APPLICATION NOTE

Detection of multiplexed GFP reporters in primary articular chondrocyte cultures using Cellometer image cytometer.

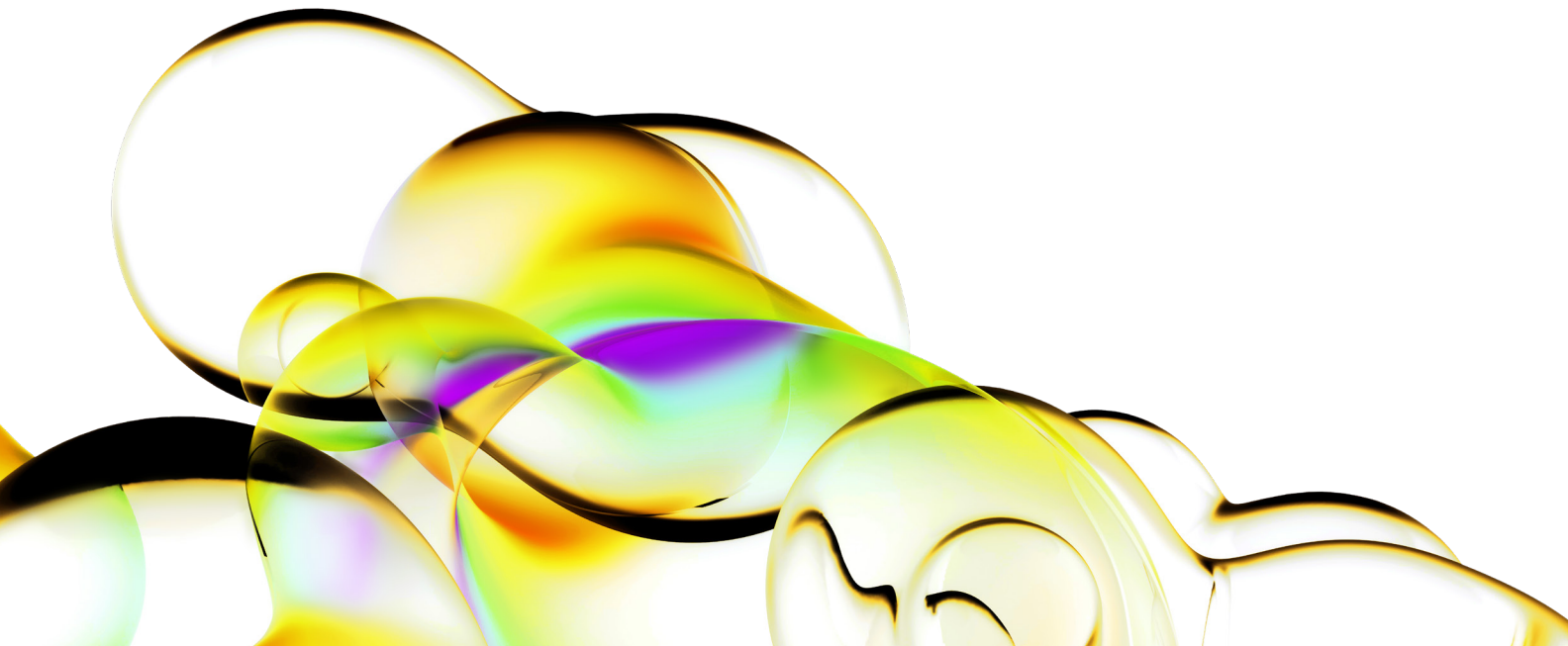
Introduction

In this work, we have developed an image cytometry method for detecting and monitoring the cell expansion and differentiation of articular chondrocytes in primary culture. First, the feasibility of utilizing image cytometry for detection of fluorescent is shown by comparing measured fluorescent positive cell populations to flow cytometry. Next, articular chondrocyte cultures were established in multi-well plates from either single or Cyan/eGFP double reporter mouse lines and grown for 20 days to test the utility of the fluorescence-based image cytometry system. We show that image cytometry is capable of measuring the positive cell populations. Here, we demonstrate an image cytometry method that is useful for a timecourse characterization of cell growth, proliferation, and differentiation of a primary culture derived from a GFP reporter animal.

Materials and methods

Image cytometry instrumentation and disposable counting chamber

The Cellometer® instrumentation has been described previously [1-3]. The system utilized brightfield (BR) and dual-fluorescent (FL1 and FL2) imaging modes to quantitatively analyze and measure the fluorescence intensities of target cells. The software analyzed three image channels (BR, FL1, and FL2) and generated a fluorescent data set that was automatically exported to FCS Express™ 4 Flow Cytometry.



Preparation of reporter mice

Mice are selected from our GFP colony to represent the range of colors that are commonly used. The transgenic mice used for this purpose include a Col3.6GFPcyan, Col3.6GFPtopaz, Dkk3eGFP, Col3.6GFPsapphire, Ai9 (tdTomato) [4], Col3.6RFPmcherry, and Col3.6RFPcrimson [5]. The cultures are performed in 6-well Costar tissue culture plates using alpha MEM. All of the cultures are harvested on the same day and used to compare the functionality of image and flow cytometric analyses.

Two GFP reporter mouse lines alone or in combination are used to study the transition of one reporter color to the other in a population of cells within a primary cell culture derived from articular cartilage. The first reporter expressed an eGFP driven by a Dkk3 promoter within the context of a BAC construct. The second was a GFPcyan reporter driven by a fragment of the mouse Col2A1 gene [6, 7]. The animals are intercrossed and offspring expressing both transgenes are identified by fluorescence microscopy of tail snips.

Image cytometric detection of fluorescent proteins

In order to show the capability of image cytometry, the detection method was first compared to a standard flow cytometer system. For image cytometric detection, appropriate fluorescence optics modules (FOM) were selected for each type of fluorescent protein. (excitation/ emission), VB-535-302 (376 ± 10 / 534 ± 20 nm), VB-480-402 (430±10 / 479±20 nm), VB-535-402 (475±20 / 535±20 nm), VB-535-403 (497 ± 8 / 535 ± 11 nm), VB-605-502 (525 ± 32 / 605 ± 22 nm), VB-625-502 (550 ± 25 / 625 ± 20 nm), and VB- 695-602 (628 ± 20 / 692 ± 20 nm) were used to detect the fluorescent proteins Sapphire, Cyan, eGFP, Topaz, tdTomato, mCherry, and Crimson, respectively. The Cellometer software automatically analyzed the captured images and exported the fluorescent intensity data into FCS Express™ 4 Flow Cytometry for population analysis. Each sample analysis is performed in duplicate.

Flow cytometry detection of fluorescent proteins

Single cell suspensions from sister wells of the bone marrow stromal cultures utilized for image cytometric analysis were prepared and independently read on a Beckton Dickinson Aria II cell sorter. For the purpose of this analysis, samples were screened with each laser: the 488 nm laser was used to excite eGFP, Topaz, and Sapphire; the 633 nm laser was used to excite Crimson; the 407 nm laser was used to excite Cyan; and the 561 nm laser was used to excite mCherry and tdTomato. For eGFP, Topaz, and Sapphire detection, a 505LP filter was used followed by a 530/30 filter. For Crimson detection, a 660/20 filter was used. For Cyan detection, a 450/50 filter was used. For mCherry detection, a 600LP filter was used followed by a 610/20 filter. For tdTomato, a 570LP filter was used followed by a 582/15 filter.

Histology

The expression pattern of the two reporters was assessed in articular cartilage using a cryohistological technique that preserves the GFP signal in adult tissues [8, 9]. Briefly, formaldehyde fixed non-decalcified tissues were equilibrated overnight and embedded in Cryomatrix for sectioning. The sections were cut at 5–7 µm and examined with a microscope using a cyan fluorescent protein filter (CFP, Chroma Cat 49001ET, EX: 436/20, EM: 480/40), a yellow fluorescent protein filter (eYFP, Chroma Cat 49003ET, EX: 500/20, EM: 535/30) filter, and a mCherry filter for the alizarin complexone staining (mCherry, Chroma Cat 49009ET, EX: 560/40, EM: 630/75). Using Photoshop (Adobe), the signal histogram of each filter image file was intensity-adjusted to occupy 50% of the 256-pixel intensity.

Articular chondrocyte cell culture preparation

Details on animal sacrifice, tissue harvest, and cell resuspension can be found in detail here [10]. The cells are pelleted by centrifugation and resuspended in PBS from which it is pipetted into a disposable counting chamber for analysis in the image cytometer.

Time-course study to evaluate cyan and eGFP Expression, concentration, and cell size using image cytometry

Two separate experiments were performed. The first was designed to validate that the imaging condition discriminated between single and double color cells throughout the length of the culture. Cultures from four separate mouse lines developed in a CD1 background (eGFP Negative (CD1), Dkk3eGFP, Col2A1GFPcyan, Dkk3eGFP/Col2A1GFPcyan) were established in alpha MEM. The second experiment assessed the impact of adjusting the medium to contain 4 mM β-glycerol phosphate, 50 µg/mL ascorbic acid, and 10 nM dexamethasone relative to non-supplemented culture media. In this case, only the Dkk3eGFP/Col2A1GFPcyan double transgenic animals were employed. The cells were grown in standard medium and then switched to the supplemented medium or maintained in control medium.

At each time point during the 20-day proliferative period, cells were collected and analyzed in the image cytometer. Under brightfield analysis, the concentration and cell diameter were automatically generated.

Under fluorescent image analysis, the fluorescence intensities of every cell were measured and exported to FCS Express 4 for population analysis.

Results

- The cell population results show comparable percentages between the two methods (flow %/image %): Cyan (29/31), eGFP 394 (45/ 38), Topaz (11/11), Sapphire (47/27), tdTomato (44/45), mCherry (13/11), and Crimson (31/34), which indicated that image cytometry could has similar performance capabilties as a standard flow cytometer.
- Sapphire was the only fluorescent protein that did not correspond between image and flow cytometry, which could be due to the different excitation and emission wavelengths between the two systems.

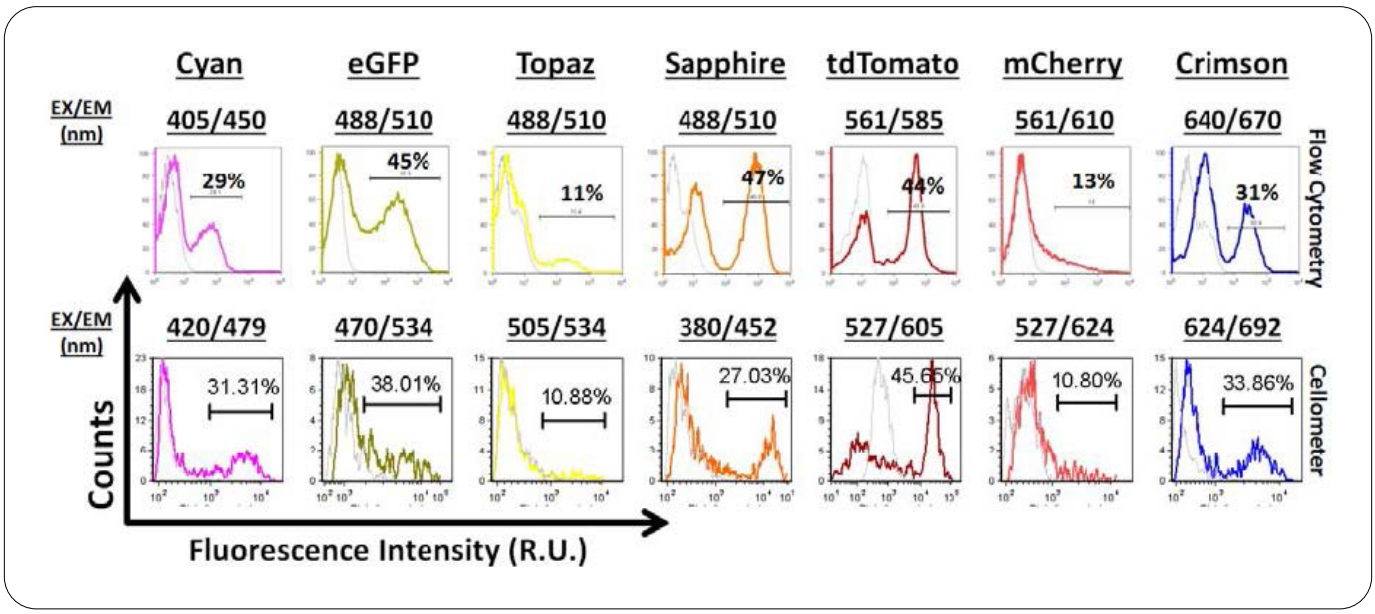


Figure 1: Comparison of image and flow cytometry results

- Epifluorescent image of a non-decalcified section taken as a coronal section through the knee of a Col2A1-GFPcayn/Dkk3-eGFP reporter mouse. (a) Full view showed the strong Dkk3-eGFP signal in adjacent ligaments and the meniscus. Within the articular cartilage, the Dkk3eGFP cells were located just beneath the articular surface, while the Col2A1GFPcyan cells are positioned deeper in the structure. The transition of cells from Dkk3 to Col2A1 is appreciated as lighter blue color in the merged image.

- (b) An enlarged version of (a) is shown here. The silver color is accumulated mineral, green is Dkk3, blue is Col2A1 and red was alizarin complexone, a systemically administered mineralization dye that labels actively mineralizing bone and cartilage. The growth plate cartilage generated the strongest blue and red signal, and the blue color of the surrounding is skeletal is autofluorescence.
- (c) and (d) Show the weaker blue and red signal in the articular cartilage (arrows) and the tissue used to initiate the chondrocyte cultures. Note that osteoblasts were not labeled by these reporters and that growth plate chondrocytes would not have been exposed to the digestive enzyme mixture. Because the meniscus and ligaments were dissected away from the remaining bone surface, articular cartilage would be the primary contributor to the cell culture.

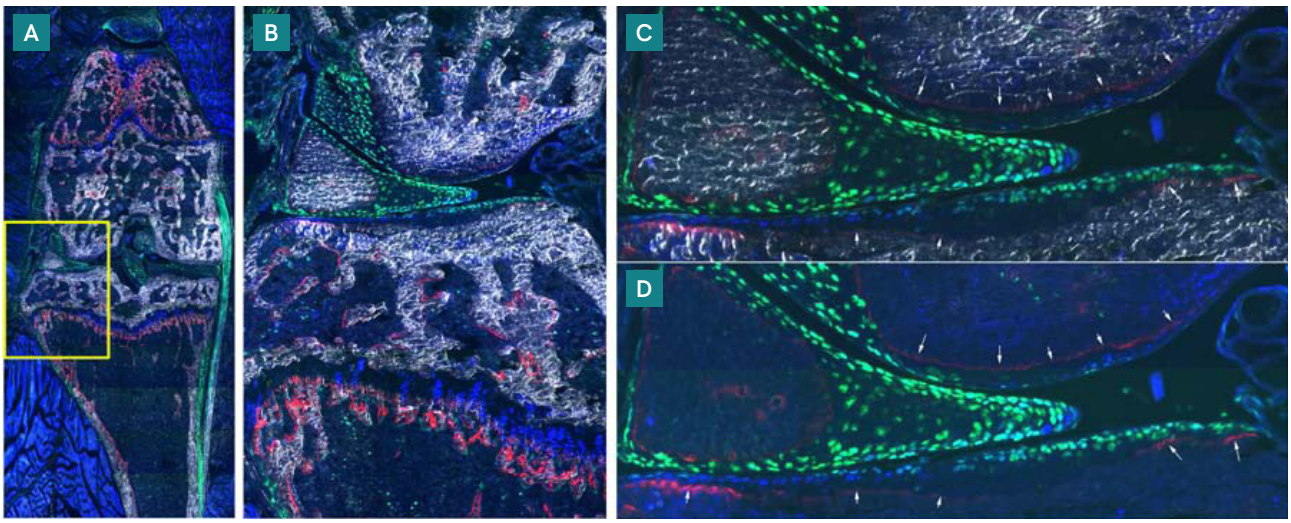


Figure 2: Histological Characterization of the Dkk3eGFP and Col2A1GFPcyan in Articular Cartilage

- (a-d) These graphs represent cultures grown under control conditions with a) eGFP Negative (CD1), b) Dkk3eGFP only, c) Col2A1GFPcyan only, and d) Dkk3eGFP/Col2A1GFPcyan double trans-genic mice.
- The Dkk3eGFP culture (b) showed a gradual increase from 2 to 29% of cell in the culture by day 14, after which the percentage began to decrease.
- In the single reporter culture (c), Col2A1GFPcyan remained constant at about 15 % from day 2 forward, while in the double reporter, a high baseline of Col2A1GFPcyan cells (10%) (reached as high as 40% and gradually decreased to 20 %) is observed. This pattern would suggest that either another cell source (growth plate chondrocytes) that expresses Col2A1GFPcyan but not Dkk3 may have contaminated the initial culture, or that the plated Dkk3eGFP/Col2A1GFPcyan double population rapidly lost the Dkk3 component as suggested in (f). The color from this excessive population is gradually lost either due to apoptosis or progression to the hypertrophic level of differentiation.
- In the double reporter culture (d), Dkk3EGFP only cells never exceeded 12% of the total cell number with the difference being due to the increase in Dkk3eGFP/ Col2A1GFPcyan double population, which in turn accounted for approximately 15%. There may be a discrepancy in the Col2A1GFPcyan cells between the single and double reporter combinations.
- (e) This graph represents cultures switched to differentiation medium at day 6 of culture. At day 8 of culture, the medium is supplemented to promote chondrogenic progression to terminal differentiation. This change resulted in an:

- increase of the double positive Dkk3eGFP/ Col2A1GFPcyan cells from 15 to 30%;
- a decrease in the Dkk3eGFP only percentage from 12 to 8%;
- and a smaller increase in the Col2A1GFPcyan population from 12 to 20%. These changes were consistent with progression of differentiation from Dkk3eGFP only → Dkk3eGFP/Col2A1GFPcyan → Col2A1GFPcyan only [11].

- (f) This graph demonstrates the calculated cell concentrations of the culture that was grown under differentiating conditions, where there is an obvious increase in the double color cell type. The Dkk3eGFP/ Col2A1GFPcyan population increases almost six-fold while the single color and non-GFP population increase about three-fold in number. After day 16 of culture, the number begins to fall suggesting the culture is losing viability.

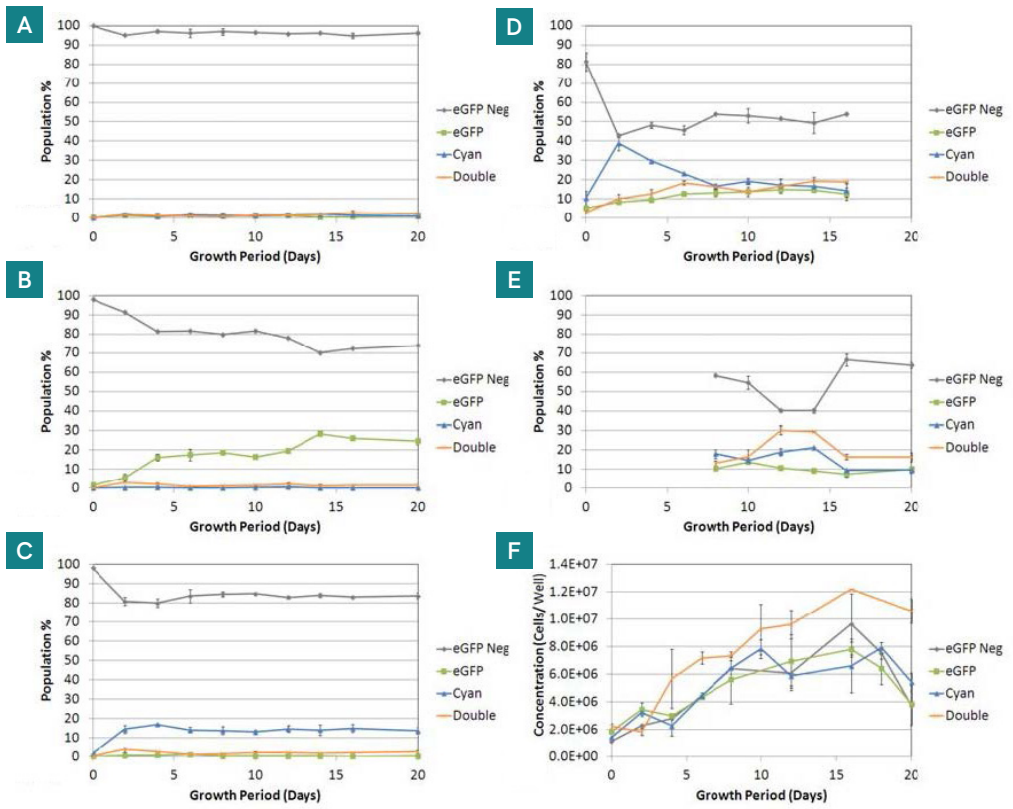


Figure 3: Articular chondrocyte under various culture conditions

Conclusion

- The fluorescent image cytometry method described here can capture multiple image data so that a series of wells, any of which may produce as few as 500 cells, is still adequate for an accurate reading.
- The application of utilizing fluorescence-based image cytometry for primary cell culture of articular chondrocytes illustrates its utility for establishing culture conditions and monitoring cell differentiation over time.
- For example, the possibility of contamination of cells not of articular cartilage origin, such as growth plate chondrocytes, is identified through this method because an inconsistency in reporter combination early in the culture period. Similarly, the loss of GFP activity and cell number after day 16 in culture indicates the limitation to the length that the culture remains viable.

- What is not answered in the current study is which GFP population was actively dividing and providing the progenitor source for subsequent differentiation. The observation that the supplemented medium increased the proportion of the Dkk3eGFP/ Col2A1GFPcyan double positive cells without a comparable decrease in the Dkk3eGFP only population suggests that the Dkk3eGFP population is being replenished either from a non-GFP progenitor or by proliferation within the Dkk3eGFP population. Future studies combining fluorescent markers of DNA replication with the GFP markers of differentiation should be able to make this distinction.
- In summary, the use of GFP reporters, as markers of lineage differentiation, allows for the analysis of articular chondrocyte proliferation and differentiation.
- Slight modifications easily adapt this assay to image cytometry analysis, which provides an efficient and simple method that conforms to the daily workflow of a cell culture-based laboratory.

References

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