Application Note

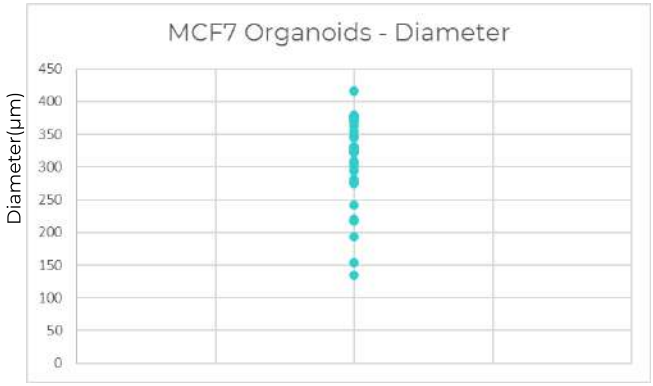
Quantifying heterogeneity in 3D cell culture

INTRODUCTION

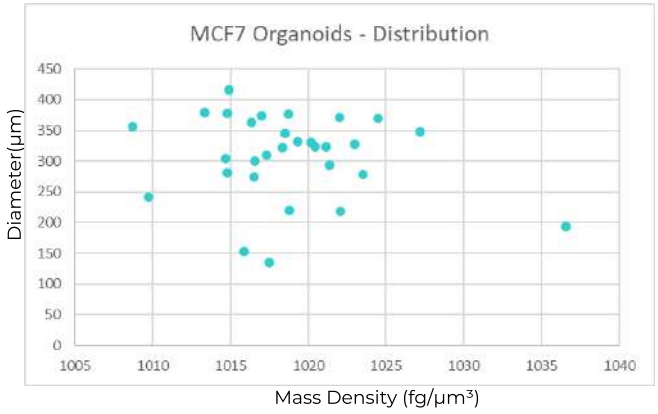
Heterogeneity in 3D cell cultures is a critical aspect that influences the accuracy and reliability of *in-vitro* models mimicking the complexity of tissues and organs in vivo. While heterogeneity provides a more representative portrayal of physiological conditions, it concurrently poses challenges in data interpretation and experimental consistency. This study introduces a novel, non-disruptive, and label-free approach to quantify heterogeneity in 3D cell cultures, aiming to provide quantitative insights into structural characteristics of the 3D models. This approach promises to enhance the utility of 3D cell cultures in disease modeling, drug testing, and tissue engineering by providing valuable quantitative data on the heterogeneity within the culture.

Shifting the size paradigm

Quantifying heterogeneity in 3D cellular models presents a significant challenge due to the inherent complexity introduced by the third dimension. Unlike traditional 2D cell cultures, where variables primarily relate to cell behavior and interactions on a flat surface, 3D models bring an additional layer of intricacy. This arises from the spatial aspects of the cellular organization within the model, the presence of extracellular matrix, and the potential for cavities or voids, all of which contribute to the structural and organizational diversity observed in 3D cellular systems.
In the pursuit of consistent and homogeneous populations of organoids, one of the prevailing strategies employed today revolves around ensuring that organoids exhibit similarity in size. This is motivated by the notion that size uniformity is a key determinant of cellular homogeneity within a population.



This singular focus, however, falls short in addressing the multifaceted nature of heterogeneity within these models. The issue lies in the misconception that uniform size alone can guarantee standardization.
In reality, several other critical factors, including compaction, cellular organization, organoid weight, significantly influence variations on any treatment or test within 3D cellular models.



When assessing the same organoids, beyond merely measuring diameters, but also incorporating mass density, it affords a more profound insight into the structure and organization of the examined organoid. The inclusion of this biomarker allows for a clearer understanding of the true distribution exhibited by organoids when measuring variables that account for factors present in 3D structures.

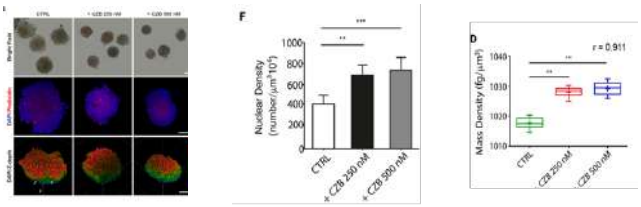
Mass Density in nutshell

The mass density in organoids, provide a general quantification of organoid structure.
The variations in mass density are primarily influenced by three factors:

1. Cell density
2. Extracellular matrix concentration
3. Presence or absence of cavities

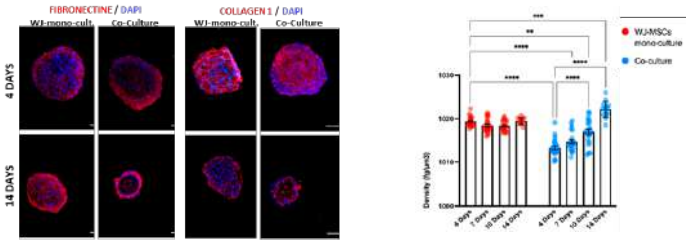
CELL DENSITY

The mass density of an organoid is heavily contingent upon the number of cells per unit volume. Higher cell densities result in increased mass density due to the accumulation of cellular material.



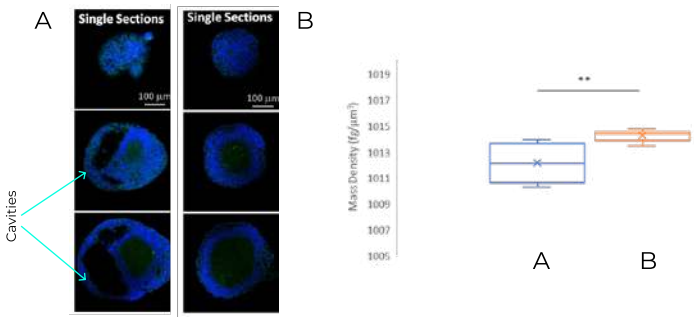
EXTRACELLULAR MATRIX CONCENTRATION

Mass density is also influenced by the concentration of the extracellular matrix (ECM), a complex network of proteins and molecules that provide structural support and regulate cellular behavior. Greater ECM concentration can lead to increased mass density, as it adds non-cellular mass to the organoid.



PRESENCE OR ABSENCE OF CAVITIES

The existence of cavities or void spaces within the organoid can significantly impact mass density. Presence of cavities reduces mass density, as these regions contain less cellular and ECM material, while their absence leads to a higher mass density.



We delved into the intricacies of organoid architecture, emphasizing how factors such as cell density, extracellular matrix composition, and the presence of cavities collectively contribute to the overall structural and maturation characteristics of organoids. It's evident that these variables and their interplay significantly influence the outcomes of tests or treatments conducted on the organoids.

Now, an essential question arises: how can we gain a comprehensive understanding of the structural state of organoids without the practical constraint of relying on daily confocal microscopy assessments?

Using Mass Density as Biomarker.

Quantify the structural heretogenicity of organoids in daily activities.

CASE STUDY
Assessment of a Quality Control Method for Evaluating Operator-Introduced Error in the Generation of Breast Cancer Tumor Organoids

In this case study, we will examine how operator influence can be assessed during the organoid generation process. This study will consider, correlate, and combine variations in both size and structural aspects. Furthermore, we will evaluate whether the organoids produced are similar or comparable to a database.

The procedure unfolds in three main phases:

1. **Generate a Reference Database:** In this phase, the generation of organoids using a standard protocol is imperative to establish a reference database.

2. **Define Acceptability Thresholds**: This phase involves a statistical analysis of the obtained database, leading to the definition of acceptability rules.

3. **Organoids Testing and Assess Quality**: The final step entails testing your organoids and evaluating their quality in adherence to the predefined acceptability criteria.

1. Generate a Reference Database

To establish a robust reference database for Breast Cancer organoids using the MCF7 cell line, a meticulous approach was taken, adhering to key criteria and methodologies. A minimum of 40 organoids were generated to ensure statistical robustness. The protocol was standardized, comprehensively documented for materials, methods, reagents, and plasticware to ensure consistency. The same operator handled the entire process to reduce operator-dependent variability, and stringent quality control measures were implemented, encompassing contamination checks, cell viability assessments, and organoid morphology evaluations. Comprehensive documentation was maintained, recording deviations from the standardized protocol.

DATABASE	MASS DENSITY	DIAMETER
MEAN	1018,79	307,83
SD	5,28	69,27
CORRELATION	-0,17	

2. Define Acceptability Thresholds:

In this phase, a statistical analysis of the dataset is carried out to establish acceptability thresholds for both diameter and Mass density. The definition of the thresholds employs a multivariate approach, considering key statistical parameters such as mean, standard deviation, variance, and covariance for both diameter and Mass density measurements.

THRESHOLD DETERMINATION:

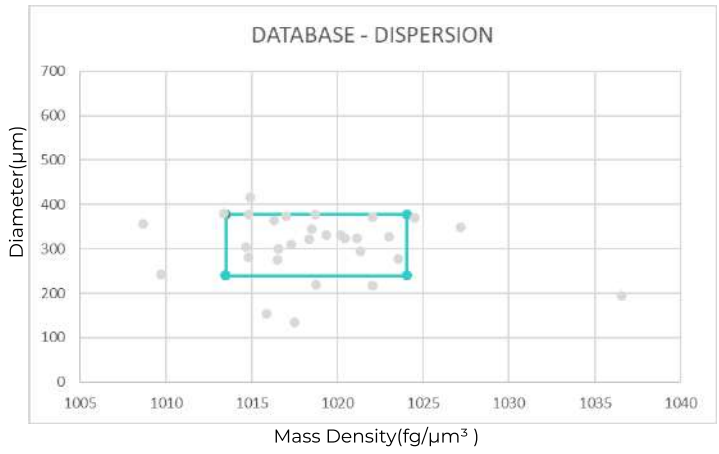
- Diameter Threshold: For this database, we opted to define the diameter thresholds based on the mean and standard deviation, thus identifying the maximum and minimum values. This approach, consistent with a normal distribution, enables us to pinpoint the organoids falling within one standard deviation, often referred to as Sigma=1.
- Density Threshold: For this database, we opted to define the Mass density thresholds based on the mean and standard deviation, thus identifying the maximum and minimum values. This approach, consistent with a normal distribution, enables us to pinpoint the organoids falling within one standard deviation, often referred to as Sigma=1.
- Combined Threshold: A combined threshold is defined, representing an area in the parameter space where both Diameter and Mass density measurements are most likely to be concentrated. This combined threshold identifies the region in which organoids exhibit characteristics considered acceptable.

Threshold Rules	
MASS DENSITY	DIAMETER
1024,1	377
1013,5	239

Acceptability Criteria			
	DATABASE		
Mass density	80%	>=	<=
Diameter	73%	>=	<=
Combined	60%	>=	<=

Finally, it outlines the acceptability criteria: for a population to be deemed satisfactory, it must exhibit:

- 80% of samples falling within the Mass Density threshold.
- 70% of samples falling within the Diameter threshold.
- 60% of samples in the combined threshold (the area defined by the intersection of the Diameter and Mass Density thresholds).



3. Organoids Testing and Assess Quality:

DATABASE

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OPERATOR X

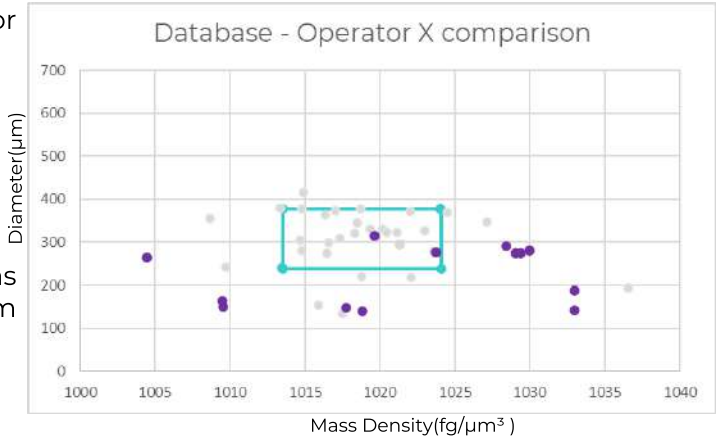
OPERATOR X	MASS DENSITY	DIAMETER
MEAN	1020,71	214,42
SD	9,92	70,40
CORRELATION	0,12	

N. of Samples	13	
N. Of sample in mass density threshold	4	31%
N. Of sample in diameter threshold	7	54%
N. Of sample in combined threshold	2	15%

This panel, describes the samples from Operator X comparted to the database.
In particular, 13 organoids were measured, with:

- 4 falling within the Mass Density threshold,
- 7 within the Diameter threshold.
- 2 within the combined threshold.

This clearly demonstrates that Operator X has generated samples that significantly deviate from the predefined standard.



W8 QC SCORE

	Database	Operator X
Mass density	80%	31%
Diameter	73%	54%
Combined	60%	15%

As a result, these samples do not meet the acceptability criteria, and are therefore deemed unacceptable.

CONCLUSIONS

These concluding remarks of the application note delineate a straightforward procedure designed for seamless integration into daily routines, facilitating a qualitative assessment of organoid preparation. The technique employed for the assessments is the W8 Physical Cytometer.

W8 QC SCORE, a database-dependent methodology that enables the assessment of organoid reproducibility, while mitigating errors originated from side effects biases, thereby enhancing the reliability of outcomes.

Once the database is validated, customized statistical analysis and acceptability criteria can be applied based on the scientific requirements of the user. The CellDynamics Team will be available to generate a tailored solution. Once the W8 QC SCORE criteria are defined, the quality control check necessitates 1-hour per single population, with 20 min commitment of the user.