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Elevate MSC Quality Standards With a Combined Live-Cell Analysis and HTS Cytometry Approach

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Abstract

Mesenchymal stem cells (MSCs) are pivotal in regenerative medicine and cell therapy due to their multipotent nature and immunomodulatory functions. Ensuring the quality of MSCs is crucial for their therapeutic efficacy, particularly in high-risk clinical applications. Challenges in evaluating MSC quality arise from donor variability, culture conditions, and passage numbers. Traditional endpoint assays fall short in providing dynamic insights, necessitating advanced technologies including live-cell analysis and high-throughput screening (HTS) cytometry, which offer real-time, high-throughput, and multiparametric analysis capabilities, addressing these challenges effectively.

Here, these techniques are used to demonstrate that MSC growth dynamics slow with age, with later passages showing reduced proliferation and increased senescence, as indicated by senescence-associated β-Galactosidase (SA-β-Gal) activity and morphological changes. Marker expression remains relatively stable, emphasizing the importance of utilizing multiparametric techniques for a holistic view of MSC quality.

Introduction

Mesenchymal stem cells (MSCs) are multipotent cells that give rise to multiple tissue lineages including bone, cartilage, and fat. They are also a major component of the bone marrow hematopoietic stem cell niche and have both paracrine and immunomodulatory functions. As such, they are a key target cell type for cell therapies and tissue engineering research, including for mitigating adverse effects associated with cancer treatments,[1] in neuro-regeneration during treatment for ischemic stroke,[2] and as the source of MSC-derived exosomes in fracture healing.[3]

The therapeutic efficacy of MSCs is highly dependent on their quality. To this end, ensuring the quality of MSC cultures before administration as a cell therapy, either in stem cell or differentiated form, is of fundamental importance.

Accordingly, the minimum criteria as defined by the International Society for Cellular Therapy (ISCT)[4] states that MSCs must be plastic-adherent when maintained in standard culture conditions, have a distinct marker expression profile (CD105, CD73 and CD90 positive, CD45 and CD34 negative), and have tri-lineage differentiation potential. Consistency within the population of interest, and retention of these properties along with self-renewal capacity all need to be confirmed before cells can be used for downstream applications.

During MSC culture, it is important to understand growth dynamics, multipotency, and level of senescence to ensure the highest quality MSCs are taken forward for subsequent applications, especially in a high-risk clinical situation such as cell therapy. General consensus defines cell senescence as a permanent state of growth arrest without concurrent cell death. Biologically, it serves as a protective mechanism against the proliferation of damaged cells which could lead to tumorigenesis. However, in the context of cell therapy and quality control, senescent MSCs are undesirable due to their reduced efficacy, impaired differentiation potential, and altered secretory profile which could lead to unwanted pro-inflammatory or dysregulated microenvironments. Indicators of cellular senescence include increased doubling time, altered cell morphology, and senescence-associated β-Galactosidase (SA-β-Gal) activity.

Evaluating MSC quality presents several challenges. Firstly, MSCs can be derived from different donors and tissues, which leads to inherent variability of samples, influenced by donor age, health status, and genetic background. Additionally, variations in culture conditions, passage number, and the heterogeneity of MSC populations add further complications. Traditional methods for assessing MSC quality are often endpoint assays, providing limited information on dynamic processes. The use of advanced technologies such as the Incucyte® Live-Cell Analysis System and iQue® (HTS) cytometer platform can help overcome some of these challenges by providing real-time, rapid, and multiparametric analysis capabilities.

Materials and Methods

Media for Routine Cell Culture

Materials	Supplier	Cat. No.
MSC NutriStem® XF Medium	Sartorius	05-200-1A
MSC NutriStem® XF Supplement Mix	Sartorius	05-201-1U
NutriCoat™ Attachment Solution	Sartorius	05-760-1-15
Bone marrow Mesenchymal stem cells RoosterVial™-hBM	RoosterBio	MSC-003

Table 1. Cell Culture Media and Reagents Used for Culturing MSCs

Surface Marker Expression Panel Reagents

	Product Name	Supplier	Cat. No.	Final Concentration
Viability	Zombie Violet™ Fixable Viability Kit	BioLegend	423113	1:200
MSC marker panel	PE | Dazzle™ 594 anti-human CD90 (Thy1) Antibody	BioLegend	328133	1:100
	PE | Cyanine7 anti-human CD105 Antibody	BioLegend	323217	1:100
	APC anti-human CD73 (Ecto-5'-nucleotidase) Antibody	BioLegend	344005	1:100
	Brilliant Violet 570™ anti-human CD45 Antibody	BioLegend	304033	1:100
	FITC anti-human CD34 Antibody	Invitrogen	MA5-16925	1:100
Senescence	Senescence Assay Kit (Beta Galactosidase, Fluorescence)	Abcam	ab228562	1:333

Table 2. Reagents Used for Cell Analysis Using the iQue® HTS Cytometer

MSC Culture

Prior to seeding into culture vessels for routine culture and experiments, plates and flasks were pre-coated with NutriCoat™ Attachment Solution. The solution was diluted 1:500 in PBS according to the manufacturer's instructions. The diluted coating solution was added to the culture vessel and incubated for 1 hour in a humidified CO_2 incubator (37°C). Prior to cell seeding, the solution was aspirated, and cell culture media containing a cell suspension was quickly added to ensure that the coating did not dry out.

MSCs were obtained from RoosterBio and cultured in MSC Nutristem® XF basal medium. They were seeded at a density of 3K cells/cm^2 in tissue culture flasks and passaged at 80% confluency and analyzed using the Incucyte® System. Cell confluence was measured using integrated Incucyte® AI Confluence Analysis.

Surface Marker Expression

MSCs were harvested at passage and analyzed for expression of markers CD34, CD45, CD73, CD90, and CD105. Following harvest, cells were washed in PBS and stained for viability using Zombie Violet™ for 20 minutes in the dark at room temperature. The cells were seeded into a 96-well V-bottom plate at 20K/well and washed twice with PBS + 2% FBS before staining with the MSC marker antibody cocktail as described in Table 2 for 30 minutes at room temperature. The cells were washed twice by centrifugation (5 min at 1000 × g) using PBS + 2% FBS. Cells were then resuspended in 20 µL of PBS + 2% FBS and analyzed on the iQue® HTS Cytometer. Data was analyzed using integrated iQue Forecyt® Software.

MSC Senescence Analysis

MSCs were seeded into a 24-well plate at 10K/well and cultured until 80% confluent. The media was removed and replenished with media containing 1.5 µL Senescence Dye per 500 µL media. Following a 2 hour incubation at 37°C, the cells were scanned in the Incucyte to collect images for quantification, washed twice in Assay Buffer XXVII | Wash Buffer, then harvested by trypsinization, washed, and resuspended for analysis on the iQue® HTS Platform.

Unstained cells were used as a negative control. Label-free quantification of images was performed using the Incucyte® AI Cell Health Analysis Software Module, which enables AI-based segmentation of heterogenous cell morphologies to give univariate morphology metrics (AI Cell Segmentation).

Results

Reduction in MSC Growth Rate is Linked to Cellular Age
One key measure of MSC functionality is their proliferative capacity. Monitoring percentage confluence over time provides valuable insights into the growth dynamics of MSCs across different passages and under various culture conditions.

Live-cell analysis can be utilized to compare the proliferation of MSCs during routine culture under different conditions and over multiple passages. Cells were initially thawed in commercially available medium, then cultured in parallel in the commercial medium and in MSC NutriStem® XF medium. In MSCs cultured in a commercially available MSC medium (Figure 1A), the time required to reach confluence increased noticeably with passage number. Specifically, cells at passages 1 and 2 (P1 and P2) achieved 80% confluence within 3 days.

In contrast, cells at passage 3 (P3) required over 6 days to reach the same confluence, and cells at passage 4 (P4) only reached 70% confluence after 12 days.

For comparison, MSCs cultured in MSC NutriStem® XF medium (Figure 1B) exhibited a similar trend of slowing growth rates as the cells aged. Cells at P2 and P3 maintained a high proliferative capacity, rapidly reaching confluence within 4 days. Mid-passage cells (P4 – P6) showed a decline in growth rate, achieving 80% confluence within 6 – 7 days. Late passage cells (P7) demonstrated a significant reduction in proliferation, with confluence remaining below 50% even after 7 days of culture. This suggests that these cells may have entered a state of growth arrest or senescence.

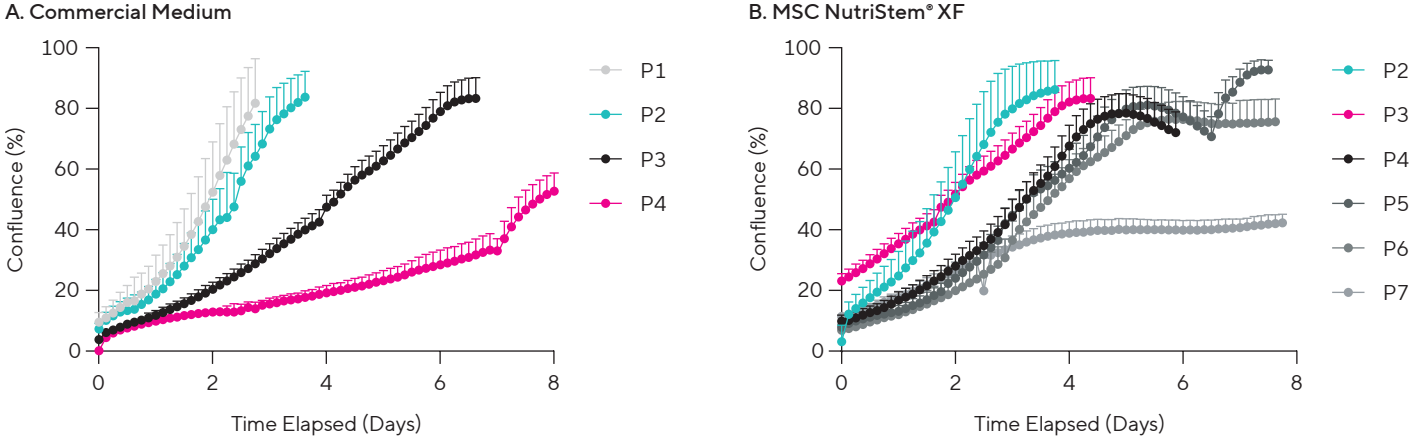

Figure 1. MSC Percentage Confluence Over Time During Routine Culture, Evaluated Using Live-Cell Analysis.
(A) MSCs cultured in a commercially available medium. (B) MSCs cultured in MSC NutriStem® XF medium.

Expression of MSC Identifying Markers Does Not Significantly Alter With Age

As mentioned previously, MSCs can be defined as having a distinct expression pattern for five markers, i.e., positive for CD73, CD90, and CD105, and negative for CD34 and CD45. Analysis of the percentage positive population for each of these markers for MSCs at passages 1–6 (Figure 2) show that the expected positive markers remain highly expressed even by older cells.

The levels of CD34 and CD45 positivity are slightly elevated in the later passages, suggesting some loss of multipotency. However, the minimal changes in marker expression suggest that this is insufficient alone to determine cell quality and should be evaluated in combination with other factors.

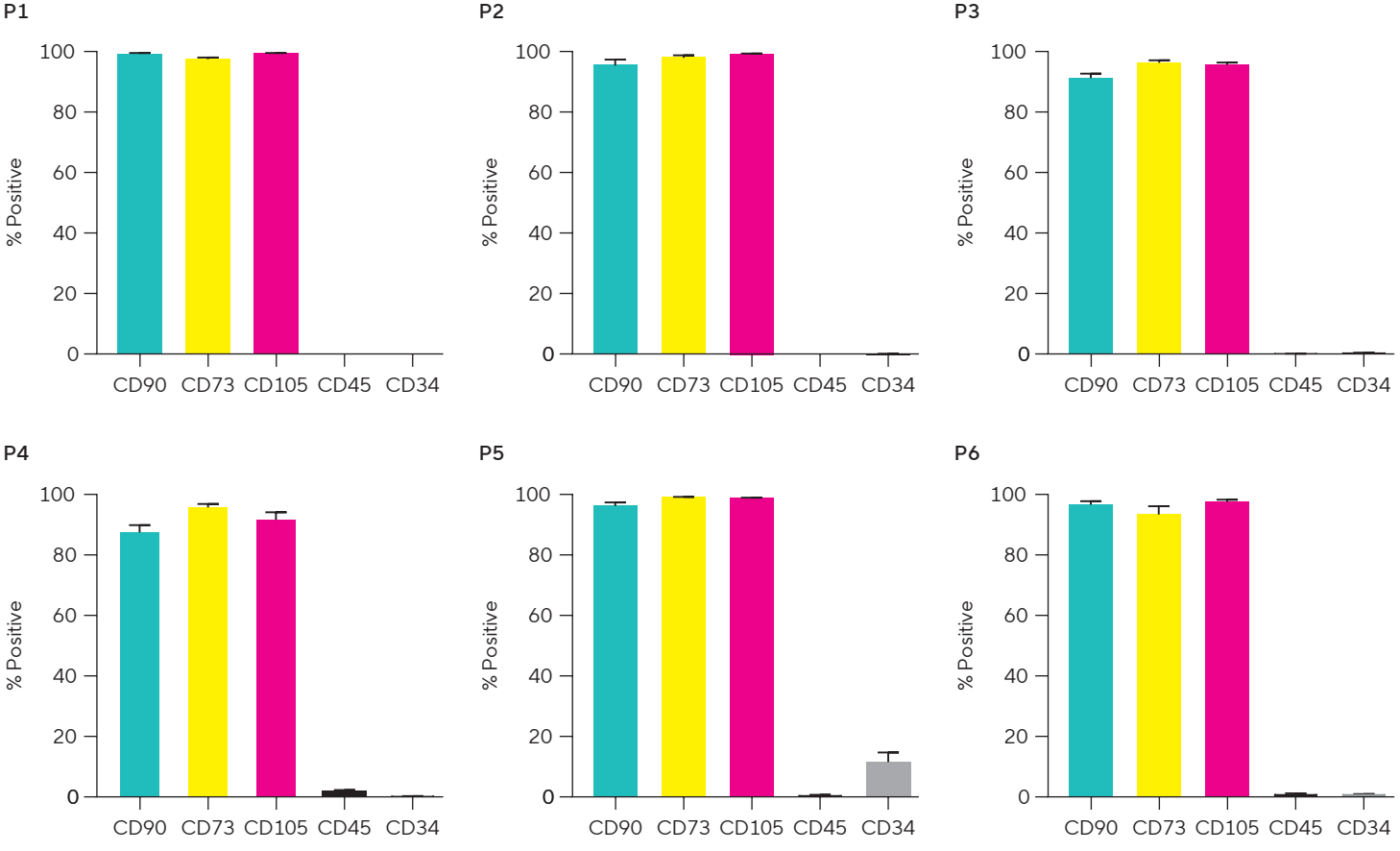

Figure 2. Expression of MSC Marker Panel Over Multiple Passages. Percentage positive populations are shown for each marker, as measured using HTS cytometry. Data shows percentage populations of an average of flasks at each passage, cultured in both Nutristem® and in commercial medium.

Multiparametric Evaluation Demonstrates That MSC Senescence Increases With Age

Evaluating MSC morphology can also provide valuable insights into cell status. MSCs tend to become larger and more irregular in shape as they age, indicated by a flattened, elongated shape with increased cytoplasmic area and granularity. In contrast, young MSCs are typically spindle-shaped and have a high nucleus-to-cytoplasm ratio.[5]

From the images, this difference is evident with cells in the earlier passages exhibiting smaller cell bodies which tend to increase gradually in size as the cells age through to P6 (Figure 3A). This morphology can also be indicative of a senescent state.

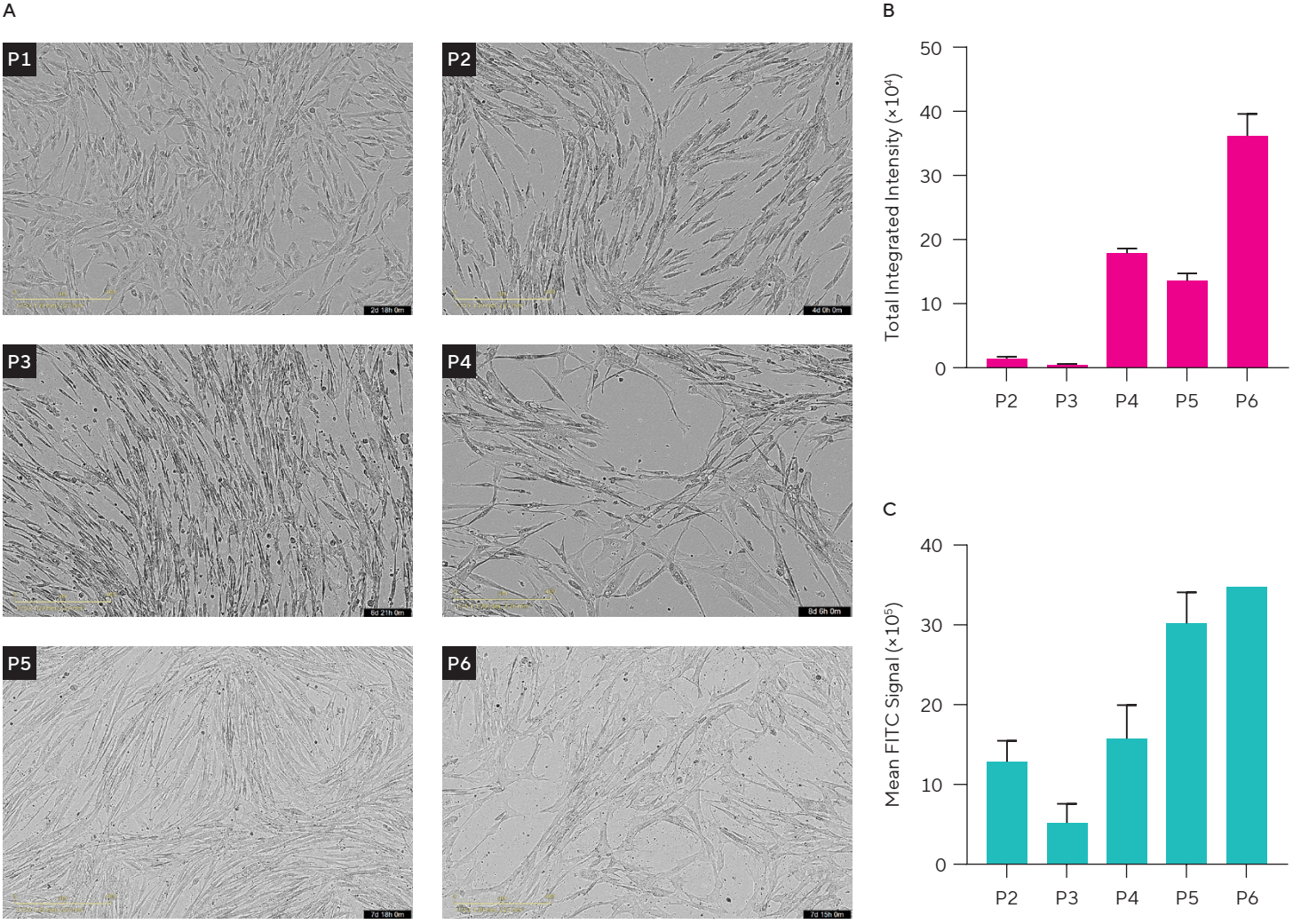

Figure 3. MSC Morphology and Quantification of Senescence Over Multiple Passages. (A) Images of MSCs at passages 1–6 at point of harvest. (B) Quantification of MSC senescence by analysis of fluorescent SA-β-Gal activity using live-cell analysis. (C) Quantification of MSC senescence by analysis of fluorescent SA-β-Gal activity of single cells using HTS cytometry.

Cellular senescence can be quantified through measuring SA-β-Gal activity at pH 6. In this study, fluorescent SA-β-Gal has been measured using both live-cell imaging (Figure 3B) and single cell fluorescence detection using HTS cytometry (Figure 3C). The data sets presented here were generated using the same samples. Although both analysis methods show similar trends, with fluorescent SA-β-Gal activity increasing with increased passage number as expected, the data obtained using live-cell analysis portray a clearer difference between the early and late passage cells. This could be linked to the different detection systems in each instrument; the optical path (including filters, lenses, and detectors), and the calibration or sensitivity level may affect the amount of fluorescence detected.

Label-free analysis can aid in the evaluation of MSC quality and hence senescence, by quantifying cell morphology through various metrics. Analysis of the images demonstrate both average cell area (Figure 4A) and eccentricity (Figure 4B) change as passage number and the age of the cells increase.

The data presented was taken from cells that were at the same percentage confluence (30%) to account for the changes in cell morphology and barriers to efficient cell segmentation as cells reach high confluence.

In accordance with expectations, these metrics indicate an increased cell size and increased irregularity in cell shape as the cells approach the Hayflick limit—the number of times a cell will divide before proliferation stops. The Hayflick limit is a particular consideration in the use of MSCs for therapeutic purposes, as it affects their proliferative capacity and longevity. These metrics provide researchers, looking to preserve valuable cellular material, with the potential to quantify the quality of MSCs label-free without having to employ labor-intensive and destructive protocols.

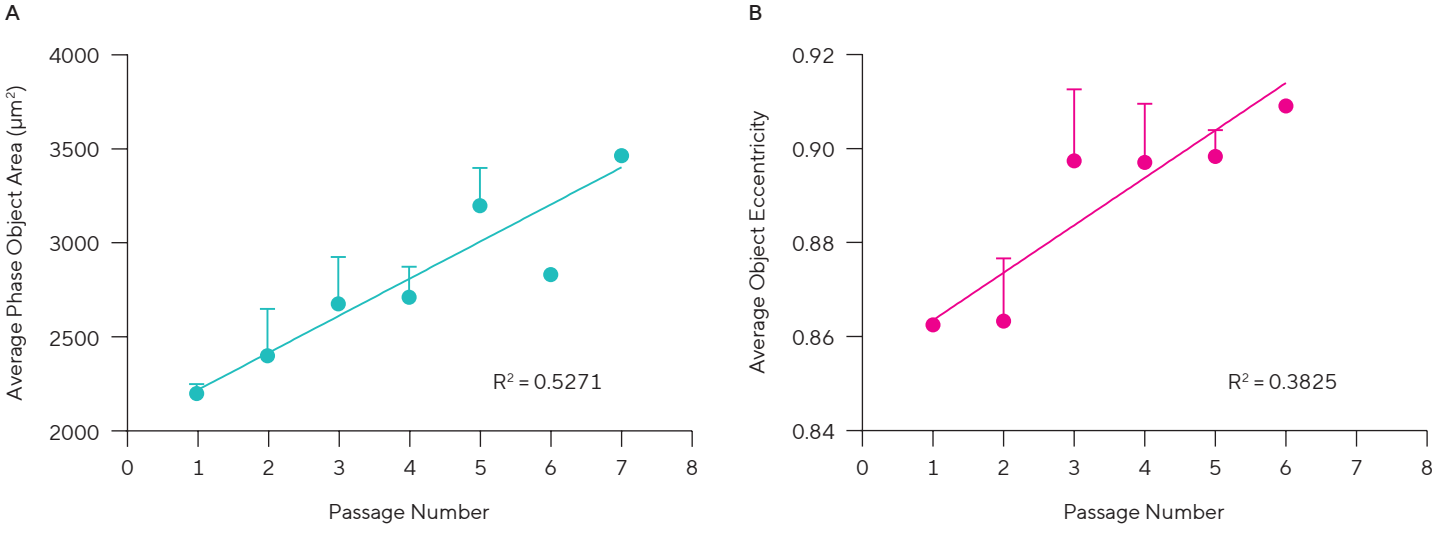

Figure 4. Quantification of MSC Morphological Characteristics Using Live-Cell Analysis. (A) Average cell area at 30% confluence. (B) Average cell eccentricity at 30% confluence.

Summary and Outlook

In conclusion, the combined use of live-cell analysis and HTS cytometry represents a powerful and effective strategy for the thorough evaluation of MSC quality and functionality. This integrated workflow not only improves the standardization and reproducibility of MSC assessments but also supports the advancement of MSC research and clinical applications.

By leveraging the strengths of both the Incucyte® System and iQue® HTS Platform, researchers can obtain comprehensive and high-resolution data on various aspects of MSC biology. The ability to quantify growth dynamics, cellular morphology, senescence, and marker expression in real-time and using minimal sample volumes is particularly advantageous.

The non-destructive nature of live-cell assays ensures that valuable cell populations are preserved for further use, enhancing the efficiency and effectiveness of MSC-based research and therapies.

Furthermore, the insights gained from this integrated workflow can inform the optimization of culture conditions, passage strategies, and expansion protocols, ultimately leading to improved outcomes in MSC-based clinical applications. By ensuring that MSCs meet stringent quality criteria, researchers can enhance the reliability and efficacy of MSC-based treatments, contributing to the advancement of regenerative medicine and cell therapy.

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