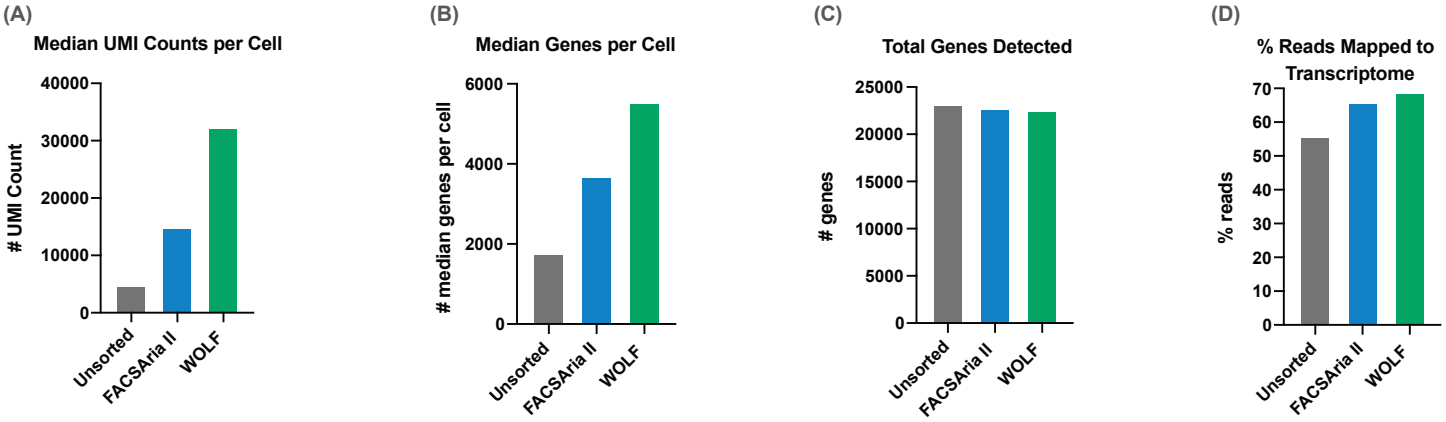


Figure 2. Cell Ranger Summary Results Comparing Microfluidic Sorting to Droplet Sorting to Unsorted: The WOLF Cell Sorter showed the best results, relative to the BD FACSAria II and Unsorted controls in **(A)** Median UMI Counts per Cell and **(B)** Median Genes per Cell. All three approaches appeared to reach a ceiling effect in **(C)** Total Genes Detected. Sorting by either droplet or microfluidic technologies was superior to not sorting in **(D)** % Reads Mapped to Transcriptome.

of PBS+0.05% BSA to increase cell recovery. All samples were then centrifuged and re-suspended at ~1000 cells/µL. An estimated 10,000 cells were then loaded into the 10x Chromium Controller. cDNA libraries were generated using the Chromium Next GEM Single Cell 3' Library Construction Kit v3.1 and sequenced with the Illumina NovaSeq 6000 Sequencing System. All samples were analyzed with the 10x Genomics Cell Ranger pipeline to evaluate the sample and sequencing quality.

Results

The Cell Ranger summary produces several key metrics about the RNA and sequencing quality of a sample. The WOLF sorted sample 'Median UMI Counts per Cell' were increased nearly 7-fold increase (32,239) over the unsorted population (4,668) and 2-fold over the BD FACSAria II sorted population (14,949) (Figure 2A). A similar result was observed for 'Median Genes per Cell', where the WOLF had a 3-fold increase over the Unsorted cells, while the BD FACSAria II sorted sample had only 2-fold more than the Unsorted cells (Figure 2B). These results reveal that a higher library complexity is achieved when debris and dead cells are removed from the sample. Interestingly, we found a similar number of 'Total Genes Detected' in all three conditions (Figure 2C), perhaps due a ceiling effect of sampling so many cells. Lastly, the '% Reads Mapped to Transcriptome' shows the percentage of the reads that uniquely map to a specific gene. A low percentage could indicate the presence of fragmented RNA that cannot be unequivocally mapped. Our results show an improved percentage of reads that mapped to the transcriptome in both the Aria (65.8%) and WOLF (68.8%) after sorting (Figure 2D).

Conclusion

This experiment demonstrates that using a cell sorter upstream of your 10x Genomics applications results in improved library complexity. Although library complexity was improved in the both the WOLF and Aria sorted sample, it was more improved in the WOLF sample, enabling you to get more data per cell. Furthermore, even though these results showed that there was a similar number of genes detected, there was still a higher percentage of reads that mapped uniquely to a specific gene. This suggests that there was less contamination in the sorted samples, which means you can have more confidence in the data. In summary, these results show that the WOLF serves as a robust cell sorter to use when preparing your samples for the 10x Genomics applications.

For more information, visit nanocellect.com or email info@nanocellect.com

© 2020 NanoCellect Biomedical, Inc. All rights reserved.

9525 Towne Centre Dr., Suite 150, San Diego, CA 92121
(877) 745-7678 info@nanocellect.com

nanocellect Biomedical, Inc.

Application Note

WOLF Exceeds Limiting Dilution Cell Cloning Efficiency

Aly Krasny and José Morachis, Ph.D.

Introduction

Monoclonal cell line generation from small-scale to high-throughput labs

Generating monoclonal cell lines is essential for a variety of scientific applications. From ensuring uniform gene expression in genetically engineered disease research lines to stabilizing production of antibodies for industrial manufacturers, there is a ubiquitous need for labs to isolate single cells for monoclonal cell line expansion. However, despite the demand for quick and cost-effective options, most labs still utilize the manual and inefficient cell cloning method known as limiting dilution (LD).

Manual limiting dilution methods limit productivity

According to Poisson distribution statistics,[1] when cells are diluted and dispensed at 1 cell/well, 37% of wells should contain a single cell. More dilute concentrations can further eliminate doublets, with expected frequencies of 19% to 30% single-cell wells for 0.25 to 0.50 cells/well concentrations, respectively. These statistics are often inflated by the assumption that all viable clones have the same random chance of monoclonality,[2] however, even under perfect conditions, 2/3 of each plate is still rendered useless. This wastes a large amount of supplies and time to produce sufficient monoclonal colony numbers.

Improve time, cost, and expression efficiency with the WOLF

The NanoCellect WOLF Cell Sorter significantly reduces all these resources wasted by the limiting dilution method. The WOLF and N1 single cell dispenser use sterile disposable microfluidic cartridges to analyze, sort, and dispense single cells into 96- or 384-well plates in as little as 8 minutes with over 90% efficiency.[3] The WOLF is small enough to fit within a biosafety cabinet to maintain sterility; it provides 2 scatter and 3 fluorescent channels for selection of your cells of interest. Below, Chinese Hamster Ovarian (CHO) cells, the leading cell line used by industrial manufacturers for protein production, are used to demonstrate the substantially higher efficiency of the WOLF when compared with a range of limiting dilution concentrations.

Methods

CHO-K1 cells were mixed 50:50 with CHO-K1-GFP cells to mimic a 50% effective transfection and stained with a viability dye (7-AAD) to indicate dead cells. Sort gates were set on the viable singlets with the top 10% of GFP fluorescence intensity (Figure 1A). Single cells were dispensed by the N1 into two 96-

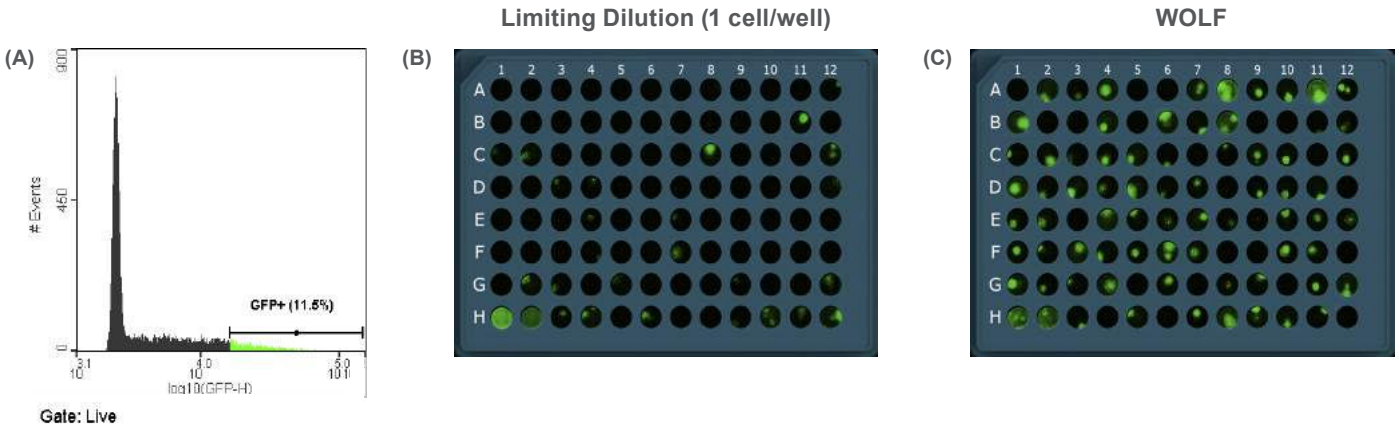


Figure 1. Top GFP expressing cells were sorted, plated, and imaged for outgrowth on Day 14. (A) A 50:50 mix of CHO-K1/CHO-K1-GFP+ mix was stained with 7-AAD viability dye at 1x10^5 cells/mL, sorted on 7AAD- singlets with the top 11.5% fluorescence intensity, and plated to two 96-well plates. **(B)** Limiting dilution and **(C)** WOLF plates were imaged for brightfield and green fluorescence and evaluated for number of GFP+ colonies, eliminating wells with multiple GFP+ colonies, GFP- colonies, and artifacts. Wells H1 and H2 contain focusing controls.

© 2020 NanoCellect Biomedical, Inc. All rights reserved.
APN-017 2019-09
9525 Towne Centre Dr., Suite 150, San Diego, CA 92121
(877) 745-7678 info@nanocellect.com

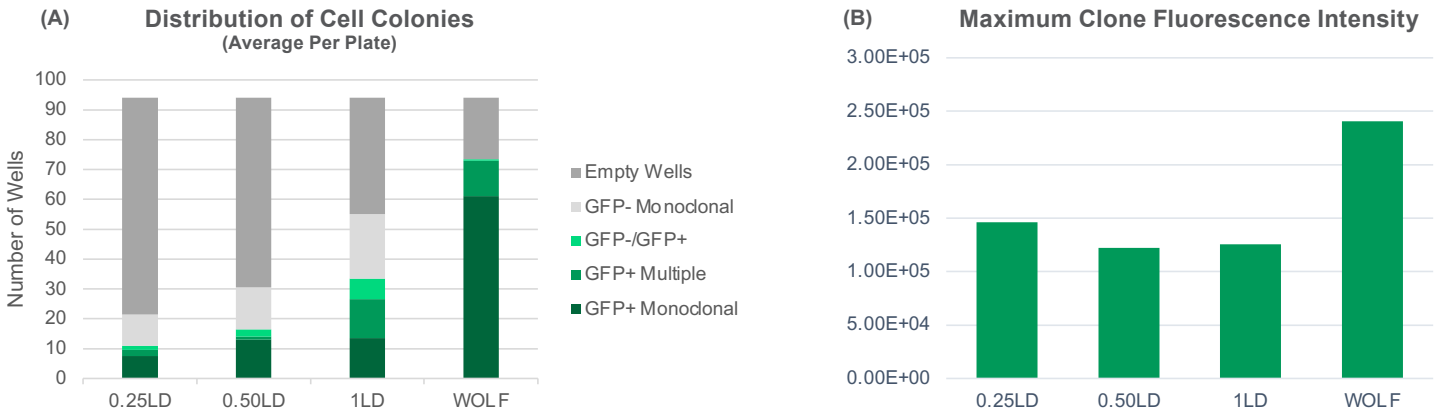


Figure 2. Count and characterization of cell colonies: (A) Colony counts were averaged between both plates of each condition (2 focusing wells not included). **(B)** The fluorescence intensity for each well was background corrected, summed for all cell fluorescence in the well, and evaluated to identify the well with maximum sum of fluorescence for each condition.

well tissue culture-treated plates filled with Ham's F-12 media, supplemented with 10% FBS and 2x Antibiotic-Antimycotic. The 50:50 mix of CHO-K1 and CHO-K1-GFP cells was also diluted and plated at 0.25, 0.5, and 1 cell/well into two 96-well plates per condition and all plates were incubated for two weeks. Extra cells were added to wells H1 and H2 to facilitate focusing prior to imaging each plate. Brightfield and green fluorescence images were captured for each plate on Day 0 and Day 14 on the SYNENTEC's NyOne™ (Figures 1B, 1C).

Results

WOLF increases monoclonal colony count 8-fold and doubles expression level

After culturing the cells for 14 days, plate images were manually evaluated for clonal outgrowth. Compared to the limiting dilution method, there was a significant increase (up to 8-fold) in the abundance of GFP+ monoclonal colonies dispensed by the WOLF (Figure 2A). The colony quality was also improved, with maximum clone fluorescence intensity almost twice as high as the limiting dilution clones (Figure 2B).

Eliminate the need for downstream characterization

Dispensing with the WOLF decreased GFP- colony outgrowth 30-fold, yielding only a single colony across two plates (Figure 2A). With this low level of off-target interference, downstream clone characterization needs may be decreased or even eliminated.

Conclusion

Using the WOLF for single cell cloning assays results in a significant decrease in plating time, supplies needed, and increases the gene expression levels in the lead bioproduction cell line. In conclusion, the WOLF Cell Sorter can drastically increase efficiency and productivity of any cell cloning workflow.

For more information, visit nanocellect.com or email info@nanocellect.com

References

1. Coller, H. A.; Coller, B. S. Poisson statistical analysis of repetitive subcloning by the limiting dilution technique as a way of assessing hybridoma monoclonality.Methods Enzymol. 121:412: 1986.
2. Underwood, P. A.; Bean, P. A. Hazards of limiting-dilution method of cloning hybridomas. J. Immunol. Methods 68:89; 1988.
3. Krasny, A., Chen, H., Daniells, W., Bozek, E., Leary, B., Alaynick, W., & Morachis, J. M. (2019). Single-Cell Cloning for Cell Line Development. Retrieved from https://nanocellect.com/scientific-content/

© 2020 NanoCellect Biomedical, Inc. All rights reserved.

9525 Towne Centre Dr., Suite 150, San Diego, CA 92121
(877) 745-7678 info@nanocellect.com

nanocellect Biomedical, Inc.

Application Note

Maximizing Protein Production with WOLF Bulk Sorting

Aly Krasny, Walker Daniels, José M. Morachis, Ph.D.

Introduction

Cell-based protein production systems, in addition to providing commercial lab products such as detection antibodies, are becoming increasingly important for the development and manufacture of biotherapeutics.[1] In efforts to boost protein production, focus is often placed on improving bioreactor systems. However, there is also room for improvement upstream in selecting better, high-producing cells.

The WOLF Cell Sorter makes it possible to significantly enrich a cell population for high expressors with its bulk sorting capabilities. The easy-to-use WOLFViewer software, small footprint (2ft^3), and affordable price point make it a good fit for labs with either limited or advanced experience in flow cytometry. The instrument's ability to enhance protein expression is shown below with the most commonly used cell line in bioproduction, Chinese Hamster Ovarian (CHO) cells.

Method

CHO-K1 cells were mixed 50:50 with CHO-K1-GFP cells to mimic a mixed population of GFP expression levels.Final concentration was 3x10^5 cells/mL. Cells were stained with a 7-AAD viability dye (Bio-Rad ReadiDrop) to eliminate dead cells, and doublets were eliminated with a FSC-H v FSH-W singlet gate. Viable singlets with the top ~15% of GFP+ expression were sorted. Cells were returned to culture for at least one week in Ham's F-12 media, supplemented with 10% FBS and 2x antibiotic-antimycotic. This was repeated four times with the cell outgrowth from each previous week's sort.

Conclusion

Over 5 generations of sorting, the WOLF increased GFP+ production ~18-fold. This upstream process optimization could quickly boost the presence of antibody-producing cells in a heterogenous population, which could also be used upstream of monoclonal selection without spending substantial time and money learning to operate a large, complex instrument. The WOLF makes the powerful technology of fluorescence-activated cell sorting accessible and simple for any lab to add it to their workflow.

For more information, visit nanocellect.com or email info@nanocellect.com

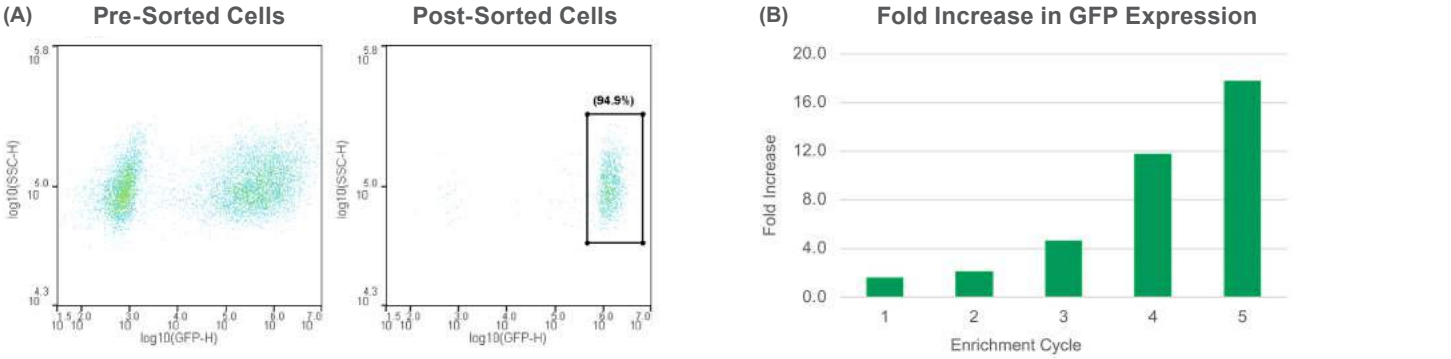


Figure 1. Top GFP-expressing cells isolated during successive bulk sort and 1-week outgrowth cycles yield increasing GFP fluorescence. (A) CHO-GFP cells were mixed 50:50 with CHO-K1 cells for a final 3x10^5 cells/mL concentration. 7-AAD negative singlets with the top 10-15% GFP fluorescence were sorted for 94.9% purity of high-expressors. **(B)** After one week of outgrowth, GFP median fluorescence intensity (MFI) values were measured for the sorted population and background corrected with CHO-K1 MFI for auto-fluorescence and inter-assay variability. Expression increased 17.7-fold (4.88x10^5 to 8.66x10^6 MFI) over 5 cycles.

References

1. Jayapal KP, Wlaschin KF, Hu W-S, Yap MGS. Recombinant protein therapeutics from CHO cells-20 years and counting. Chem Eng Prog. 2007;103:40–47.

© 2020 NanoCellect Biomedical, Inc. All rights reserved.
APN-018 2019-09
9525 Towne Centre Dr., Suite 150, San Diego, CA 92121
(877) 745-7678 info@nanocellect.com

nanocellect Biomedical, Inc.

Application Note

Increase HEK293T Single-Cell Clonal Outgrowth Under Standard Conditions

Adonary Munoz, Aly Krasny

Introduction

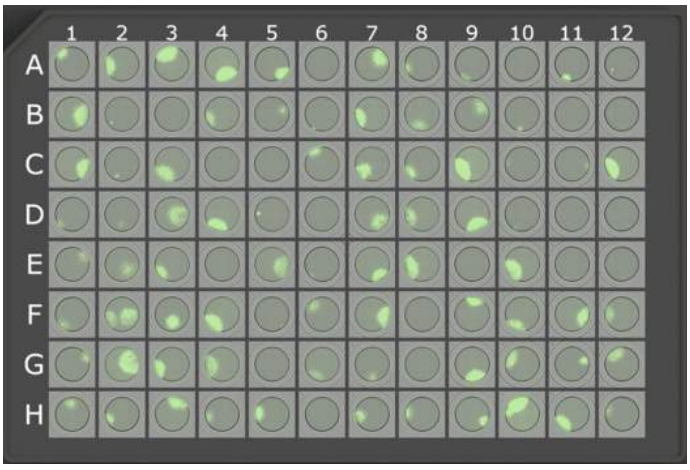
The HEK293T cell line is increasingly used in biotherapeutic protein production due to its human origin, but often becomes very fragile after transfection. The WOLF Cell Sorter and N1 Single Cell Dispenser offer a gentle platform to efficiently sort and dispense single transfected cells for high, targeted monoclonal outgrowth and production.

Methods like limiting dilution or low throughput and cell printers do not offer selectivity of the transfected population and some may be harsh on cells. Traditional fluorescent-activated cell sorters solve some of these problems, but are also harsh with high-pressure sorting mechanisms that can limit colony outgrowth and require use of conditioned media or coated plates.[1,2] Below, we show that the WOLF and N1 can successfully sort GFP-transfected HEK293T cells with high outgrowth, free of specialty supplies or conditions.

Method

Adherent HEK293T-GFP cells were diluted to 1x10^{5}cells/mL in HBSS (Gibco, catalog# 14175095)/2%FBS (GenClone, catalog #25-514H) for sorting. Untransfected HEK293T cells served as the negative control for GFP+ gating in the FL1 channel. GFP+ events were sorted and dispensed into three 96-well plates and three 384-well plates, respectively pre-filled with 200µL and 50µL of DMEM (GenClone, catalog #25-501)/10%FBS/1%anti-anti (Gibco, catalog #15240-062). This was repeated twice more, for a total of three cartridges and 9 plates of each type. The sorted cells were then placed undisturbed in an incubator at 37°C with 5% CO_2. On Day 14, outgrowth was imaged with the NYONE® (SYNENTEC) and GFP+ colonies were counted (Figure 1).

(A)



(B)

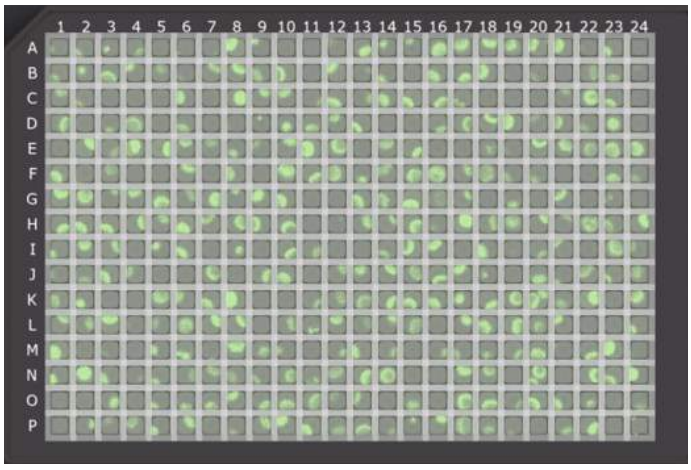


Figure 1. HEK293 GFP Outgrowth Images: (A) One of the 96-well plates and **(B)** 384-well plates imaged with the NyONE at day 14 to check for outgrowth based on GFP+ fluorescence.

© 2020 NanoCellect Biomedical, Inc. All rights reserved.
APN-022 2020-12
9525 Towne Centre Dr., Suite 150, San Diego, CA 92121
(877) 745-7678 info@nanocellect.com

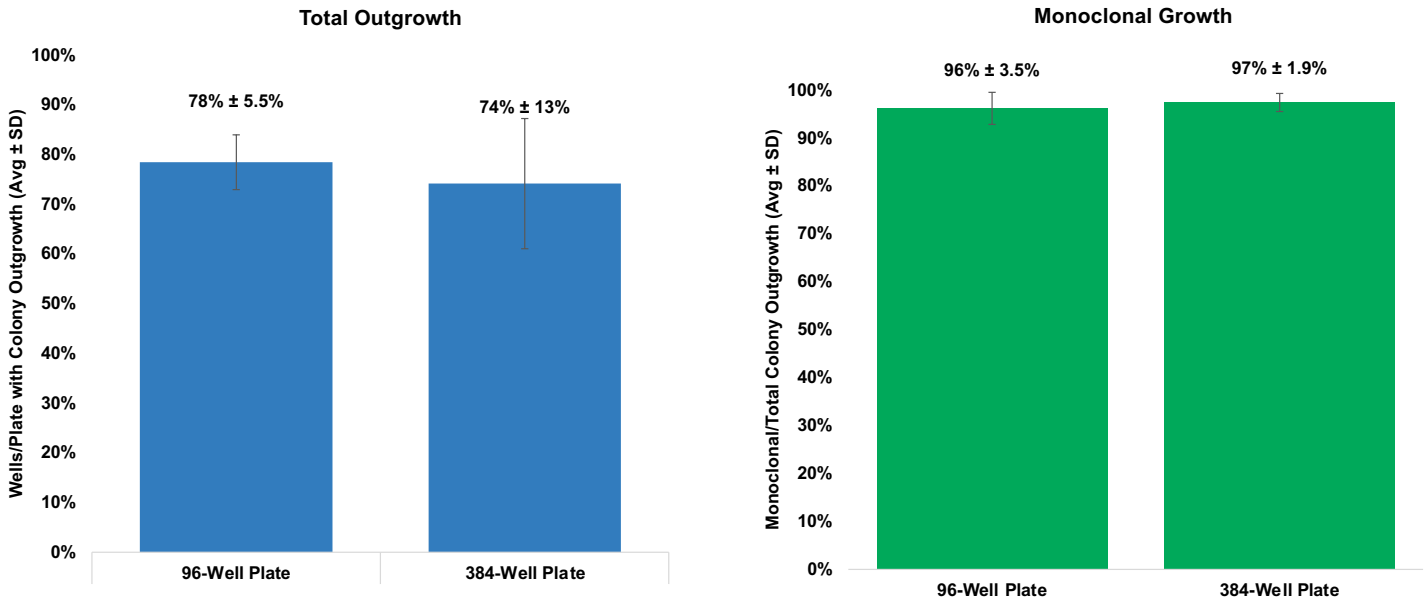


Figure 2. Summary of HEK293 GFP Outgrowth: (A) Total outgrowth of HEK293-GFP cells averaged 78%±5.5% and 74%±13% for 96-well and 384-well plates respectively (n=9 for each plate type). **(B)** From the wells containing colonies, 96%±3.5% and 97%±1.9% were single colonies for 96-well plates and 384-well plates, respectively.

Results

The WOLF was able to produce >70% monoclonal HEK293T GFP+ colonies in either plate without specialized media, plates, or growth conditions. On average, 78% of 96-well plates and 74% of 384-well plates contained GFP+ colonies (Figure 2A). Of those colonies, 96% on 96-well plates and 97% on 384-well plates were monoclonal (Figure 2B).

Conclusion

The WOLF significantly outperformed outgrowth expectations of limiting dilution.[3] It yielded far more HEK293T-GFP outgrowth than the <10% quoted for a cell printer without specialized media.[4] Traditional sorters, though able to enrich for GFP+ cells, would likely produce similar results to the cell printer due to their harsh sorting conditions.

Even though these were stable HEK293T lines, the baseline outgrowth performance indicates the WOLF is the ideal platform for cell line development of sensitive transfected HEK lines.

For more information, visit nanocellect.com or email info@nanocellect.com

References

1. Liste-Calleja, L., Lecina, M., & Cairó, J. J. (2013). HEK293 cell culture media study: increasing cell density for different bioprocess applications. BMC Proceedings, 7(Suppl 6), P51. https://doi.org/10.1186/1753-6561-7-S6-P51
2. Yu, S. P., & Kerchner, G. A. (1998). Endogenous voltage-gated potassium channels in human embryonic kidney (HEK293) cells. Journal of neuroscience research, 52(5), 612–617. https://doi.org/10.1002/(SICI)1097-4547(19980601)52:5<612::AID-JNR13>3.0.CO;2-3
3. Krasny, A. & Morachis, J. M. (2019). WOLF Exceeds Limiting Dilution Cell Cloning Efficiency. Retrieved from https://nanocellect.com/scientific-content/
4. Solentim. (2020). Clonality and improved outgrowth for HEK 293 cells when developing stable producer cell lines for viral vector production in gene therapy. Retrieved from https://www.solentim.com/resources/clone-screening-resources-application-notes/

© 2020 NanoCellect Biomedical, Inc. All rights reserved.

9525 Towne Centre Dr., Suite 150, San Diego, CA 92121
(877) 745-7678 info@nanocellect.com

nanocellect Biomedical, Inc.

Technical Note

Fast Plating Mode Increases Single-Cell Cloning Throughput

Adonary Munoz, Aly Krasny, José M. Morachis, Ph.D., and Michael Benchimol, Ph.D.

Introduction

Single-cell genomics, antibody discovery, cell line development, and gene editing can all benefit from single-cell plating as part of their respective workflows. NanoCellect's WOLF Cell Sorter and N1 Single Cell Dispenser are established tools for each of these workflows. The N1 dispenses single cells into 96- and 384-well plates with complete sterility and high viability. Under standard settings, it completes a 96-well plate in 9 minutes and a 384-well plate in 33 minutes. However, these settings have now been optimized to reduce plating times by three-fold and increase the overall productivity of single-cell dispensing. Here, this new Fast Plating Mode performance was verified.

Method

15 µm Dragon Green Beads (Bangs Labs, #FSDG009) were prepared at 1.0x105 beads/mL in PBS. Beads were sorted based on scatter and dispensed with Fast Plating Mode into three 96-well and three 384-well plates. This was repeated for a total of 5 cartridges. Control plates were dispensed with standard settings at the beginning and end of the experiment for each cartridge. Plates were imaged and single-bead wells were counted using the NyOne® (SYNENTEC). Single-bead dispensing efficiency (single-bead wells/total wells) was averaged for each mode and plate type.

Results

Fast Plating Mode reduced dispense time at least three-fold – 384-well plating dropped from 33 to 9 minutes and 96-well plating dropped from 9 to 3 minutes. In an eight-hour day, Fast Plating Mode could dispense 38 more 384-well or 107 more 96-well plates than Standard Mode (Figure 1C). The efficiency is still more than double the theoretical efficiency of manual limiting dilution1 and, at this higher speed, could yield up to a 216% improvement in daily single-cell cloning throughput.

Conclusion

The WOLF Cell Sorter and the N1 Single Cell Dispenser can now dispense single cells even faster with a limited impact on efficiency. With Fast Plating Mode, the user can substantially increase their productivity for a variety of downstream applications

For more information, visit nanocellect.com or email info@nanocellect.com

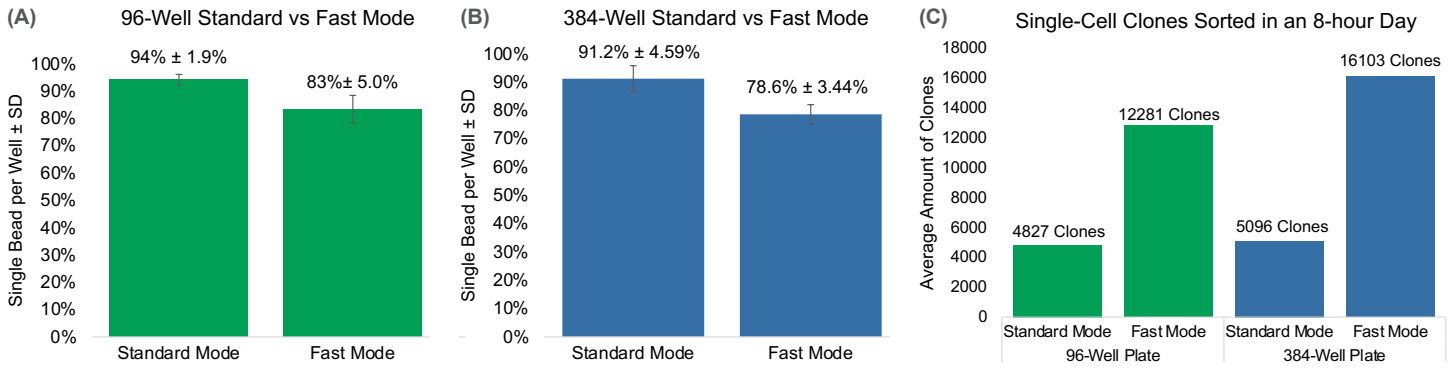


Figure 1. Fast Plating Mode can dispense 154.4-216.0% more clones with only 11-12.6% decrease in plating efficiency: **(A)** Single-bead plating efficiency decreased 11% for 96-well plates and **(B)** 12.6% for 384-well plates with Fast Plating Mode. **(C)** In a standard workday, at 3 minutes/plate and 83% efficiency, Fast Plating Mode could theoretically dispense 7,545 more single-cell clones to 96-well plates than Standard Mode at 9 minutes/plate, 94% efficiency. Similarly, at 9 minutes/plate instead of 33 minutes/plate, Fast Plating Mode could dispense 11,007 more clones to 384-well plates.

References

1. Krasny, A. & Morachis, J. M. (2019). WOLF Exceeds Limiting Dilution Cell Cloning Efficiency. Retrieved from https://nanocellect.com/scientific-content/

© 2020 NanoCellect Biomedical, Inc. All rights reserved.

9525 Towne Centre Dr., Suite 150, San Diego, CA 92121
(877) 745-7678 info@nanocellect.com

nanocellect
Biomedical, Inc.

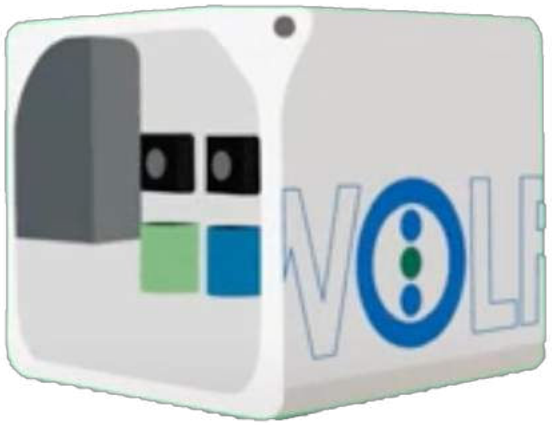
Happy Cells. Better Science.

Microfluidic Single Cell Sorting and Cloning

William Alaynick, PhD
Founder, VP International Sales

Overview

- **Introduction**
- **NanoCellect**
- **Resources and Support**
- **The WOLF**
- **Applications**



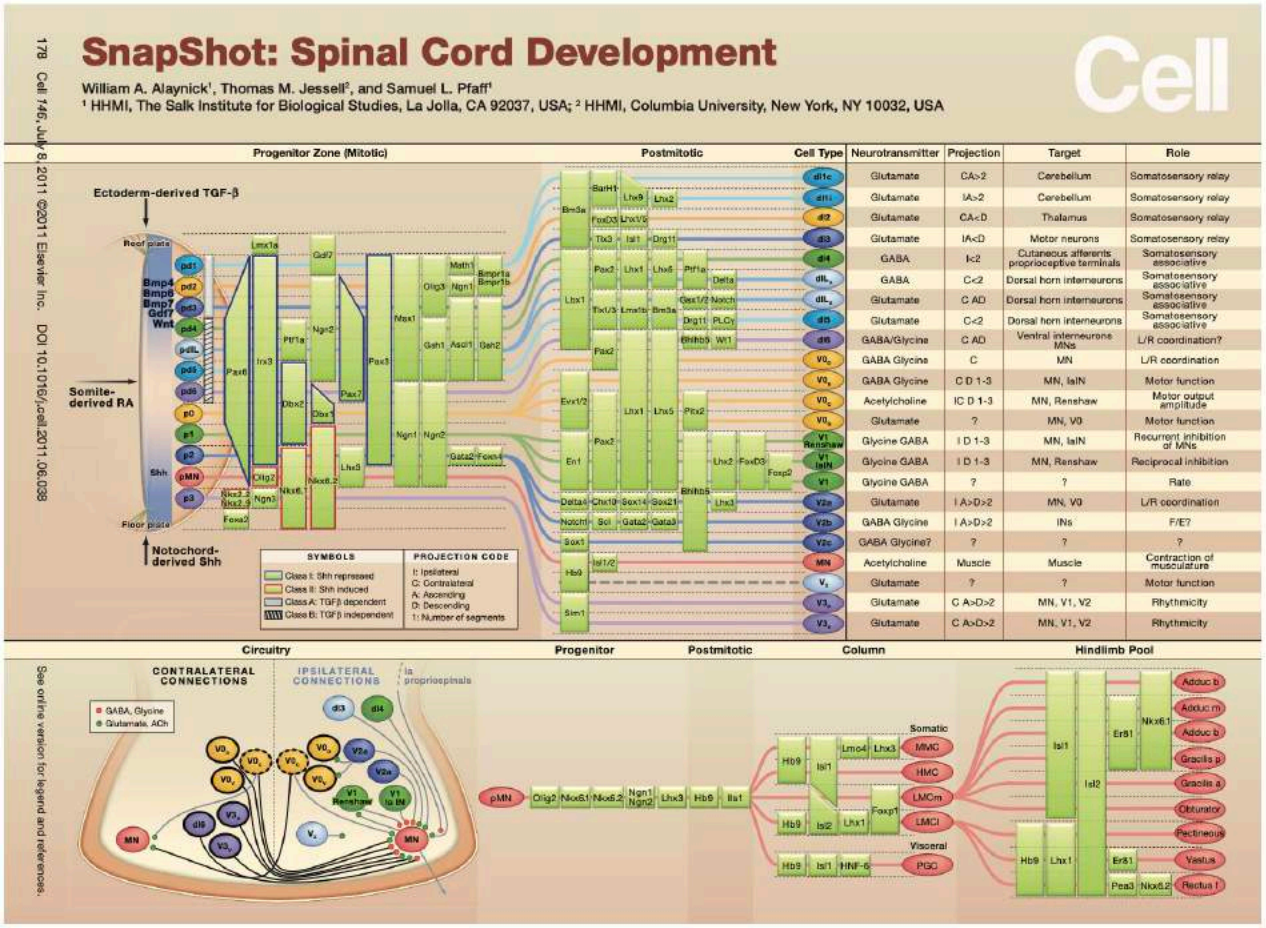
Strictly Confidential

© Copyright 2020 NanoCellect Biomedical. All rights reserved.

nanocellect Biomedical, Inc.

My Background: Academia to Industry

- ’98-’01: Tech HHMI UW
- ’01-’06: PhD HHMI/Salk/UCSD
- ‘06-’11: Post Doc HHMI/Salk
- ‘09-: Founder, NanoCellect
- ‘15-: Founder, Arima Genomics



Strictly Confidential

© Copyright 2020 NanoCellect Biomedical. All rights reserved.

nanocellect Biomedical, Inc.

NanoCellect's History

NanoCellect was founded in late 2009 as a spinout from UCSD.

The proprietary technology was validated by 20 NIH grants.

Series A & B rounds raised over $16M, and are backed by the following strategic investors:

illumina VENTURES™

Agilent Technologies

ANZU PARTNERS

FusionX ventures

VERTICAL VENTURE PARTNERS

Strictly Confidential

© Copyright 2020 NanoCellect Biomedical. All rights reserved.

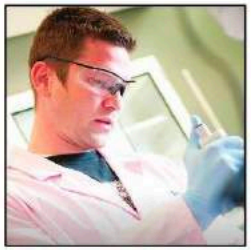
nanocellect Biomedical, Inc.

Global Customers

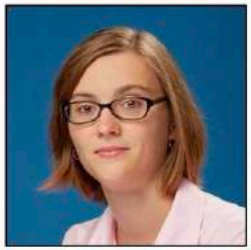
Customer Success Team

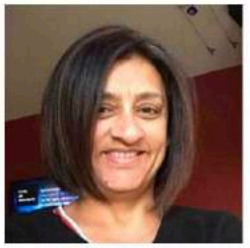
Field Application Scientists

Michal Bonar PhD


Jerome Karpiak PhD

Beth Leary PhD


Alena Bartakova MD

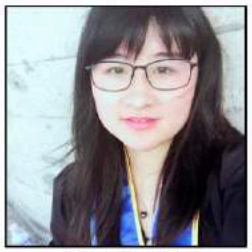

Bela Desai PhD

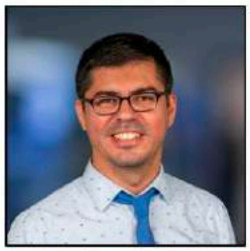
Tom Chu, PhD

Application Scientists

Nicole Jagnandan PhD

Aly Krasny BS


Lin Xia MS


Jose Morachis PhD

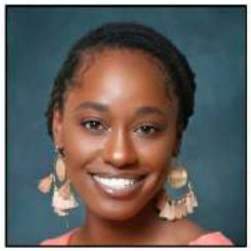
Will Alaynick PhD

Technical Support

Elena Torre PhD

Rebekah Whitty BS

Paul DiGregorio PhD


Jainabah Phillips BS

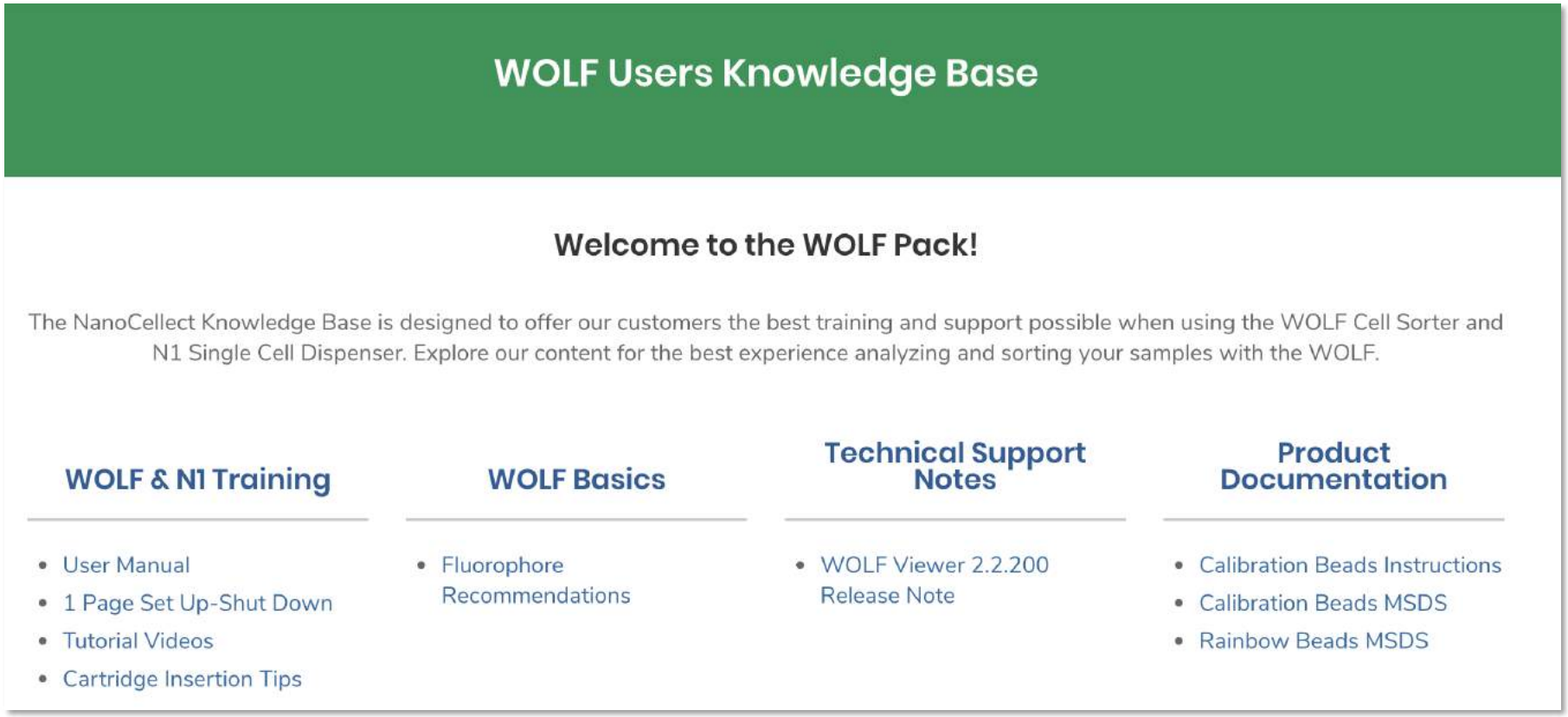
Ashley Lo BS

Strictly Confidential

© Copyright 2020 NanoCellect Biomedical. All rights reserved.

nanocellect Biomedical, Inc.

Extensive Online Resources



WOLF Users Knowledge Base

Welcome to the WOLF Pack!

The NanoCellect Knowledge Base is designed to offer our customers the best training and support possible when using the WOLF Cell Sorter and N1 Single Cell Dispenser. Explore our content for the best experience analyzing and sorting your samples with the WOLF.

WOLF & N1 Training

- User Manual
- 1 Page Set Up-Shut Down
- Tutorial Videos
- Cartridge Insertion Tips

WOLF Basics

- Fluorophore Recommendations

Technical Support Notes

- WOLF Viewer 2.2.200 Release Note

Product Documentation

- Calibration Beads Instructions
- Calibration Beads MSDS
- Rainbow Beads MSDS

Strictly Confidential

© Copyright 2020 NanoCellect Biomedical. All rights reserved.

nanocellect Biomedical, Inc.

Webinars

Available for on-demand viewing at nanocellect.com

Compatible Products
10x GENOMICS

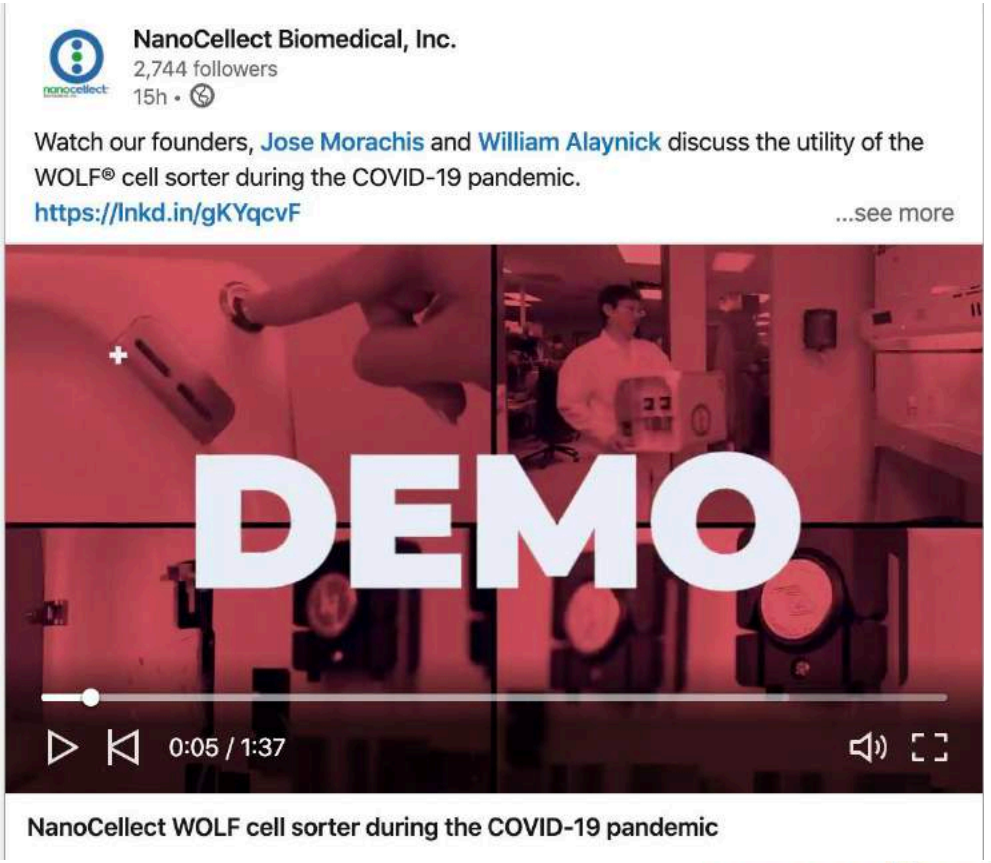
- July 9th: Labroots webinar with NanoCellect scheduled
- Engagement with 10x field team to position WOLF alongside the Chromium

Strictly Confidential
© Copyright 2020 NanoCellect Biomedical. All rights reserved.
nanocellect Biomedical, Inc.

Covid19 Stay Home Demo

- Overview of the WOLF with co-founders, Will Alaynick, PhD and Jose Morachis, PhD

- Available at nanocellect.com

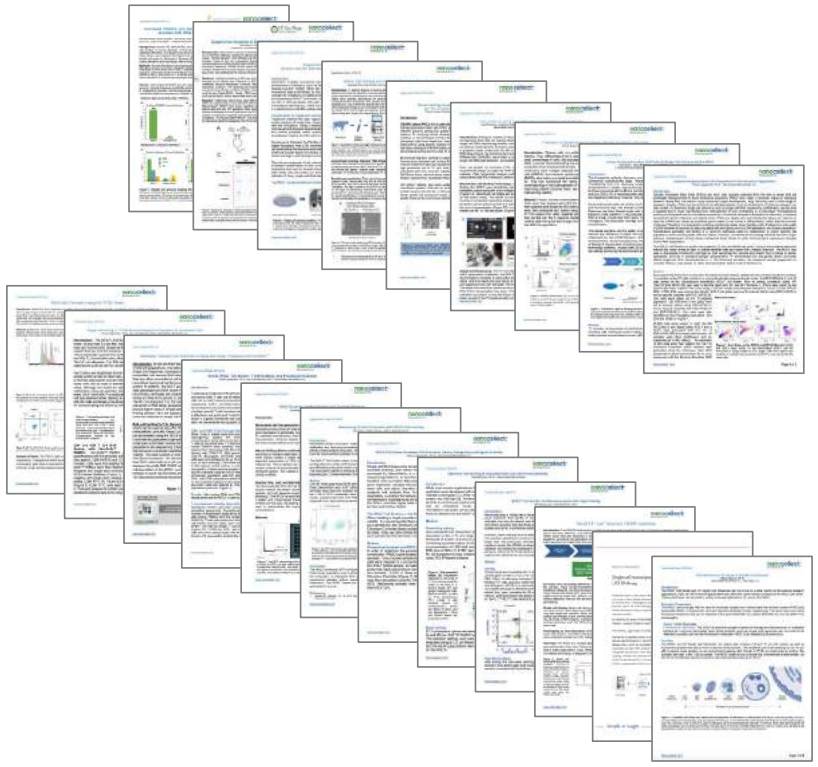


NanoCellect Confidential Information
© Copyright 2020 NanoCellect Biomedical. All rights reserved.

Application Notes

25 Application and Technical Notes

A001-Viability and Karotype of CRISPR-modified hiPSCs
A002-Epigenomic Analysis of Sorted Retinal Ganglion Cells using ATAC-seq
A003 Single-Cell Cloning for Cell Line Development
A004-Ab Disc from Single Bovine B Cells
A005-Gentle Sorting Accelerates Generation of Genome-Edited iPSCs
A006-High Viablilty cell sorting and 3'RNA-seq
A007-Purification of Multiple Myeloma Plasma Cells with the WOLF Cell Sorter
A009-Isolate Antibody-Secreting CHO Cells for Single-Cell Cloning with the WOLF
A010-iPSC for 10x Genomics Applications
T001-Multicolor Cytometry using the WOLF Sorter
T002-Single-cell Sorting of T cells from Lyophilized Cells
T003-Seamlessly Transition from Cell Sorter to SC Library Prep
T004-T cell Isolation and Functional Analysis
T005-The WOLF Exceeds Limiting Dilution Cell Cloning Efficiency
T006-Maximizing Protein Production with WOLF Bulk Sorting
T007-WOLF upstream of Chromium Controller
T008-Small Particle
T009-Neutrophil Purity and Viability
T0010-Performance Envelope of the WOLF
W001-The WOLF "cuts" time from CRISPR workflows
W002-Single-cell transcriptomics combining FACS with plate-based UPX RNA-seq



© Copyright 2020 NanoCellect Biomedical. All rights reserved.

It's Tough to get the Right Cells

Cell sorting has all the following challenges:

- **Poor sterility**
- **Long wait times** and **inconvenient travel** to a core facility
- **High costs** of constantly running more samples
- **Low efficiency** in workflow for single cell dispensing
- **Difficult to use** a complex instrument
- **Dead, dying, and high debris sample** output from high pressure instruments

Strictly Confidential

© Copyright 2020 NanoCellect Biomedical. All rights reserved.

nanocellect Biomedical, Inc.

Meet the WOLF

High viability: The industry leader for gentle cell sorting at <2 psi

Small Footprint: fits in a hood easily

100% **sterile** & **disposable** fluidics path and cartridge. **Safe**. Zero aerosols, no sample carryover.

Simple and **intuitive** software. Ready to use after 1 day of training.

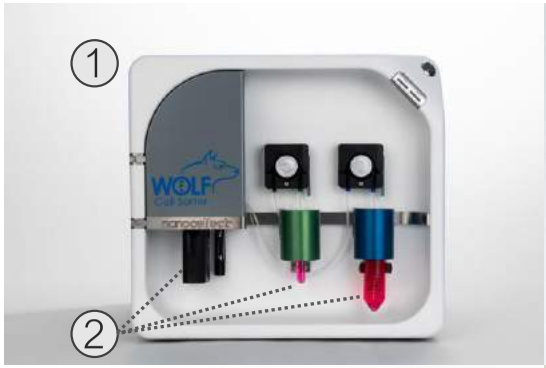
Fraction of the price compared to others

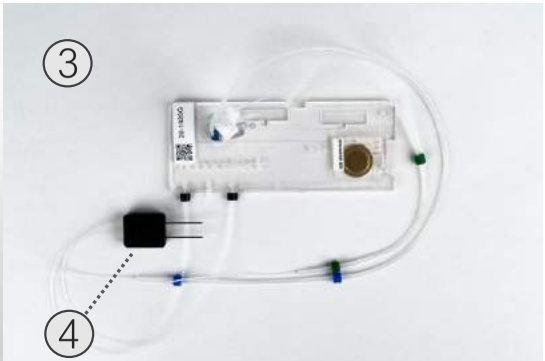
Strictly Confidential

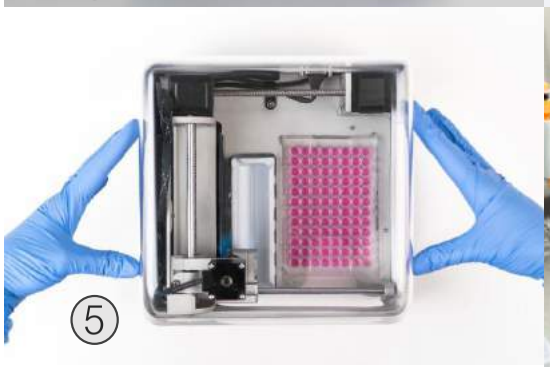
© Copyright 2020 NanoCellect Biomedical. All rights reserved.

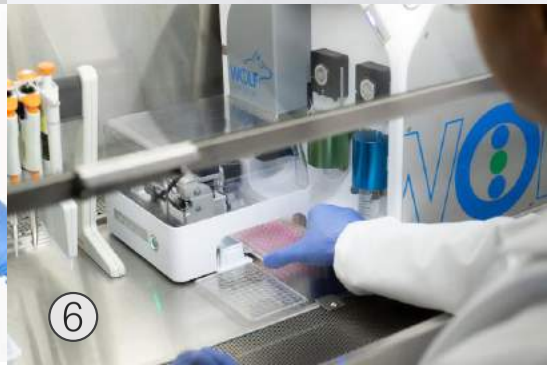
nanocellect Biomedical, Inc.

The WOLF Workflow: Simple and Easy









① WOLF Cell Sorter

② Sample collection, sample and sheath input

③ Microfluidic Cartridge

④ Single cell dispensing head

⑤ N1 Single Cell Dispenser

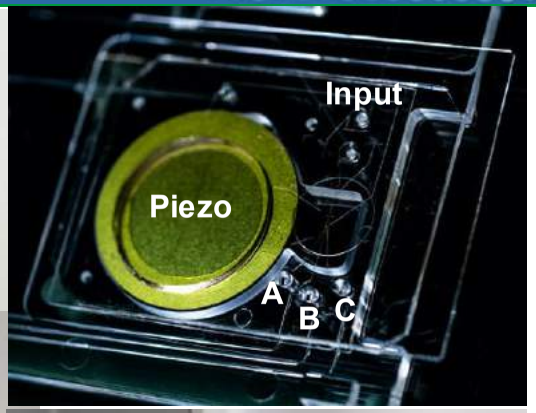
⑥ Entire system inside a TC hood

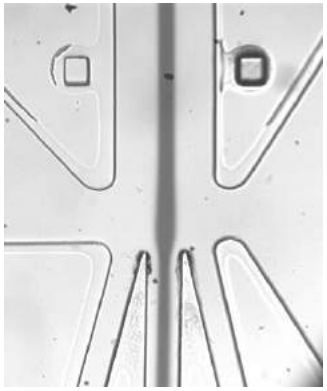
Strictly Confidential

© Copyright 2020 NanoCellect Biomedical. All rights reserved.

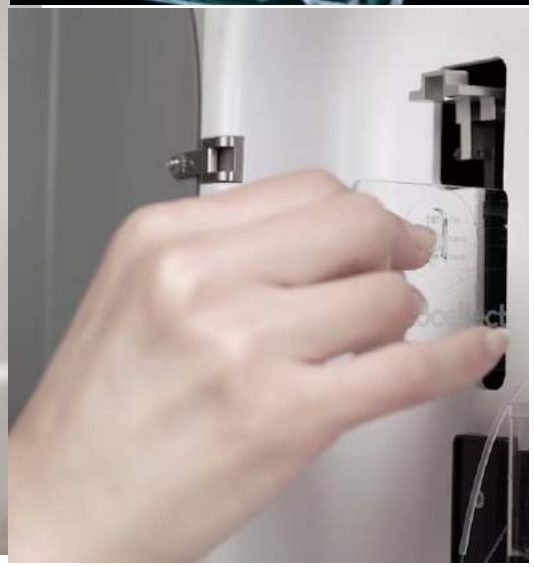
nanocellect Biomedical, Inc.

Key Innovation is the Cartridge





Ultra low sorting pressure at <2 psi
300 sorts per second

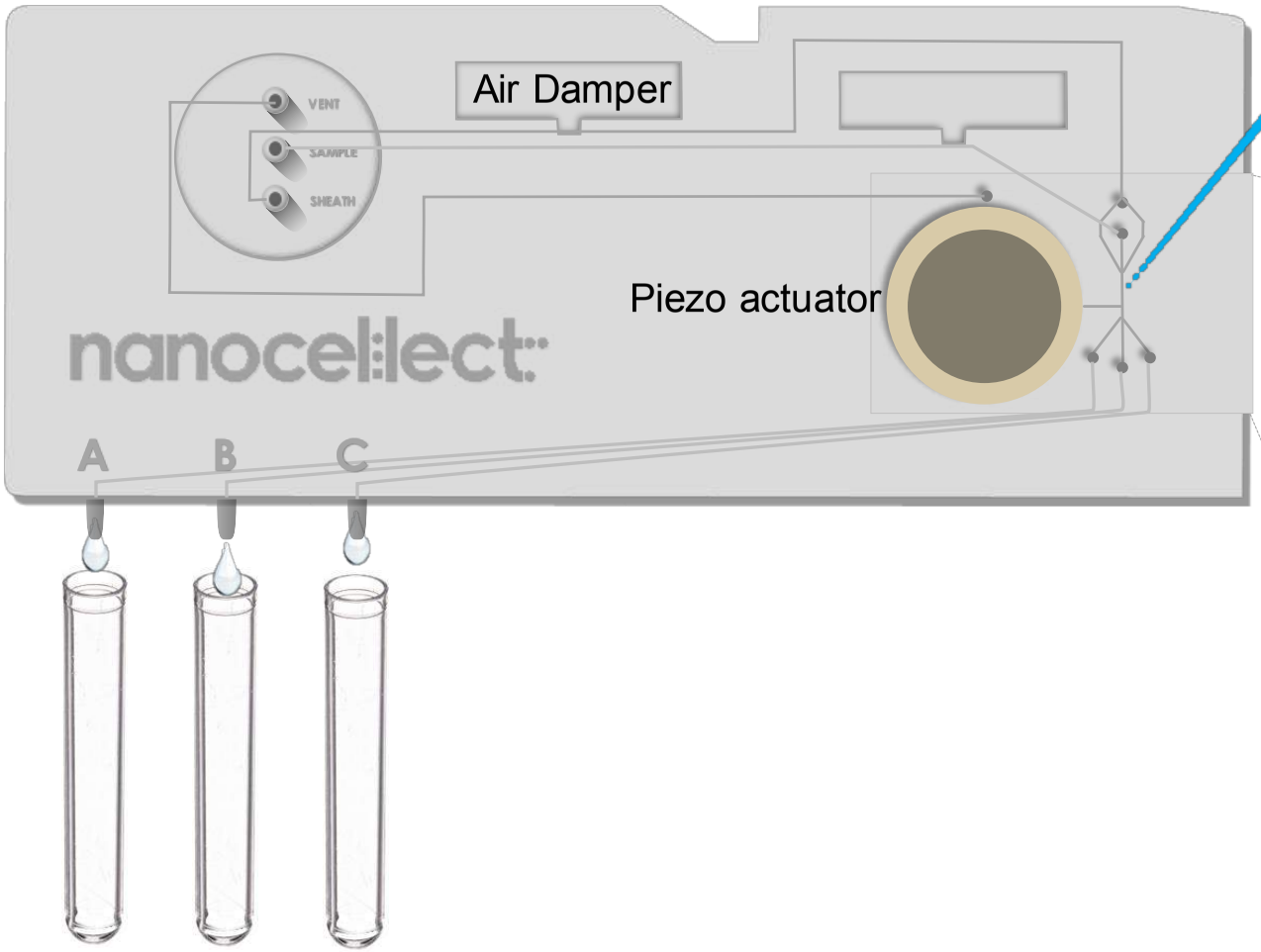


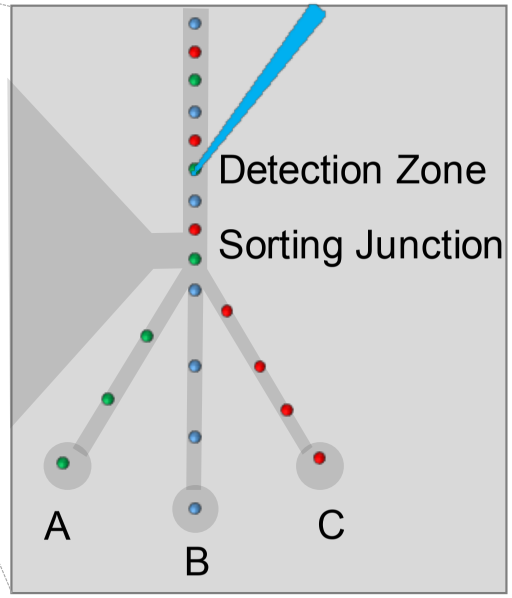
Strictly Confidential

© Copyright 2020 NanoCellect Biomedical. All rights reserved.

nanocellect Biomedical, Inc.

Cartridge Details





Strictly Confidential

© Copyright 2020 NanoCellect Biomedical. All rights reserved.

nanocellect Biomedical, Inc.

Droplet Sorters Damage Cells

Cuvette

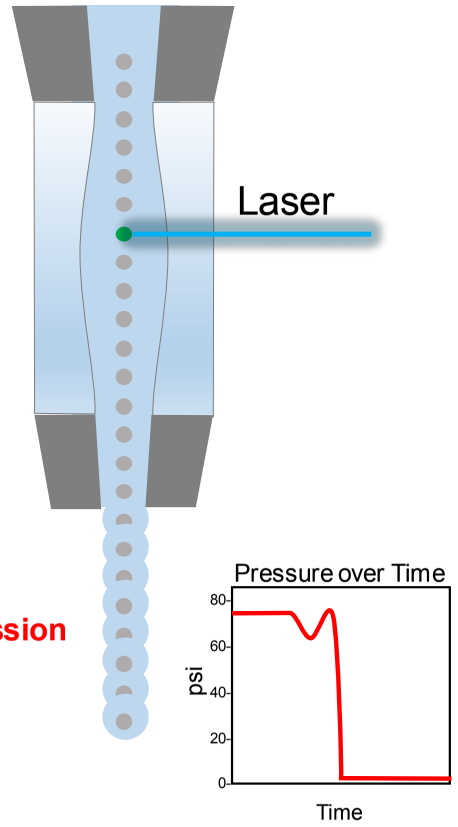
Jet-in-Air

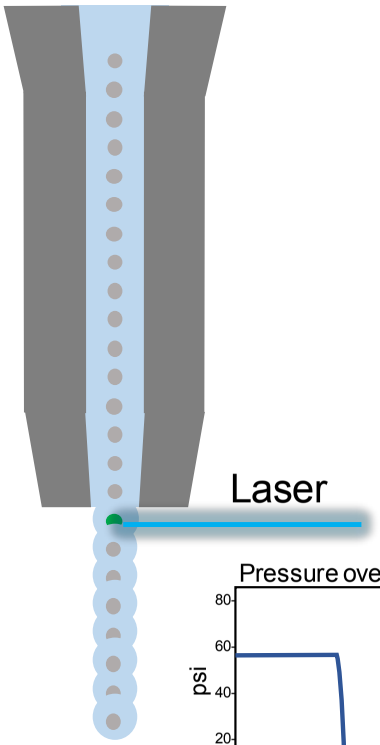
WOLF

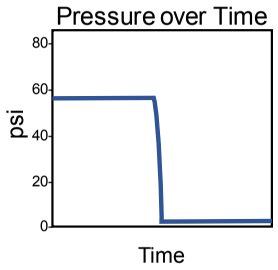
Flared Cuvette

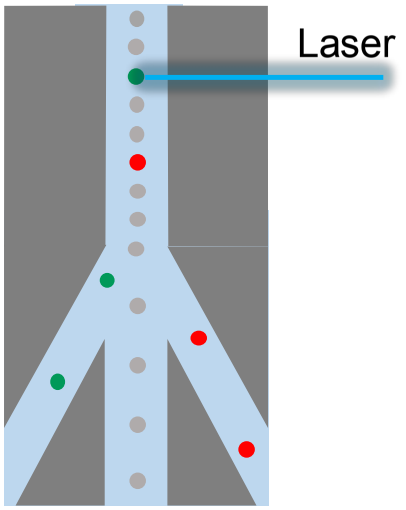
Nozzle Shear

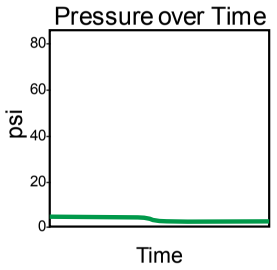
Decompression Shock









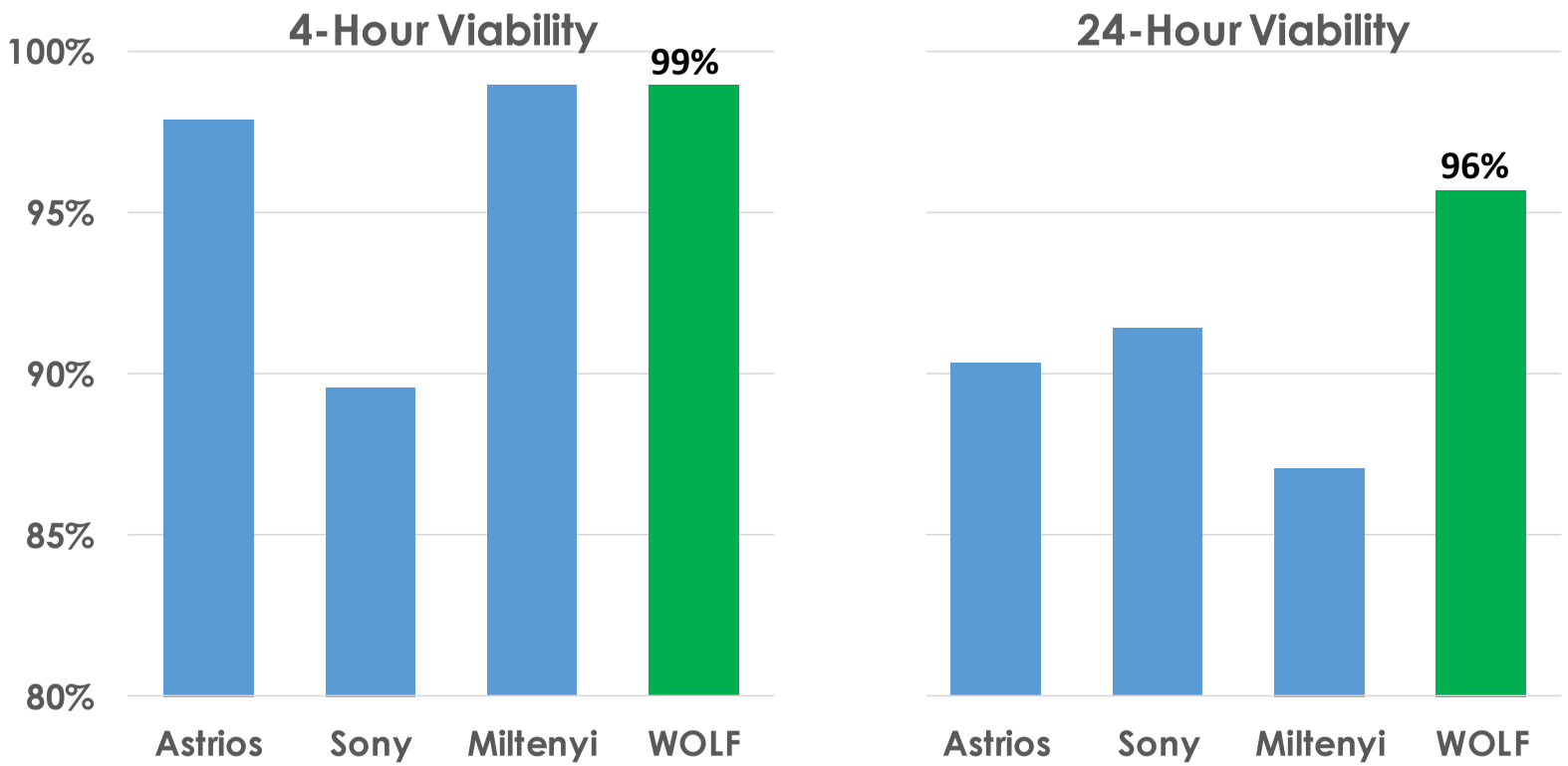


Strictly Confidential

© Copyright 2020 NanoCellect Biomedical. All rights reserved.

nanocellect Biomedical, Inc.

WOLF has Highest Post-Sort Viability



K562 cells. PI for dead and Calcein AM for live normalized to unsorted controls. Analyzed on a Beckman Coulter CytoFlex at 4 and 24 h post-sort.

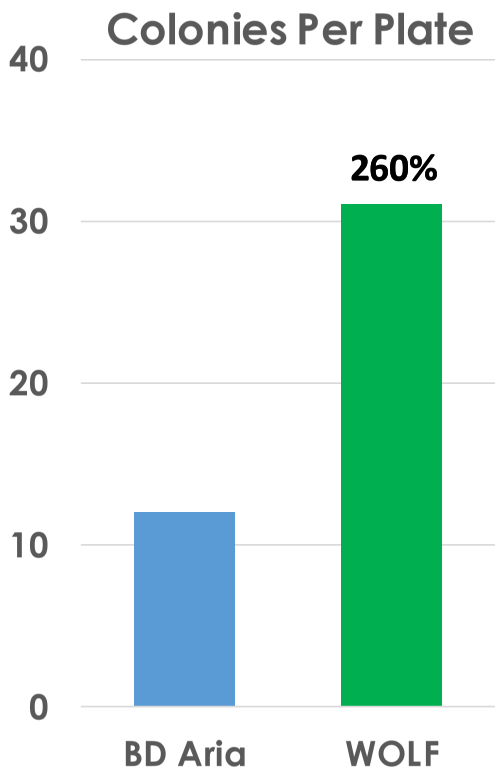
Adapted from Broad's Flow Core results presented at CYTO 2019

Strictly Confidential

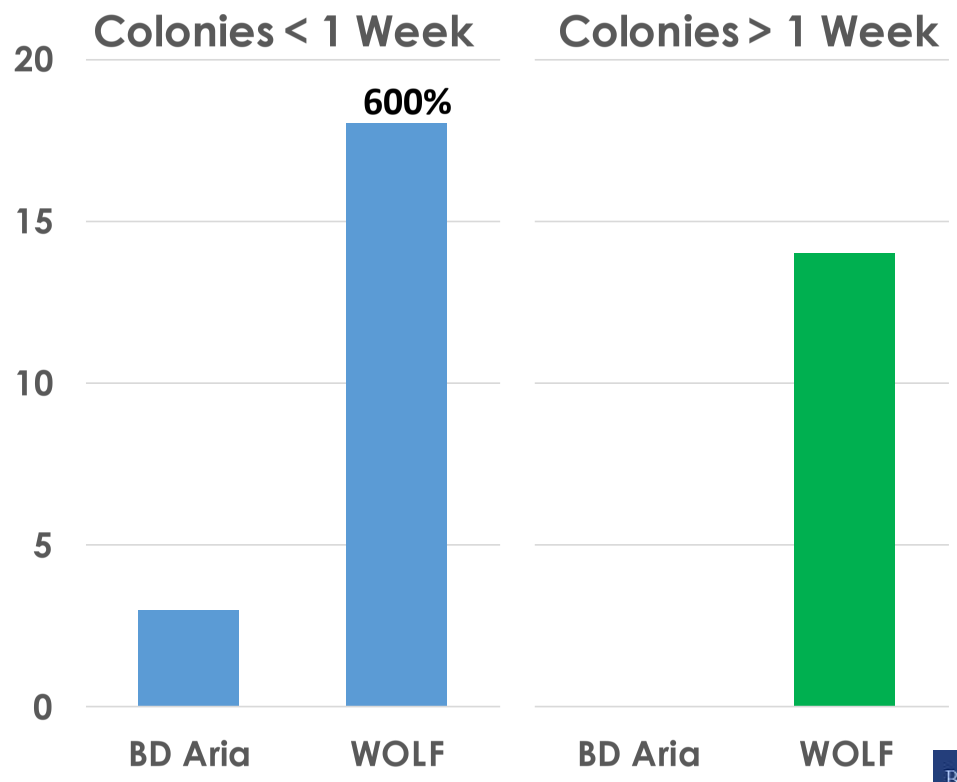
© Copyright 2020 NanoCellect Biomedical. All rights reserved.

nanocellect Biomedical, Inc.

WOLF has Higher Viability in Sensitive Cells



Top 10 Pharma



Academic, iPSCs

Baylor College of Medicine

Strictly Confidential

© Copyright 2020 NanoCellect Biomedical. All rights reserved.

nanocellect Biomedical, Inc.

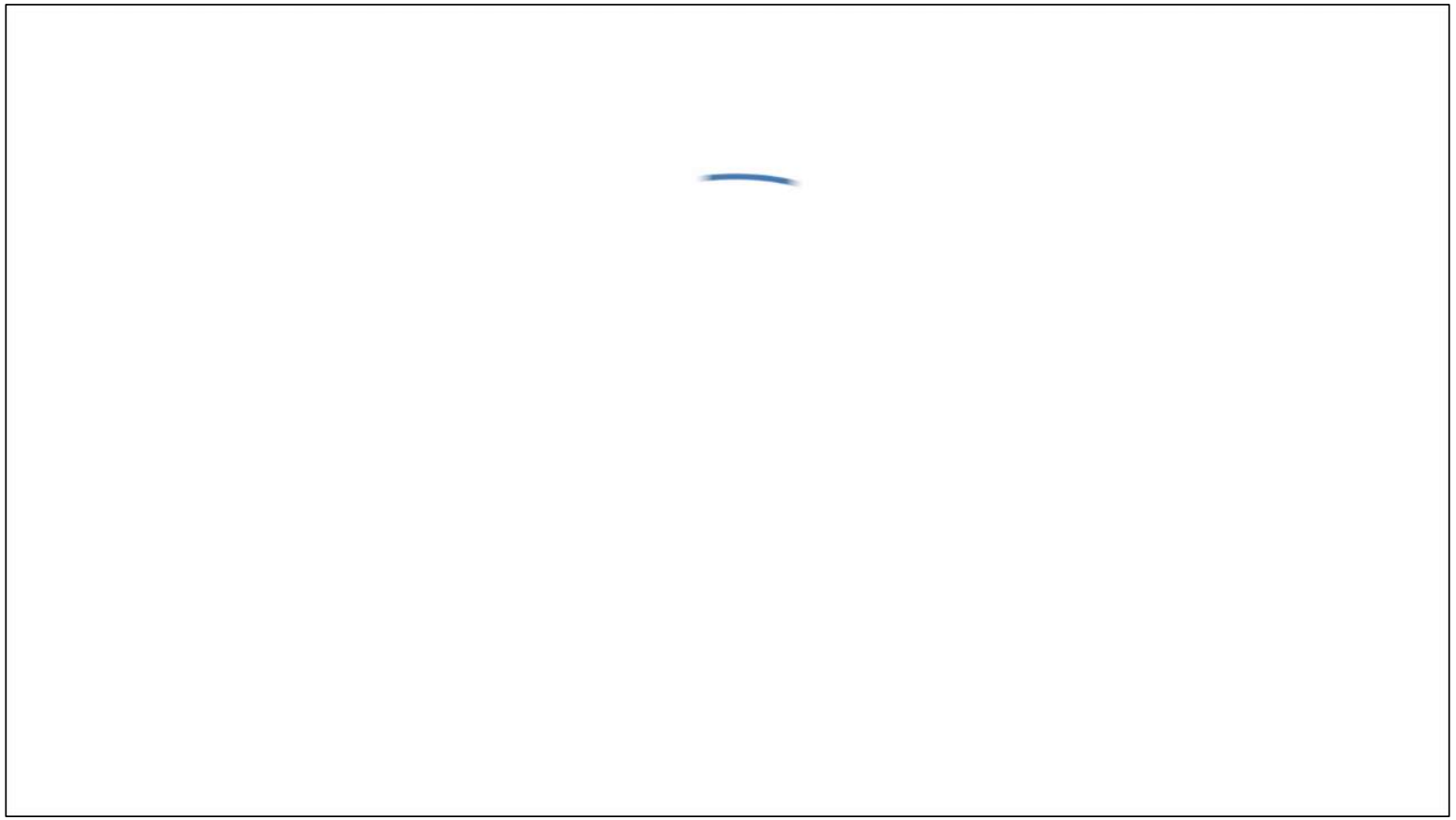
The N1 Single-Cell Dispenser

Strictly Confidential

© Copyright 2020 NanoCellect Biomedical. All rights reserved.

nanocellect Biomedical, Inc.

The N1 Single-Cell Dispenser

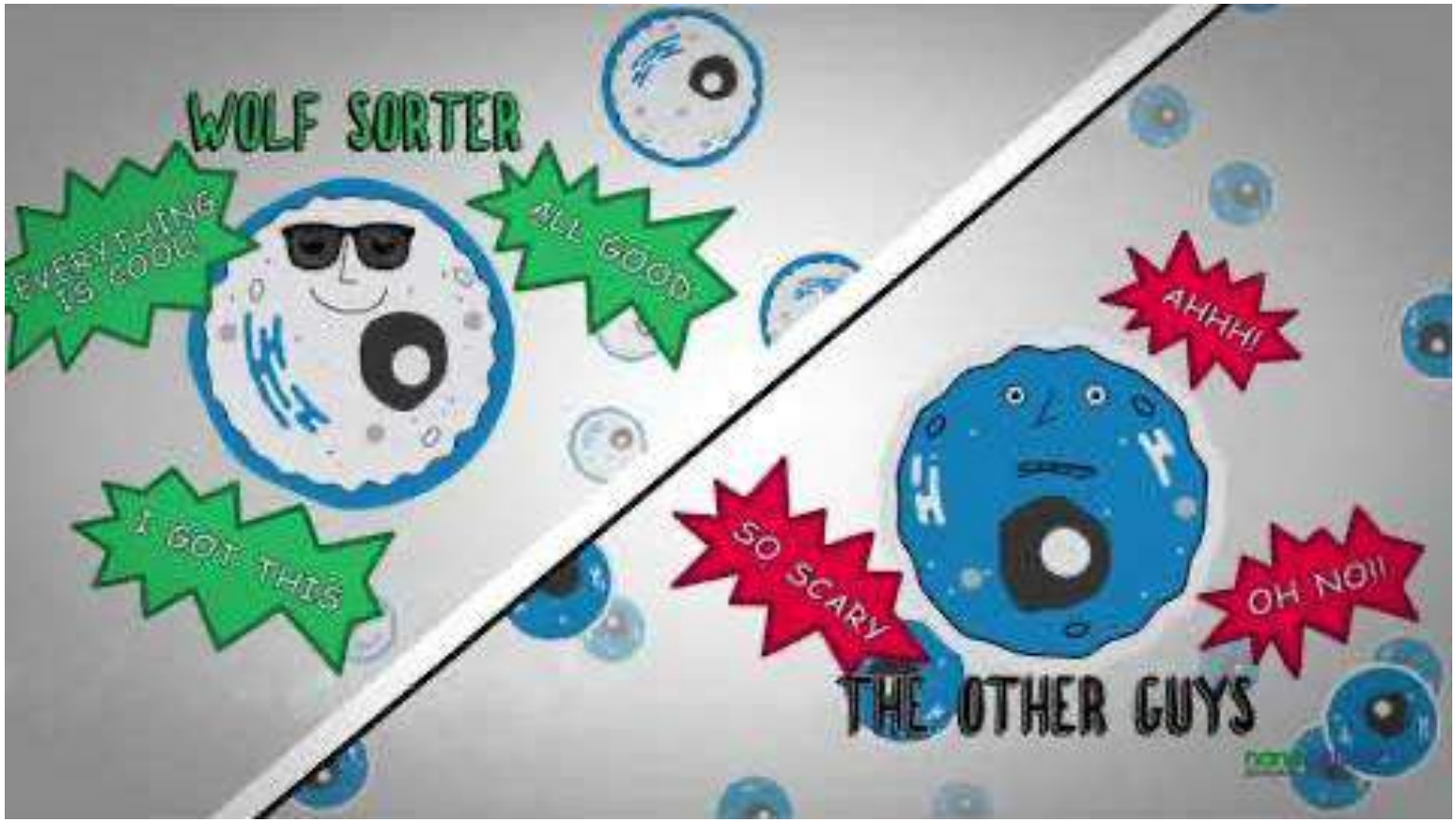


Strictly Confidential

© Copyright 2020 NanoCellect Biomedical. All rights reserved.

nanocellect Biomedical, Inc.

Happy Cells, Better Science



Strictly Confidential

© Copyright 2020 NanoCellect Biomedical. All rights reserved.

nanocellect Biomedical, Inc.

Happy Cells, Better Science

WOLF Cell Sorter

SMALL & EASY TO USE
- User-friendly
- Intuitive software

SAFEST
- No contamination
- No aerosols
- Sterile operation

• GENTLE
- Highest viability

LOW COST
- Fewer approvals required

Strictly Confidential
© Copyright 2020 NanoCellect Biomedical. All rights reserved.

Stay Sterile with the WOLF and N1

The WOLF and N1 easily fit in standard Biosafety Cabinets

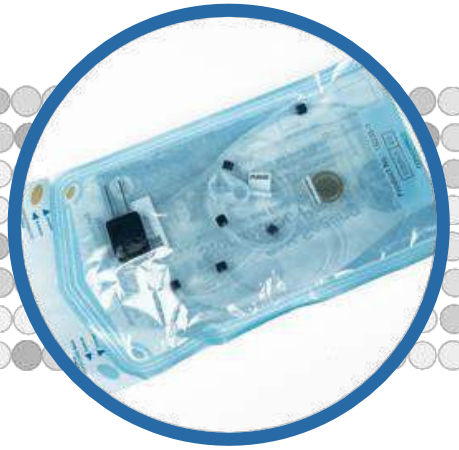
Strictly Confidential

© Copyright 2020 NanoCellect Biomedical. All rights reserved.

nanocellect Biomedical, Inc.

Stay Sterile with the WOLF and N1

All input and output tubing is sterile and included with each cartridge



Ethylene Oxide Sterilized Disposable Cartridges

100% disposable fluidic pathway

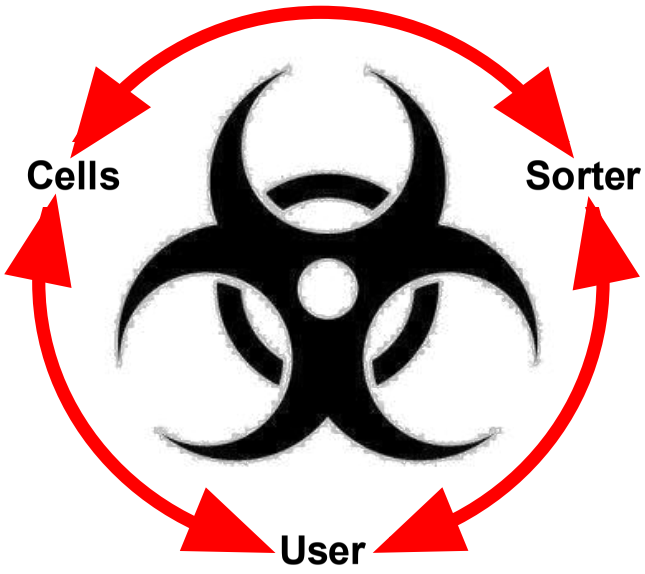
Strictly Confidential

© Copyright 2020 NanoCellect Biomedical. All rights reserved.

nanocellect Biomedical, Inc.

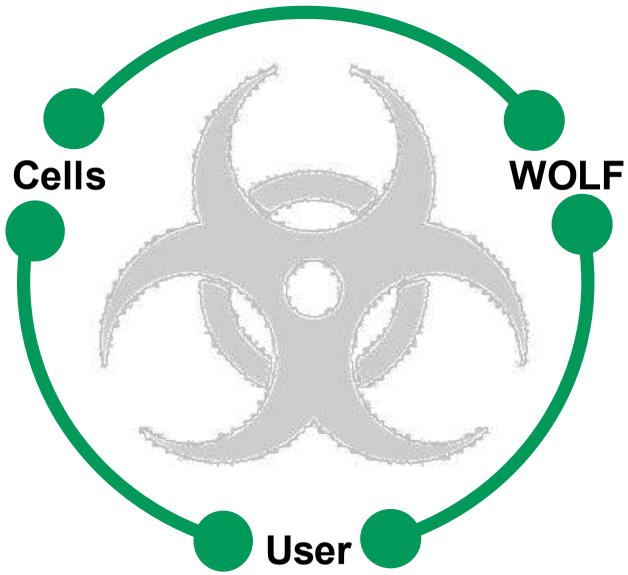
NanoCellect Protects Cells, Machine, and User

Aseptic: dilute and wash



Becton-Dickinson, Beckman-Coulter, Sony, Bio-Rad, ThermoFisher

Sterile: definitively eliminate microbes



NanoCellect

Strictly Confidential

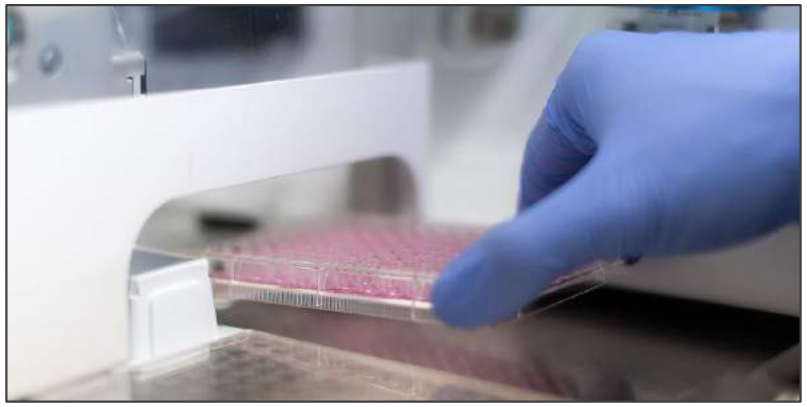
© Copyright 2020 NanoCellect Biomedical. All rights reserved.

nanocellect Biomedical, Inc.

Sort Into a Variety of Plate Types

The WOLF can sort into:

- **Flat-Bottom**
- **Round-Bottom**
- **V-Bottom**
- **Half-Area**
- **PCR**
- **Any plate with alignment tool**



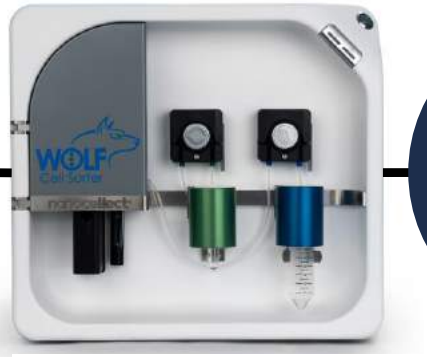
Strictly Confidential

© Copyright 2020 NanoCellect Biomedical. All rights reserved.

nanocellect Biomedical, Inc.

Cell Line Development Workflow

Transfection



Isolation & sterile plate seeding

Image Single Cells

Clone Expansion, Fed-Batch Culture

Process Development

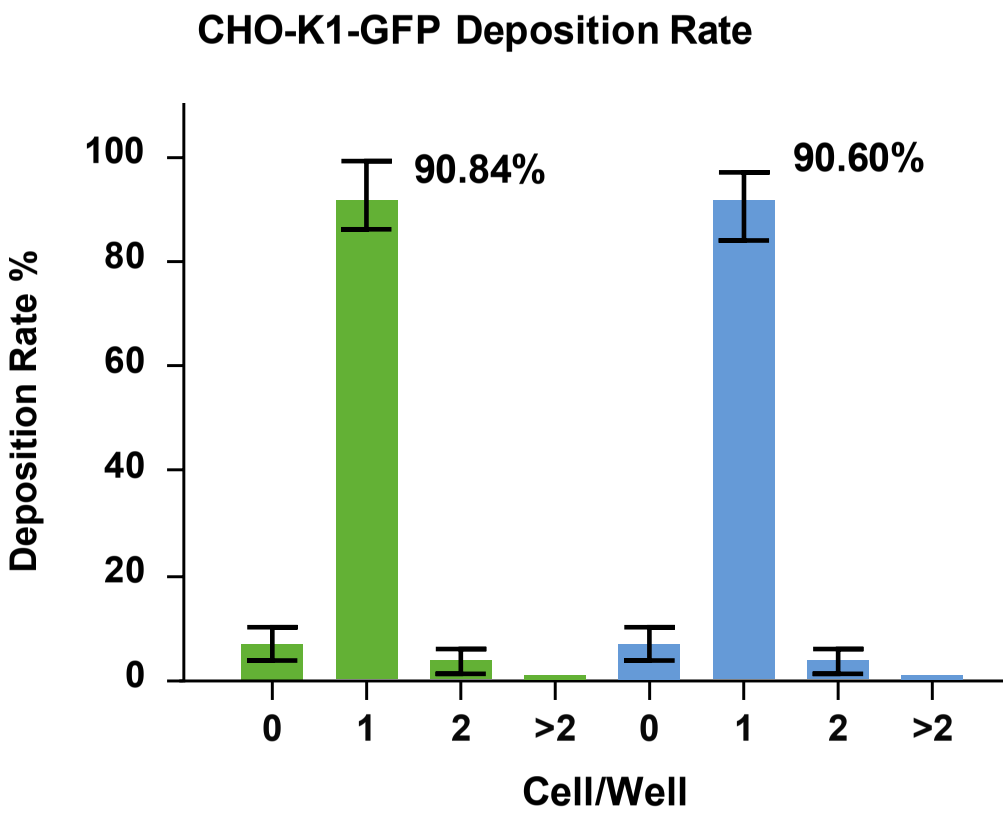
Cell Banking

Strictly Confidential

© Copyright 2020 NanoCellect Biomedical. All rights reserved.

nanocellect Biomedical, Inc.

WOLF + N1 Results in >90% Single-Cells



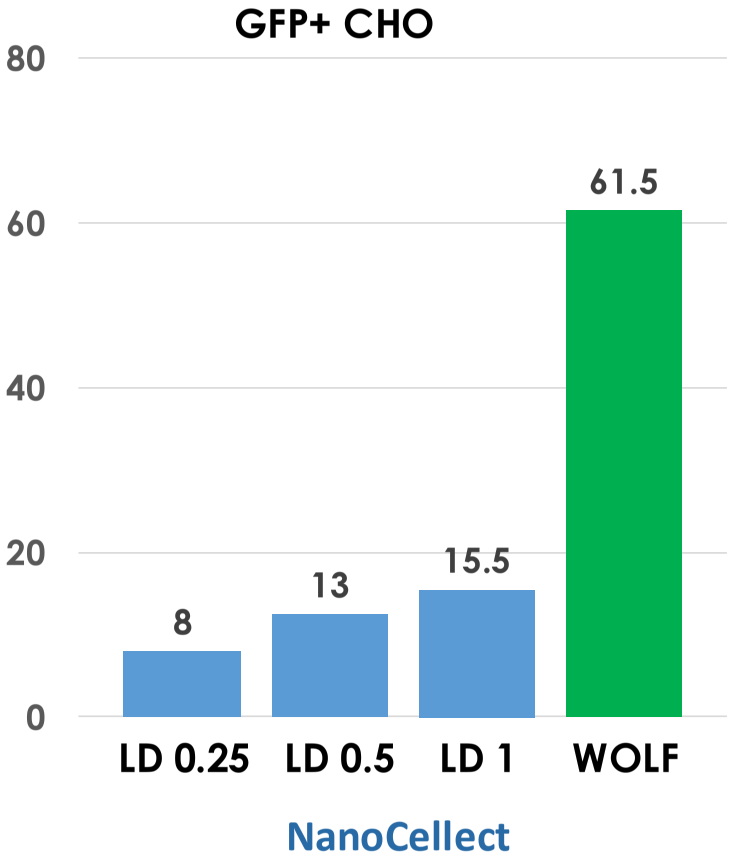
96-well: n=25, 384-well: n=14. Data analyzed with Synentec NyOne Plate Imager

Strictly Confidential

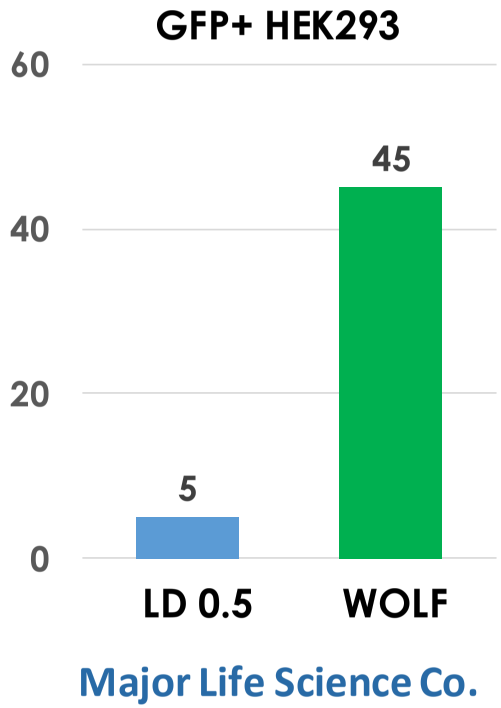
© Copyright 2020 NanoCellect Biomedical. All rights reserved.

nanocellect Biomedical, Inc.

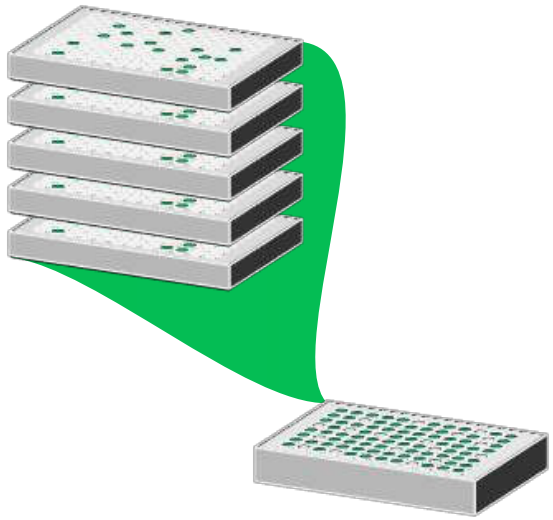
WOLF Increases GFP+ Monoclonal Plating Density



NanoCellect



Major Life Science Co.



4-8X improvement in GFP+ monoclonal colonies per plate

Strictly Confidential

© Copyright 2020 NanoCellect Biomedical. All rights reserved.

nanocellect Biomedical, Inc.

WOLF Improves Limiting Dilution Workflows

WOLF

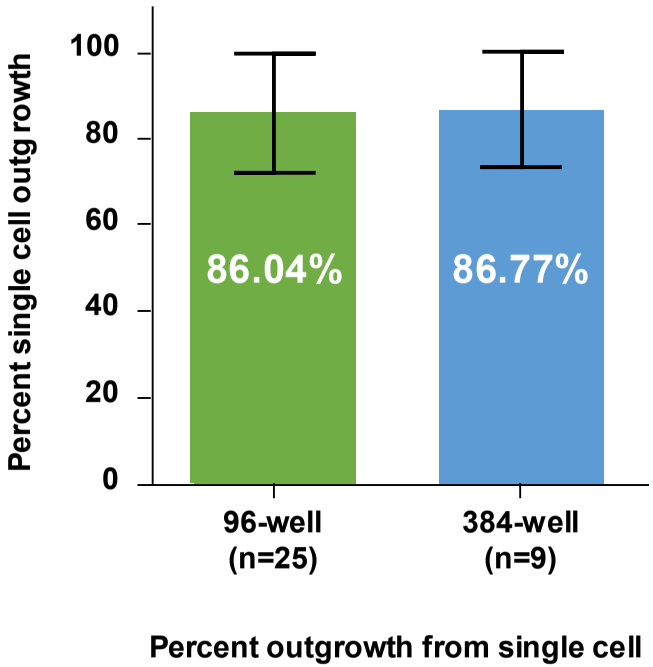
	Culture Day 0	Culture Day 1	Culture Day 3	Culture Day 7	Culture Day 14	
	5 Lines	5 Lines	5 Lines	5 Lines	5 Lines	1,000 clones
	25 Lines	25 Lines	25 Lines	25 Lines	25 Lines	5,000 clones
	Imaging Day 0	Imaging Day 1	Imaging Day 3	Imaging Day 7	Imaging Day 14	
	100 minutes	100 minutes	100 minutes	100 minutes	100 minutes	8.3 hours
	20 minutes	20 minutes	20 minutes	20 minutes	20 minutes	1.7 hours

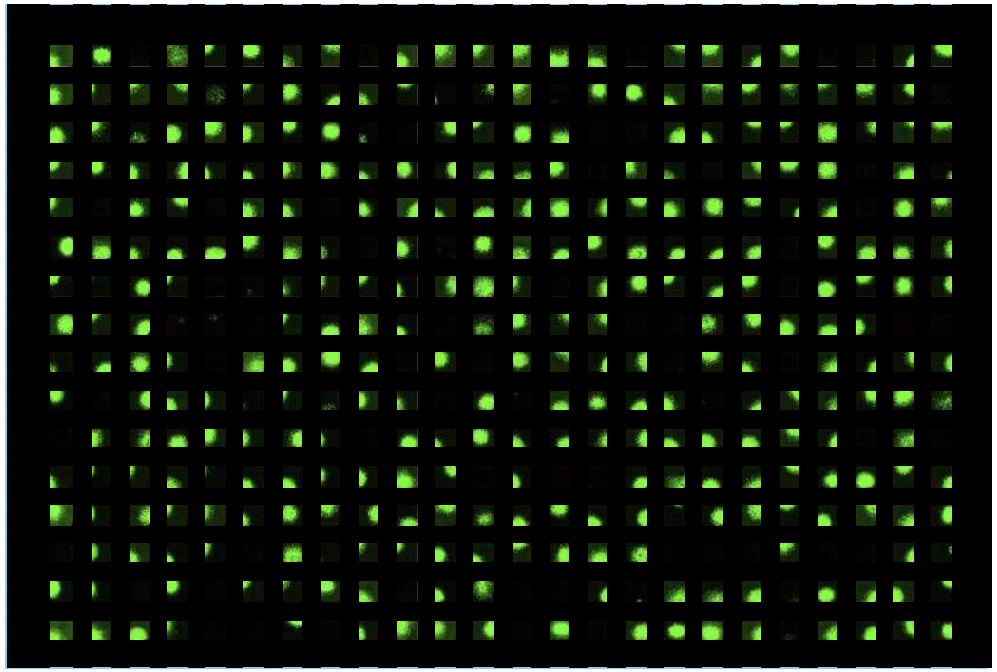
Strictly Confidential

© Copyright 2020 NanoCellect Biomedical. All rights reserved.

nanocellect Biomedical, Inc.

High Density Monoclonal Plating





CHO-K1 GFP+ cells analyzed with Synentec NyOne Plate Imager

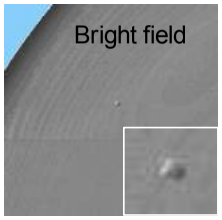
Strictly Confidential

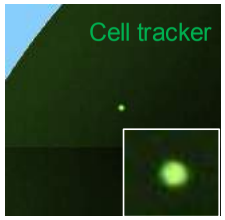
© Copyright 2020 NanoCellect Biomedical. All rights reserved.

nanocellect Biomedical, Inc.

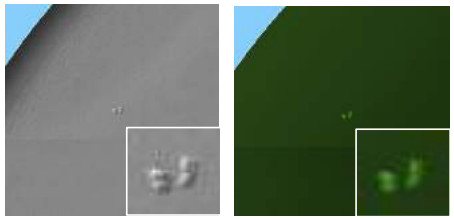
N1 Performance: 80+% Monoclonal Outgrowth

Day 0

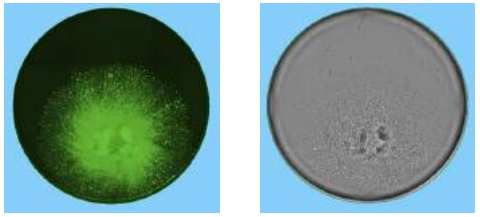




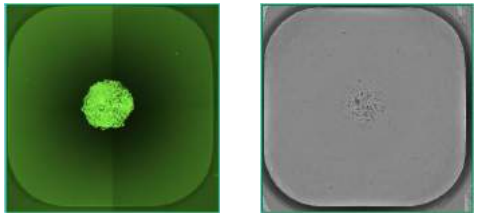
Day 1



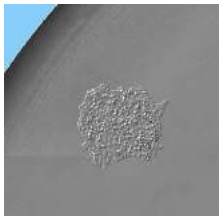
CHO-K1 GFP+ 96-well



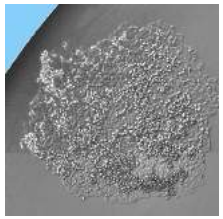
CHO-K1 GFP+ 384-well



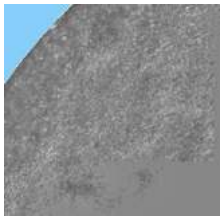
Day 5

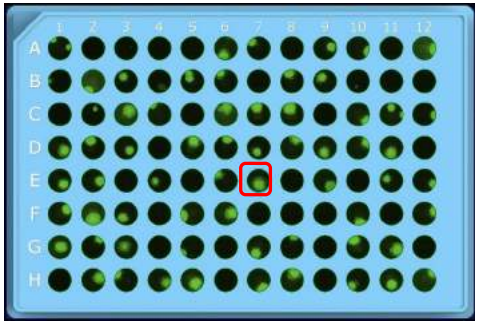


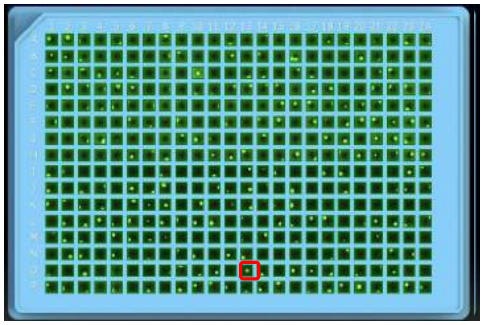
Day 7



Day 14







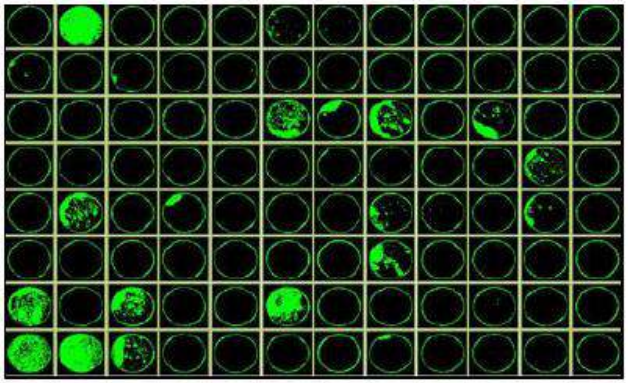
Strictly Confidential

© Copyright 2020 NanoCellect Biomedical. All rights reserved.

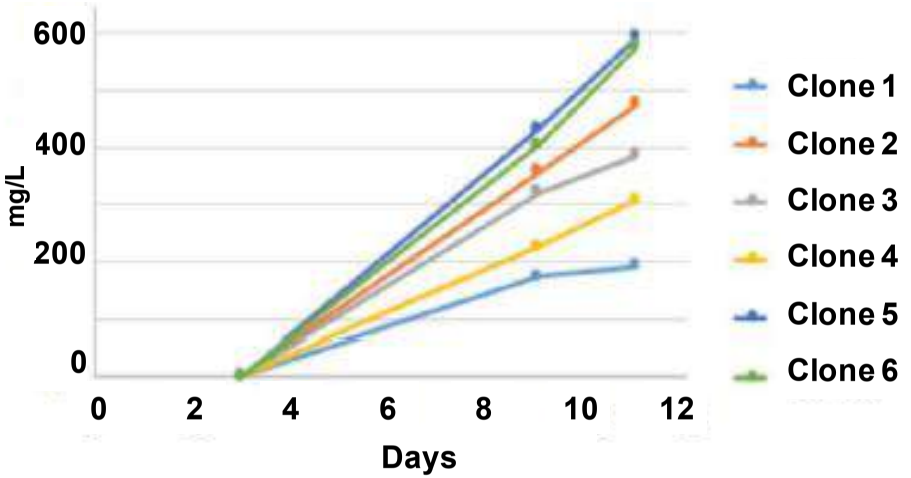
nanocellect Biomedical, Inc.

Case Study, Cell Line Development

Colonies



Antibody Production



- **Four 96-well plates resulted in 6 candidates for scale-up**

Industry Collaborator Data

Strictly Confidential

© Copyright 2020 NanoCellect Biomedical. All rights reserved.

nanocellect Biomedical, Inc.

The WOLF Sorts Cells Safely

- 0 or 1-Parameter
- Beads
- Limiting Dilution
- Cell Printer

- Multi-Parameter
- High Viability
- Plate Sorting
- Sterile

- Multi-Parameter
- Low Viability
- Plate Sorting
- Non-Sterile

Strictly Confidential

© Copyright 2020 NanoCellect Biomedical. All rights reserved.

nanocellect Biomedical, Inc.

Sample Prep for Genomics and Cloning

Other: MagSep, Limiting Dilution

Sample Prep

Other: High Speed, > 5 Parameter

WOLF: Sterile, Easy-to-Use, Gentle

Terminal Sample Preparation (Better Nucleic Acid Integrity)

Living Sample Prep/Cloning (Better Viability and Outgrowth)

Bulk Sorts

10X GENOMICS

illumina®

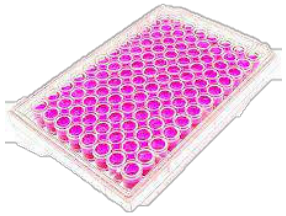
RNA-Seq
DNA-Seq
ATAC-Seq

RNA-Seq
DNA-Seq
ATAC-Seq

Plate Sorts

QIAGEN

RNA-Seq
DNA-Seq
ATAC-Seq



Cell Line Development
Biologic Drugs
Reporter Lines
Ab Discovery

Embryonic Stem Cells
ES Cells
Human ES Cells

Induced Pluripotent Stem Cells
iPSCs
hiPSCs

Strictly Confidential

© Copyright 2020 NanoCellect Biomedical. All rights reserved.

nanocellect Biomedical, Inc.

Droplet Plate Sorting vs WOLF in Cell Line Development

	SH800S Sony	FX500 Sony	MA900 Sony	FACSAriaIII BD	FACSMelody BD	Beckman-Coulter Astrios	WOLF NanoCellect
Events/sec (eps) **Sorts/sec (cps)**	100,000 12,000	100,000 12,000	100,000 12,000	100,000 10,000	34,000 10,000	100,000 10,000	5,000 300
Colors	Up to 6	Up to 6	Up to 12	Up to 28	Up to 11	Up to 30	3
Sorting	2-way	2-way	4-way	4-way	2-way	4-way	2-way
Instrument Price **Cartridge Price**	$140-200k $60	$150-200k $60	$150-220k $60	$225-$1M No cartridge	$150k-$250k No cartridge	$225-$1M No cartridge	$98.5k + $20-30k N1 $195 cartridges
Viability	+++	+++	+++	++	++	+++	++++
Scatter (large angle)	FSC (180° BSC)	FSC (180° BSC)	FSC (180° BSC)	FSC (90° SSC)	FSC (90° SSC)	FSC (90° SSC)	FSC (180° BSC)
Fits in hood	No, custom	No, custom	No, custom	No, custom	No, custom	No, custom	Yes
Portable (kg)	No, 98 kg	No, 100 kg	No, 100 kg	No, >100 kg	No, 41 kg + 36 kg	No, >100 kg	Yes, 20 kg
Sort Mechanism, Pressure Aerosol	• Cuvette sorting • ~70-20 psi • Aerosols	• Cuvette sorting • ~70-20 psi • Aerosols	• Cuvette sorting • ~70-20 psi • Aerosols	• Cuvette sorting • ~70-20 psi • Aerosols	• Cuvette sorting • ~70-20 psi • Aerosols	• Jet-in-air Sorting • ~70-20 psi • Aerosols	• Microfluidic Sorting • 1.5 psi • No Aerosols
Positives	• Ease of use • Microfluidic sorter (false)	• Disposable sterile fluidics • Microfluidic sorter (false)	• One button start (aseptic cleaning, QC, and sort). • Microfluidic sorter (false)	• High sensitivity • High speed • High parameters	• Same optics as Aria • Ease-of-use software • Form factor.	• High sensitivity • High speed • High parameters	• Ease of use • 1.5 psi • High viability • Sterile, disposable fluidics • Low biohazards
Negatives	• Non-Sterile fluidics • Droplet shear stress • Open droplet sorting • Not microfluidic sort, only microfluidic analysis • Biohazard aerosols • Fluidics cart service	• Droplet shear stress • Open droplet fluidics • Biohazard aerosols • Fluidics cart service	• Non-Sterile fluidics • Droplet shear stress • Open droplet fluidics • Biohazard aerosols • Fluidics cart service	• Non-Sterile • Highest shear stress • Open droplet fluidics • Biohazard aerosols • Complex operation • Fluidics cart service • High cost of ownership	• Non-Sterile • Highest shear stress • Open droplet fluidics • Biohazard aerosols • Complex operation • Fluidics cart service • High cost of ownership	• Non-Sterile • Droplet shear stress • Open droplet fluidics • Biohazard aerosols • Complex operation • Fluidics cart service • High cost of ownership	• Lower speeds • 5 parameters, max

Cell Printers vs WOLF for Cell Line Development

	Namo/Hana Namocell	VIPS Solentim	CloneSelect SCP	f.sight	CellenOne Scienion (Cellenion)	On-Chip SPiS	WOLF NanoCellect
Plating Speed	1 minute	10 minutes	10 minutes	10 minutes	4 minutes	60 minutes	2.5 minutes
Colors	3/2	0	0	1	0	0	3
Sorting	1-way	1-way	1-way	1-way	1-way	No	2-way
Price of instrument / price of cartridge	$80k $65	$120k $100	$160k $200	$160k $200	$150k	$100k $200	$118.5k $175
Viability	++++	++++	++++	++++	++++	++++	++++
Scatter	FSC/FSC BSC	No	No	No	No	No	FSC, BSC
Fits in hood	Yes	Yes	Yes	Yes	No	No	Yes
Portable (kg)	Yes, 10 kg	No, 70 kg	No, 30 kg	No, 30 kg	No, 205 kg	No, 100 kg	Yes, 20 kg
Selling point (claim, feature)	<2psi, small, easy, automated, chip-based (closed)	Suspension and adherent cells. Automated limiting dilutions, bright field imaging	Disposable chip, label-free sorting, high viability, Small volumes, automated limiting dilutions, bright field imaging	Disposable chip, label-free sorting, high viability, Small volumes, automated limiting dilutions, bright field imaging.	Low volume, high viability	Disposable chip, label-free sorting, high viability	Gentle 1.5psi, cell integrity (karyotype), disposable chip and tubing, ease of use
Weaknesses	Single cell speed is slow. Limited sample voumes.	No fluorescence or cytometry	No fluorescence or cytometry	No cytometry	Large footprint, expensive	No fluorescence or cytometry. Slow, at 1 hour per 96-well plate.	

Strictly Confidential

© Copyright 2020 NanoCellect Biomedical. All rights reserved.

nanocellect Biomedical, Inc.

WOLF and SONY SH800 or MA900

- Claims microfluidic sorting, but it is a droplet sorter
- Cuvette-nozzle chips are ~$40 each
- Pricing starts at ~US$140k

FEATURE	WOLF	SONY SH800	NOTES
Sterile, Disposable Detection Cuvette	Yes	Yes	WOLF: Sterile, disposable cartridge & lines
Sterile, Disposable Sample Line	Yes	Yes	WOLF: Sterile, disposable cartridge & lines
Sterile, Disposable Sheath Line	Yes	No	WOLF: Sterile, disposable cartridge & lines
Sterile, Disposable Sheath Reservoir	Yes	No	WOLF: 50 mLs of any sheath, per day
4-hour Viability	99%	91%	Broad Institute poster, ISAC 2019
24-hour Viability	96%	90%	Broad Institute poster, ISAC 2019
Fits in Standard BSL Hood	Yes	No	WOLF: 2 ft^3 44 lbs | SONY: 93 ft^3 386 lbs
Aerosol Free	Yes	No	WOLF has no biohazardous aerosols
Pressure (psi)	2	20-70	WOLF has no decompression shock
Microfluidic Sorting	Yes	No	WOLF uses low-shear microfluidic sorting

Strictly Confidential

© Copyright 2020 NanoCellect Biomedical. All rights reserved.

nanocellect Biomedical, Inc.

WOLF and BD Melody

- Same cuvette and optics as Aria
- Still a 350 lb (160kg) system, plus outboard equipment
- Requires aerosol containment hood
- Pricing starts at ~US$160k

FEATURE	WOLF	Melody	NOTES
Sterile, Disposable Detection Cuvette	Yes	No	WOLF: Sterile, disposable cartridge & lines
Sterile, Disposable Sample Line	Yes	No	WOLF: Sterile, disposable cartridge & lines
Sterile, Disposable Sheath Line	Yes	No	WOLF: Sterile, disposable cartridge & lines
Sterile, Disposable Sheath Reservoir	Yes	No	WOLF: 50 mLs of any sheath, per day
Fits in Standard BSL Hood	Yes	No	WOLF: 2 ft^3 44 lbs | Melody: 22 ft^3 349lbs
Aerosol Free	Yes	No	WOLF has no biohazardous aerosols
Pressure (psi)	2	20-70	WOLF has no decompression shock
Microfluidic Sorting	Yes	No	WOLF uses low-shear microfluidic sorting

Strictly Confidential

© Copyright 2020 NanoCellect Biomedical. All rights reserved.

nanocellect Biomedical, Inc.

WOLF and BD AriaIII

BD

FEATURE	WOLF	AriaIII	NOTES
Sterile, Disposable Detection Cuvette	Yes	No	WOLF: Sterile, disposable cartridge & lines
Sterile, Disposable Sample Line	Yes	No	WOLF: Sterile, disposable cartridge & lines
Sterile, Disposable Sheath Line	Yes	No	WOLF: Sterile, disposable cartridge & lines
Sterile, Disposable Sheath Reservoir	Yes	No	WOLF: 50 mLs of any sheath, per day
Fits in Standard BSL Hood	Yes	No	WOLF: 2 ft^3 44 lbs | Aria: 88 ft^3 1600 lbs
Aerosol Free	Yes	No	WOLF has no biohazardous aerosols
Pressure (psi)	2	20-70	WOLF has no decompression shock
Microfluidic Sorting	Yes	No	WOLF uses low-shear microfluidic sorting

Strictly Confidential

© Copyright 2020 NanoCellect Biomedical. All rights reserved.

nanocellect Biomedical, Inc.

WOLF and Beckman Coulter Astrios

FEATURE	WOLF	Astrios	NOTES
Sterile, Disposable Detection Cuvette	**Yes**	**No**	WOLF: Sterile, disposable cartridge & lines
Sterile, Disposable Sample Line	**Yes**	**No**	WOLF: Sterile, disposable cartridge & lines
Sterile, Disposable Sheath Line	**Yes**	**No**	WOLF: Sterile, disposable cartridge & lines
Sterile, Disposable Sheath Reservoir	**Yes**	**No**	WOLF: 50 mLs of any sheath, per day
4-hour Viability	**99%**	**98%**	Broad Institute poster, ISAC 2019
24-hour Viability	**96%**	**90%**	Broad Institute poster, ISAC 2019
Fits in Standard BSL Hood	**Yes**	**No**	WOLF: 2 ft^3 44 lbs | Astrios: 58 ft^3 1250 lbs
Aerosol Free	**Yes**	**No**	WOLF has no biohazardous aerosols
Pressure (psi)	**2**	**20-70**	WOLF has no decompression shock
Microfluidic Sorting	**Yes**	**No**	WOLF uses low-shear microfluidic sorting

Strictly Confidential

© Copyright 2020 NanoCellect Biomedical. All rights reserved.

WOLF and Bio-Rad S3e

FEATURE	WOLF	Bio-Rad S3e	NOTES
Sterile, Disposable Detection Cuvette	Yes	No	WOLF: Sterile, disposable cartridge & lines
Sterile, Disposable Sample Line	Yes	No	WOLF: Sterile, disposable cartridge & lines
Sterile, Disposable Sheath Line	Yes	No	WOLF: Sterile, disposable cartridge & lines
Sterile, Disposable Sheath Reservoir	Yes	No	WOLF: 50 mLs of any sheath, per day
Plate Sorting	Yes	No	Bio-Rad does only 8-well strips
Fits in Standard BSL Hood	Yes	No	WOLF: 2 ft^3 44 lbs | Bio-Rad: 10.1 ft^3 198 lbs
Aerosol Free	Yes	No	WOLF has no biohazardous aerosols
Pressure (psi)	2	20-70	WOLF has no decompression shock
Microfluidic Sorting	Yes	No	WOLF uses low-shear microfluidic sorting

Strictly Confidential

© Copyright 2020 NanoCellect Biomedical. All rights reserved.

nanocellect Biomedical, Inc.

WOLF and Miltenyi Tyto

- Claims 2-way sorting; but is 1-way.
- Claims 55,000 sorts per second, but Cell Line Development App was 142 cells per second.
- $380k in US
- Cartridges are $225 and needs two for set up.

FEATURE	WOLF	Miltenyi Tyto	NOTES
Sterile, Disposable Detection Cuvette	Yes	Yes	WOLF: Sterile, disposable cartridge & lines
Input Sample Tube Flexibility	Yes	No	WOLF: Microcentrifuge or 5 mL FACS tubes
Input Sheath Container Flexibility	Yes	No	WOLF: Up to 50 mLs of any sheath fluid
4-hour Viability	99%	99%	Broad Institute poster, ISAC 2019
24-hour Viability	96%	87%	Broad Institute poster, ISAC 2019
Fits in Standard BSL Hood	Yes	Maybe	WOLF: 2 ft^3 44 lbs | Tyto: 5.2 ft^3 133 lbs
Aerosol Free	Yes	Yes	WOLF has no biohazardous aerosols
Pressure (psi)	2	4	WOLF has lower pressure
96-well cell culture and PCR plates	Yes	No	WOLF has variable volume and speed
384-well cell culture and PCR plates	Yes	No	WOLF has variable volume and speed
Two-Way Sorting	Yes	No	WOLF has actual two-way sorting

Strictly Confidential

© Copyright 2020 NanoCellect Biomedical. All rights reserved.

nanocellect Biomedical, Inc.

WOLF and Cytena

FEATURE	WOLF	Cytena	NOTES
Sterile, Disposable Detection Cuvette	Yes	Yes	WOLF: Sterile, disposable cartridge & lines
Input Sample Tube Flexibility	Yes	No	WOLF: Microcentrifuge or 5 mL FACS tubes
Input Sheath Container Flexibility	Yes	No	WOLF: Up to 50 mLs of any sheath fluid
Fits in Standard BSL Hood	Yes	Maybe	WOLF: 2 ft^3 44 lbs | Cytena: 4 ft^3 143 lbs
Fluorescent Detection	Yes	No	WOLF has 3 fluorescent colors
Scatter Detection	Yes	No	WOLF has forward and back scatter
Flow Cytometry Analysis	Yes	No	WOLF analyzes color and size information
Threshold Gating	Yes	No	WOLF can define histogram-based gates
Polygon Gating	Yes	No	WOLF can define scatter-plot gates

Strictly Confidential

© Copyright 2020 NanoCellect Biomedical. All rights reserved.

nanocellect Biomedical, Inc.

WOLF and Namocell

Namocell

- Claims high-speed ***and*** high purity, but these are separate modes.
- Can be high-speed ***or*** high-purity
- Pricing starts at ~US$80k

FEATURE	WOLF	Namocell	NOTES
Sterile, Disposable Detection Cuvette	Yes	Yes	WOLF: Sterile, disposable cartridge & lines
Input Sample Tube Flexibility	Yes	No	WOLF: Microcentrifuge or 5 mL FACS tubes
Input Sheath Container Flexibility	Yes	No	WOLF: Up to 50 mLs of any sheath fluid
Sort into tubes?	Yes	No	WOLF can sort into 1.6 mL or 5 mL tubes, and plates
Sterile Sheath Fluidics	Yes	No	WOLF: Entire fluidics is disposable. Namo uses bottles
Fits in Standard BSL Hood	Yes	Yes	WOLF: 0.14m^2, 20kg | Namo/Hana: 0.17m^2 10kg
Fluorescent Colors Detected	Yes	Yes	WOLF: 3 | Namo 3, Hana 2
Scatter Detection	Yes	Yes	WOLF: 2 | Namo: 1; Hana 2
Flow Cytometry Analysis	Yes	Yes	WOLF: Sophisticated analysis | Namo/Hana: minimal
Threshold Gating	Yes	Yes	WOLF can define histogram-based gates
Polygon Gating	Yes	No	WOLF can define polygon, quad, square and round gates
Bulk Sorting	Yes	No	WOLF can sort into tubes or plates
Two-way sorting	Yes	No	WOLF does true-two way sorting

Strictly Confidential

© Copyright 2020 NanoCellect Biomedical. All rights reserved.

nanocellect Biomedical, Inc.

Conventional Cell Sorting & Enrichment:

FACS - Fluorescence-activated cell sorting

Quick, pure, high-throughput but…

- Harsh on cells
- Not sterile
- Safety concerns
- Requires expertise
- Often only accessible in core labs
- Large volume requirements

MACS – Magnetic-activated cell sorting (Manual and Automated)

Gentle on cells, easy to use but…

- Cells are subjected to iron particles
- Lower purity, particularly with negative isolation
- Not suitable for CRISPR or intracellular markers
- No single cell automation
- Not sterile, re-usable columns

Limiting dilution (Manual and Automated)

Simple, gentle, and sterile but…

- Statistical distribution - time and cost inefficient
- Time consuming, weeks of evaluation, reagent consuming 50-100 plates vs. 10 plates
- Low throughput
- Requires additional analytics- microscope, etc.

Strictly Confidential

© Copyright 2020 NanoCellect Biomedical. All rights reserved.

nanocellect Biomedical, Inc.

Application: Cell Line Development

Giving scientists the power to find the best cells.

Challenges:

- Conventional cell sorters produce biohazardous aerosols and are prone to sample-to-sample and microbial contamination
- Conventional sorters induce a great deal of mechanical stress, diminishing cell and nucleic acid integrity

NanoCellect offers:

- Contaminant and biohazard free via disposable, sterile microfluidic cartridges
- >2 psi sorting, allowing for high viability, healthy cells

Strictly Confidential
© Copyright 2020 NanoCellect Biomedical. All rights reserved.

Application: Genomic Sample Prep

Enabling researchers to gently sort individual cells for higher quality samples.

- Selecting and enriching only the cells of interest to avoids dying cells, debris and off-target cells—improving sequencing value and signal-to-noise.
- High cell viability and nucleic acid integrity with single cell dispensing
- Easy-to-use
  - Faster time to answer
  - Improved workflows
  - Higher efficiency

Strictly Confidential
© Copyright 2020 NanoCellect Biomedical. All rights reserved.

Application: Gene editing for CRO's

Enabling scientists with faster, better results.

Challenges:

- Conventional sorters have high shear stress, lowering cell integrity
- Lost time with inefficient workflows. Conventional sorters require an expert operators, high maintenance and dedicated facilities.

NanoCellect Offers:

- Highest cell integrity in single cell dispensing
- Ease-of-use
  - Faster time to answer
  - Improved workflows
  - Higher efficiency

Strictly Confidential
© Copyright 2020 NanoCellect Biomedical. All rights reserved.

Application: Gene editing for Biopharma

Enabling scientists to get to discovery, faster.

Challenges:

- Conventional sorters have high shear stress, lowering cell integrity
- Lost time with inefficient workflows. Conventional sorters require an expert operators, high maintenance and dedicated facilities.

NanoCellect Offers:

- High cell integrity with single cell dispensing
- Ease-of-use
  - Faster time to answer
  - Improved workflows
  - Higher efficiency

Strictly Confidential
© Copyright 2020 NanoCellect Biomedical. All rights reserved.

Benefits to Your Research

- Higher cell viability and genomic integrity.
- Sterile: Biohazard containment for human, virus, yeast, or bacteria sorting.
- Easy 96- and 384-well plate sorting for cloning and single-cell work.
- Compact and portable for your hood or bench.

Strictly Confidential

© Copyright 2020 NanoCellect Biomedical. All rights reserved.

nanocellect Biomedical, Inc.