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Cell Counting and Viability Assessments in the Process Development of Cellular Therapeutics

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With the advent of whole cell-based therapeutics has come a growing need for standardized quality control and quality assurance of the processes employed for developing and manufacturing cellular materials, similar to the controls over traditional drugs and biologicals. Cellular therapeutics present unique process and quality control challenges due to the innate complexities of living cells, making it important to use whole cell assays to provide detailed pictures of the status and consistency of cell preparations that will be used to treat patients. This article illustrates how a cellular assay from Guava Technologies addresses these issues.

Process Control — Enumeration and Characterization

One essential aspect of developing cultures for cellular therapeutics is the ability to accurately assess cell number and viability. Whether bone marrow stem cells for hematopoetic reconstitution, dendritic cells for live-cell vaccines, or engineered antitumor cytotoxic lymphocytes, in all cellular therapies the active agent is the live cell itself. Just as companies monitor the concentration and activity of therapeutic agents in pharmaceuticals, the number of cells delivered to a patient is critical in determining the therapeutic doses necessary to elicit the desired response. Moreover, the absolute number of cells is less important than knowing the number of viable, healthy cells because only these cells are likely to survive the "transplant" into a patient and perform productively. An adequate cell count for

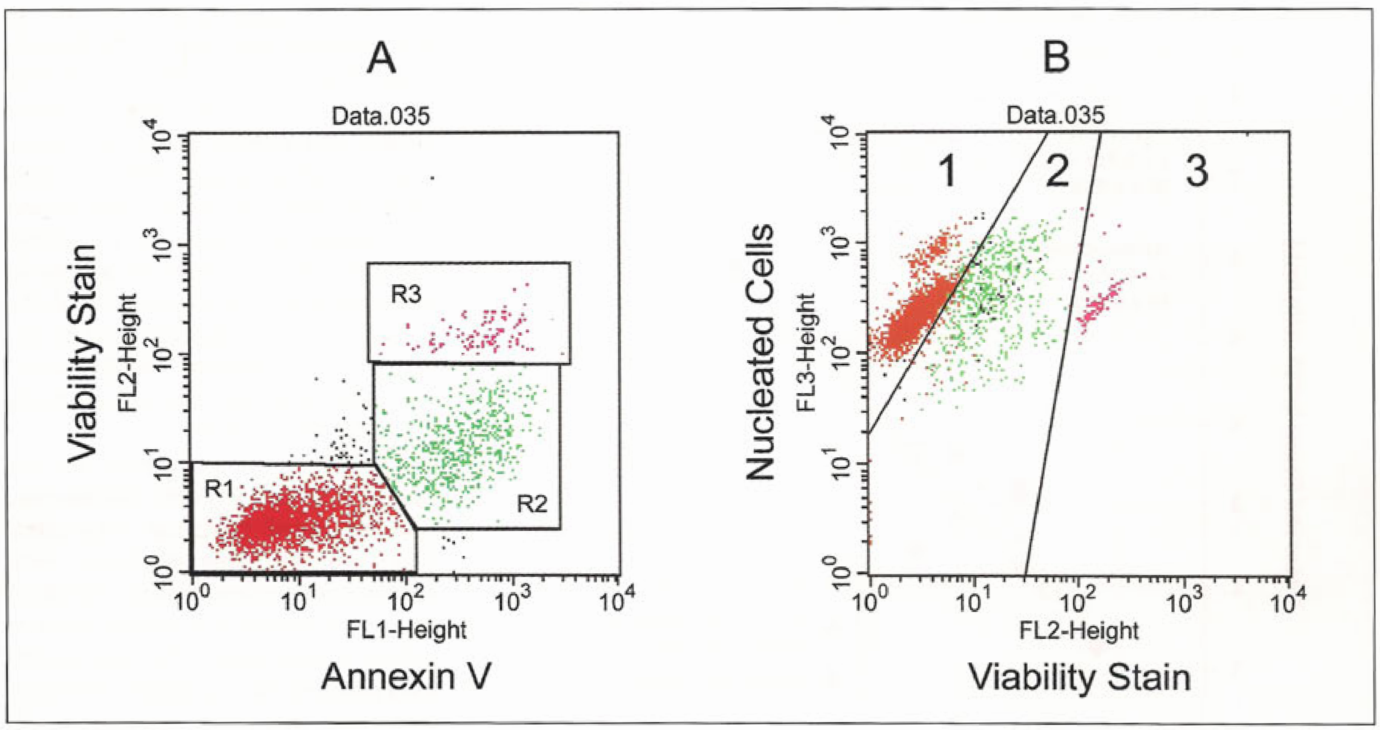

Figure 1. Intermediate viability staining marks apoptotic cells. Starved and overgrown CHO-K1 cells were separated into a single cell suspension and stained with annexin V-FITC and ViaCount dyes for viability (FL2) and nucleated cells (FL1). Data was acquired on the FACSCalibur flow cytometer with CellQuest software. Live cells are annexin V-negative, viability stain negative (R1); intermediate (apoptotic) cells are high in annexin V (R2), intermediate in viability stain; high annexin V and high viability staining indicates dead cells (R3).

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cellular therapeutic processing must distinguish between viable, dead, and apoptotic cell populations.

Cell Counting Limitations

Ironically, the most common method today for assessing cell viability and counts for these cutting-edge cellular therapies is a decades-old methodology — trypan blue exclusion — that requires manual cell counting using the embedded grid of a hemacytometer. Manual counting aside, the trypan blue method requires mastering the art of interpreting and classifying particles based on size and overall "blueness". Interpreting these multiple shades of blue or blue-gray results in subjective and imprecise cell counts, particularly when operator variability is considered. In addition, trypan blue chemistry has been reported to consistently overestimate cell viability.[1,2]

Other methods to assess cell viability include a fluorescence-based approach that monitors propidium iodide (PI) exclusion from living cells. This exclusion assay uses a fluorescent signal to distinguish healthy cells from those with compromised membrane integrity. Propidium iodide exclusion examined by flow cytometry can distinguish a cell subpopulation stained with intermediate levels of PI (the apoptotic cell population).[3] However, routine flow cytometry approaches do not allow for absolute cell counts without using reference beads, which adds cost and complexity to the assay. Furthermore, counting PI-stained cells with a fluorescent microscope has all the limitations of a trypan blue/hemacytometer count: determining whether to classify intermediate-staining cell populations as live or dead introduces subjectivity into the results.

The ViaCount Assay

These limitations suggest that an ideal cell counting assay must be accurate, ensuring adequate numbers of cells in doses; it must be precise and reproducible, eliminating operator subjectivity and variation; and it must be informative, enumerating live, dead, and apoptotic populations. In addition, the method would ideally require small cell numbers and small volumes to preserve precious cells for other development assays or therapeutic uses.

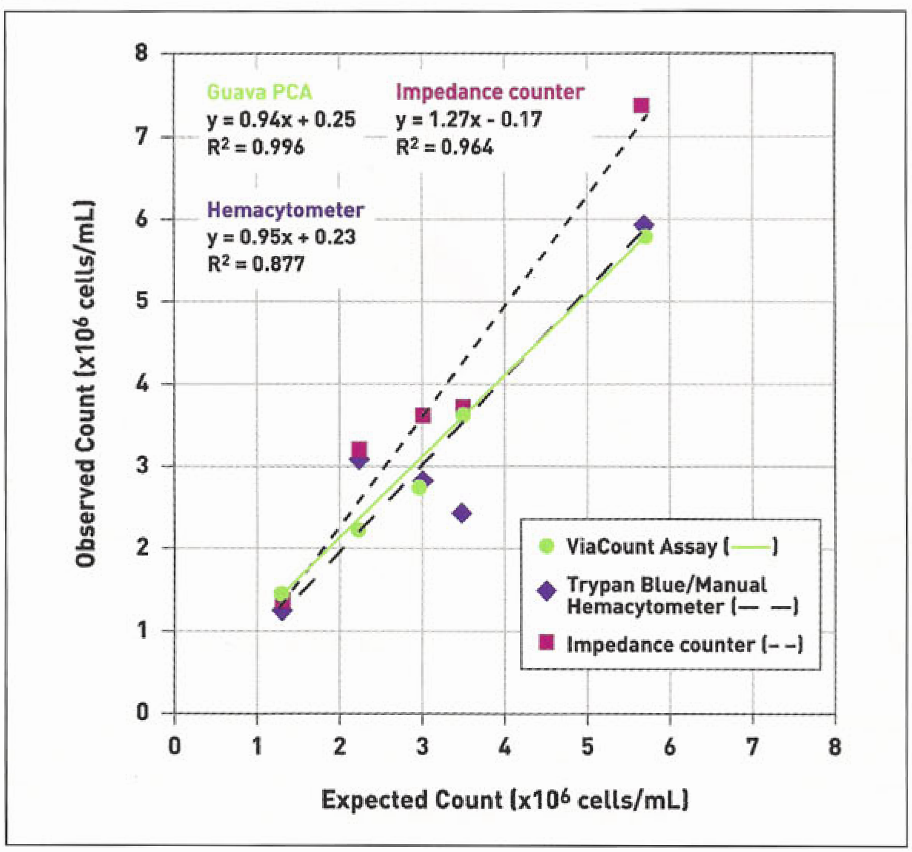

Figure 2. ViaCount's cell count measurements compared to other methods.

A cell counting and viability assay, the Guava ViaCount® assay (Guava Technologies Inc., Hayward, CA), is currently being adopted for monitoring cell health during the R&D phase of cellular therapeutics. Based on a combination of two fluorescent nucleic acid dyes, the automated assay identifies cells while excluding cellular debris and distinguishing live cells from dead. Assay results include total cell count, and cell counts and percentages for both viable and apoptotic cells.

In this assay, the first fluorochrome permeates all cells and binds DNA, marking all nucleated cells, which distinguishes them from cellular debris. The second nucleic acid dye enters only dead cells (viability stain). This combination of staining provides counts of live and dead cells while simultaneously assessing the apoptotic cell fraction based upon differential uptake of the dead cell indicator stain during apoptosis.

Cells with intermediate viability staining have also been shown to bind annexin V, indicating that they are, in fact, apoptotic cells (Fig. 1). To demonstrate this, CHO-K1 cells were stained with the ViaCount reagent (both nuclear and viability stains in a single reagent) and annexin V-FITC. Figure 1A shows that cells gated in the R2 and R3 regions were annexin V-positive, indicating they are dead or apoptotic. When the gated cells were replotted, the annexin V-positive R2 cells (green) displayed an intermediate viability staining, indicating that they are apoptotic cells (Fig. 1B, region 2).

The ViaCount assay runs on the Guava PCA and PCA-96 instruments, two automated benchtop cytometers. Based on the same platform, both instruments perform simple cell-based assays that address the process needs of cellular therapeutics. The instruments use a patented microcapillary cytometry technology that allows individual cells to be analyzed as single events without the large sample volume or cell numbers required by traditional flow cytometers. Sampling volume is as low as 20 µl on these systems compared to 1-ml samples required by other cytometers. The microvolume capability saves precious volumes of cell samples, which is particularly important for cellular therapies. In addition, the microcapillary technology provides absolute cell counts, which cannot be directly obtained with traditional flow cytometers.

Accuracy, Reliability, Precision

When the Guava ViaCount was tested for precision against the standard trypan blue/hemacytometer method, the operator-operator consistency was found to be far greater in ViaCount data. In cell concentrations ranging from 7 x 10^5 to 1.8 x 10^6 cells/ml, precision for the ViaCount assay averaged 2.6% CV (coefficient of variation), compared to a CV of 8.1% for hemacytometer counts (data not shown).

Cell counts obtained with the ViaCount assay were also shown to be accurate in comparisons with other methods. A study performed by Raven Biotechnologies, Inc. compared trypan blue exclusion on a hemacytometer, automated impedance-based cell counting, and ViaCount to a standard counting method that uses FDA-cleared reference beads (TruCOUNT™). Both trypan blue exclusion and ViaCount showed excellent correlations in total cell counts with the standard method (Fig. 2). However, ViaCount demonstrated greater linearity (R2=0.996) than the trypan blue method (R2=0.877) over the cell densities tested. Impedance-based counting deviated significantly from the other methods.

In addition to total cell counts, the study also compared viability assessments. Four different mammalian cell lines were tested using trypan blue and a hemacytometer, and ViaCount. The results were plotted against results from PI-stained cells analyzed on a flow cytometer. Figure 3 shows that hemacytometer counts were consistently higher than the ViaCount and PI methods, a trend that is more pronounced at lower viabilities.

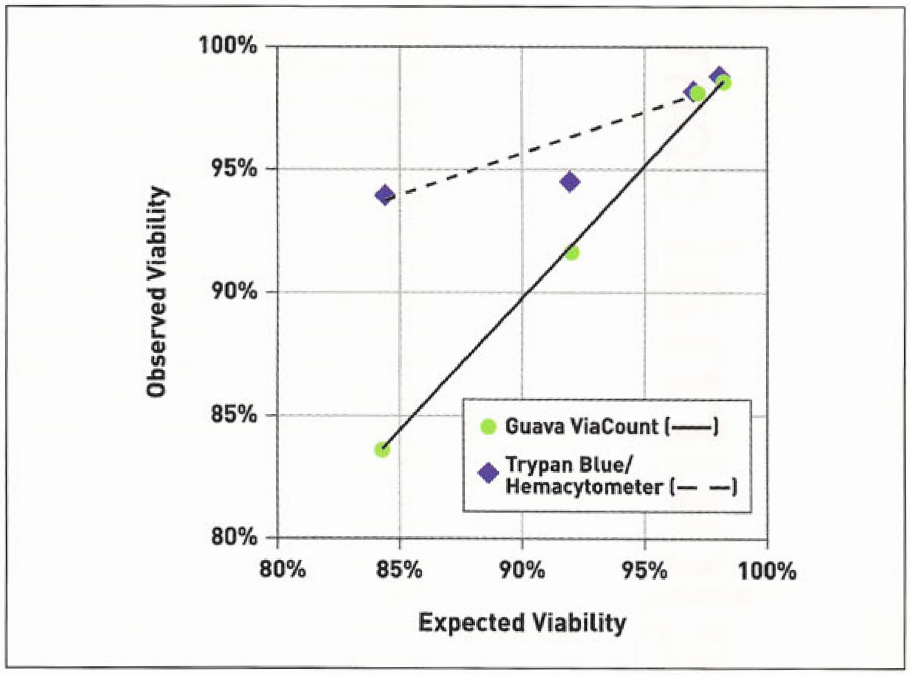

Figure 3. ViaCount viability assessments compared to those from trypan blue exclusion assays on a hemacytometer.

GMPs

In addition to its biological accuracy, the ViaCount assay has also been validated for use in a good manufacturing process (GMP) setting for human mesenchymal stem cells, demonstrating its reliability for process control.[4] Features in the ViaCount's software also allow for documentation supporting compliance with 21 CFR Part 11 regulations.[5]

Engineering Killer T Cells

The Guava ViaCount assay can be an important component of processing engineered cells for therapeutic uses. For example, Dr. Michael Jensen at the City of Hope National Medical Center (Duarte, CA) has ongoing projects using transfected cytotoxic T lymphocytes (CTLs) as anticancer therapies. The process starts with a leukapheresis, a procedure for the collection of peripheral blood mononuclear cells (PBMCs) from a cancer patient. The PBMCs are then cultured with OKT3 cells and the cytokine interleukin-2 (IL-2), which promotes outgrowth of the cytotoxic T cell population. The T cells are then transfected with DNA encoding a chimeric immunoreceptor composed of a murine single chain Fv (scFv) moiety that targets a molecule found on the tumor cells, but not on normal tissues, and a human Fc portion. The transfected T cells are grown out under drug selection then cloned. Clones that exhibit the desired functional and phenotypic characteristics are selected; these clones can then be cultured to quantities necessary for dosing patients. When reinfused into the patient, the transfected CTLs specifically target the tumor or cancerous cells via the chimeric immunoreceptor. This redirection of cytotoxic T cells through genetic manipulation results in a cancer fighting immune process providing few, if any, of the side effects of standard chemotherapy or radiation treatments.

Accurate cell counting and viability assessments of T cells are essential at many steps in this process. The Guava ViaCount assay is used daily to monitor growth of T cell cultures and clonal cultures. Counting T cell cultures is particularly difficult because T cells undergo morphological changes during their fourteen day stimulation cycle and because apoptotic cells can be found at some stages of the cycle. Despite this challenge, the Guava ViaCount assay has provided consistent, reproducible cell and viability counts throughout the T cell engineering process.

Accurate cell numbers are also essential for reproducible cellular functional assays, such as cytokine production or target cell lysis assays. Consistency in these assays depends on having quantifiable numbers of viable cells in each replicate to accurately analyze results. This consistency is now being achieved using the ViaCount assay to determine cell numbers before performing assays. Expansion of the T cells must also be monitored at each 14-day cycle of stimulation to select the clone that will grow to the numbers needed for treatment. This is reproducibly achieved through the Guava ViaCount procedure.

The control provided for cellular therapy development by accurate cell and viability assessments have been a welcome addition to the laboratory practices in the Jensen laboratory. Phase I testing of his CTLs has been completed for lymphoma and pediatric neuroblastoma. Testing is in progress for glioblastoma and is set to begin soon for B cell acute lymphoblastic leukemia (ALL).

Dendritic Cell Vaccines

Research on dendritic cell vaccines at Institute Jean Godinot (Reims, France) has also demonstrated the usefulness of the ViaCount assay in cellular therapeutic applications. Dendritic cells serve as sentinels of the immune system and play essential roles in mounting immune responses to cancer and infectious diseases. These cells are isolated from cancer patients, grown to substantial numbers, primed with cancer-specific antigens, then reinfused into the patient. As immune system stimulators, the primed dendritic cells can direct the body's defenses to attack tumors in a very specific manner.

One major difficulty in dendritic cell research is obtaining accurate cell and viability counts. Dendritic cells are difficult to culture, produce heterogenous populations, and often change physical appearance due to changes in culture conditions. These characteristics make trypan blue counts of dendritic cells highly variable and unreproducible. These difficulties are accentuated with user-user variability and with low density cell cultures.

In data to be published later this year, Drs. Jacky Bernard and Anne-Laure Millard have performed an extensive validation of dendritic cell counting methods.[6] Their study demonstrated high intra-sample variability as well as inter-operator variability for both viability data and cell counts obtained by trypan blue exclusion on dendritic cells. Coefficient of variation for these counts ranged from 15% for intra-sample cell counts to 28% inter-operator for viability counts. ViaCount measurements had 3-5% CV. At extreme lower cell densities near 10^4 cells/ml, the ViaCount measurements were replicable with approximately 10% CV while trypan blue exclusion results varied to near 95% CV.

Conclusion

These brief examinations of cellular therapy research demonstrate the essential role of accurate, reproducible cell and viability counting. Accuracy, precision, and inter-operator reproducibility are crucial to studies with cellular biopharmaceuticals. The ViaCount assay demonstrated lower variability, greater accuracy, and reproducibility in difficult counting circumstances, such as difficult cell types, lower cell numbers, or mixed cell cultures. All validation of the ViaCount assay to date has been done for development phases of cellular therapy research; however, plans are underway to validate the method for actual dosing and preparation of therapeutics to be administered to patients.

REFERENCES

1. Altman SA et al. Comparison of trypan blue dye exclusion and fluorometric assays for mammalian cell viability determinations. *Biotechnol Prog* 1993:9;671–674.
2. Mascotti K et al. HPC viability measurement: trypan blue versus acridine orange and propidium iodide. *Transfusion* 2000:40;693–696.
3. Telford WG, King LE, Fraker PJ. Comparative evaluation of several DNA binding dyes in the detection of apoptosis-associated chromatin degradation by flow cytometry. *Cytometry* 1992:13(2);137–143.
4. Yokobata K, O'Connell B, Harvey J. Validating the Guava ViaCount assay for use in a manufacturing setting. *Guava Technologies Application Note.* 2003.
5. The Guava PCA and PCA-96 systems: Support for compliance with 21 CFR Part 11. *Guava Technologies company white paper.* 2003.
6. Bernard J, Millard AL. Applications for cell culture therapy research and dendritic cell analysis. *Paper presented at Institut Gustave-Roussy.* 2003 June 10; Paris, France.