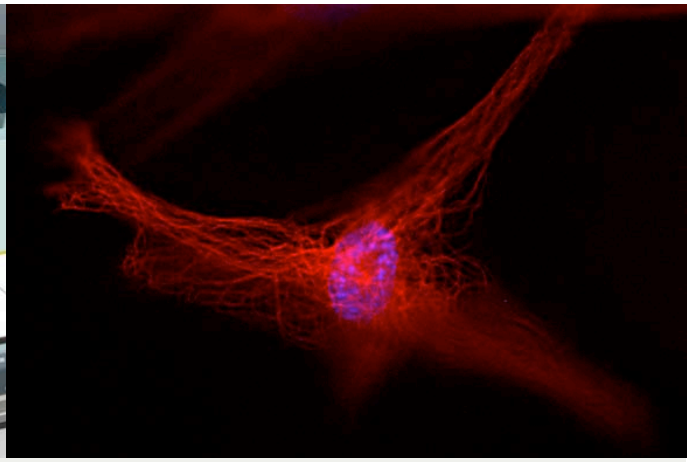

3D cell culture: a realistic and reproducible collagen-based environment

Automated preparation of human primary cell cultures using the Freedom EVO® and the RAFT™ 3D cell culture system

Introduction

Classical two-dimensional (2D) cell culture is a well-proven technique that is used widely in biological assays, drug screening and many other research areas. However, cells that are grown in 2D, *i.e.* in a monolayer, behave very differently from cells in real tissues. Consequently, researchers are increasingly turning to three-dimensional (3D) cell culture techniques, due to the improved physiological relevance of the cellular environment. 3D cell cultures offer a more *in vivo*-like response to stimuli – such as the addition of a drug candidate – more closely resembling the behavior of animal models or the human body.

In drug discovery and many other areas of research, cell culture using primary cells of human or animal origin is often desirable, or even essential. TAP Biosystems has developed a novel 3D cell culture technique, RAFT (Real Architecture for 3D Tissue), which uses the most abundant matrix protein in the body: type I collagen.

This technology allows researchers to culture cell types of their choice, including primary cells, in an *in vivo*-like environment that is essentially a hydrogel with tissue-like properties. Uniquely, the RAFT process increases the collagen concentration to physiological levels rapidly and reproducibly, while maintaining high cell viability.

Tecan and TAP Biosystems have developed an automated process for the preparation of RAFT cell cultures, aiding mixing procedures and enhancing reproducibility while maintaining excellent cell viability and reliability. Implementation on the Freedom EVO system, with its modular automation capabilities, increases experimental versatility and analytical flexibility. In this application note, we present cell viability and proliferation data for primary neonatal human dermal fibroblasts (Life Technologies) obtained over an 11-day culture period after seeding.

Materials and methods

RAFT 3D cell culture

The RAFT 3D cell culture kit contains reagents and labware for the preparation of 96-well plates for 3D cell culture. The workflow comprises five steps (Figure 1), which are automated on the Freedom EVO platform:

1. Preparing the neutralized collagen solution
2. Preparing the cell stock solution
3. Generating a cell-seeded collagen stock solution
4. Plating and gel induction
5. Finalizing of the RAFT 3D cell culture by concentration of the cell-seeded collagen to *in vivo* levels

Figure 1 Schematic drawing of the RAFT 3D cell culture plating, gel induction and concentration procedures.

Liquid handling equipment

The RAFT process was automated on a Freedom EVO liquid handling workstation equipped with an eight-channel Liquid Handling (LiHa) Arm using disposable tips, a Robotic Manipulator (RoMa) Arm, a Te-Shake® (heater-shaker), and cooled carriers (4 °C; active cooling of the reagents and the collagen solution is essential to control gel formation) for storage of reagents and microplates (Figure 2).

Figure 2 Freedom EVO equipped with carriers for automation of RAFT 3D cell culture. The RoMa Arm is holding a RAFT absorber plate, and a gel-filled cell culture plate is positioned on the Te-Shake. A cooled carrier containing cell culture plates is mounted to the right of the Te-Shake.

The collagen solution – collagen, neutralizing solution and 10x MEM – was prepared in a cooled trough, dispensing the reagents with the LiHa. Thorough mixing was achieved by gentle aspiration and dispensing, ensuring the collagen solution was homogeneous. Freshly harvested primary neonatal human dermal fibroblasts, expanded in 2D cell culture, were added to the collagen mixture to obtain a final cell density of 10,000 cells/well. The gel mixture including the cells, as well as a zero cell control for the viability assay, was plated into the RAFT 96-well cell culture plate on a cooled carrier using the LiHa. After plating, the culture plate was transferred to the Te-Shake by the RoMa for gel induction at 37 °C. After 15 min, the RAFT absorber plate was inserted into the culture plate using the RoMa, beginning a 15 min collagen concentration process. The RAFT absorber plate was then removed, and 100 µl of fresh cell culture medium added to each well with the LiHa. Cell cultures were incubated at 37 °C and 5 % CO_2, with medium exchange every two to three days.

Cell viability

Cell viability was determined with Life Technologies' 0.5 µM calcein AM (live cells) and 1.5 µM propidium iodide, analyzing the cultures on a Tecan Infinite® M200 PRO plate reader 1, 4, 8, and 11 days after seeding. Cell imaging was performed on an Olympus IX-71 inverted fluorescent microscope with Z-focus, enabling cell morphology to be observed and tracked over time in culture.

Proliferation assay

The CellTiter-Glo® Luminescent Cell Viability Assay (Promega) was used to assess cell proliferation over time, following the manufacturer's standard protocol. ATP equivalents were quantified 1, 4, 8, and 11 days after seeding, measuring luminescence on an Infinite M200 PRO plate reader.

Results

The RAFT process has been successfully automated on the Freedom EVO platform, creating a realistic and reproducible environment for primary cells. The data demonstrates excellent cell viability, ranging from 88 to 95 %, over an 11-day period. An increase in ATP equivalents from day 1 to day 11 indicates that cells proliferate within the culture (Figure 3).

Over time, the cells elongated and regained their natural morphology, showing that a 3D matrix offers a realistic, *in vivo*-like environment (Figure 4).

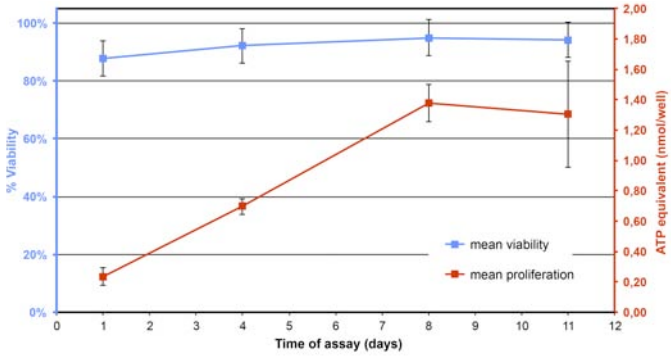

Figure 3 Viability and proliferation of primary human fibroblast cell cultures generated on the Freedom EVO. The error bars represent the standard deviation for each of the 64 samples assayed.

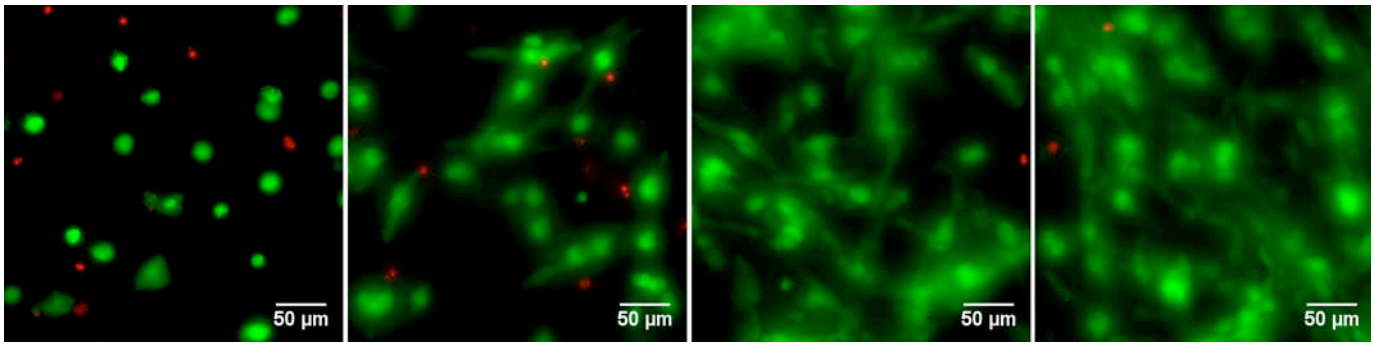

Figure 4 Primary human dermal fibroblast cultures generated in a RAFT 3D collagen environment on the Freedom EVO platform. Cells were stained with calcein AM (green – live cells) and propidium iodide (red – dead cells), and imaged on an inverted fluorescent microscope. The maximum intensity projection of a Z-stack of images, taken at 5 µm intervals across the whole RAFT culture, is shown here.

Discussion

This application note confirms the suitability of the automated 3D cell culture process for primary cells. Precise, reproducible liquid handling of highly viscous extracellular matrix proteins (type I collagen) with the Freedom EVO is crucial for the preparation of RAFT 3D cell cultures, enabling production of cultures of comparable thickness, with no bubbles and consistent cell density and distribution. Optimized liquid handling protocols ensure a high quality culture.

Automated production of one RAFT 96-well culture takes approximately one hour, which is comparable to the manual procedure. Parallel preparation of RAFT cultures on a Freedom EVO instrument is described in another application note. This reliable and robust automated process offers high reproducibility and eliminates the potential for human errors inherent in manual processing. The automation of time-consuming preparation procedures frees lab resources and improves the consistency of cell cultures.

The results presented in this application note demonstrate that it is possible to grow and assay the viability and proliferation of primary fibroblasts for at least 11 days after RAFT 3D seeding. In addition to excellent viability, the cells show good proliferation, with the population doubling several times during the period of cultivation. This result, together with the fact that cell morphology reverts to its natural appearance, makes the RAFT 3D cell culture system a reliable method for automated cell biology and drug discovery studies.

The RAFT process allows controlled concentration of the collagen, in contrast to other collagen based hydrogel cultures, which typically have a lower density and concentration of collagen. As a result these hydrogels suffer from a distortion during cell elongation. Consequently, the RAFT cultures do not suffer from the hydrogel distortion that makes further analysis difficult, or even impossible; a broad range of analytical techniques can be applied for additional analysis, as demonstrated here using Tecan's Infinite M200 PRO plate reader.

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