J Fluoresc (2014) 24:1041–1053
DOI 10.1007/s10895-014-1383-2

ORIGINAL PAPER

Discriminating Multiplexed GFP Reporters in Primary Articular Chondrocyte Cultures Using Image Cytometry

Leo Li-Ying Chan · Jianping Huang · Yusuke Hagiwara · Leonardo Aguila · David Rowe

Received: 31 December 2013 / Accepted: 20 March 2014 / Published online: 13 April 2014

Abstract Flow cytometry has become a standard tool for defining a heterogeneous cell population based on surface expressed epitopes or GFP reporters that reflect cell types or cellular differentiation. The introduction of image cytometry raised the possibility of adaptation to discriminate GFP reporters used to appreciate cell heterogeneity within the skeletal lineages. The optical filters and LEDs were optimized for the reporters used in transgenic mice expressing various fluorescent proteins. In addition, the need for compensation between eGFP and surrounding reporters due to optical cross-talk was eliminated by selecting the appropriate excitation and emission filters. Bone marrow or articular cartilage cell cultures from GFP and RFP reporter mouse lines were established to demonstrate the equivalency in functionalities of image to flow cytometry analysis. To examine the ability for monitoring primary cell differentiation, articular chondrocyte cell cultures were established from mice that were single or doubly transgenic (Dkk3eGFP and Col2A1GFPcyan), which identify the progression of superficial small articular cell to a mature chondrocyte. The instrument was able to rapidly and accurately discriminate cells that were Dkk3eGFP only, Dkk3eGFP/Col2A1GFPcyan, and Col2A1GFP, which provides a useful tool for studying the impact of culture conditions on lineage expansion and differentiation.

Keywords Articular chondrocyte · Chondrocyte differentiation · Cellometer · Image cytometry · Flow cytometry · GFP reporters · RFP reporters

Leo Li-Ying Chan and David Rowe share senior authorship.

Electronic supplementary material The online version of this article (doi:10.1007/s10895-014-1383-2) contains supplementary material, which is available to authorized users.

L. L.-Y. Chan (✉)
Department of Technology R&D, Nexcelom Bioscience LLC, 360 Merrimack St. Building 9, Lawrence, MA 01843, USA
e-mail: lchan@nexcelom.com

J. Huang · Y. Hagiwara · D. Rowe
Center for Regenerative Medicine and Skeletal Development, Department of Reconstructive Sciences, School of Dental Medicine, University of Connecticut Health Center, Farmington, CT 06030, USA

L. Aguila
Department of Immunology, School of Medicine, University of Connecticut Health Center, Farmington, CT 06030, USA

Introduction

Green fluorescent protein (GFP) reporters have become an essential experimental tool for detecting cellular heterogeneity within a larger cell population [1]. The spectrum of use extends from identifying cells that have been transfected in vitro with deoxyribonucleic acid (DNA) construct [2] to marking cells derived from a GFP reporter animal that are grown in primary culture and have achieved a defined level of differentiation [3]. In either case, the fluorescent signals can be used to assess the proportion of cells expressing the experimental feature using fluorescence-based flow cytometric analysis (FC) or by isolating these cells using fluorescence-activated cell sorting (FACS) [4]. A major effort in our laboratory is defining lineage branch points and stages of progression to full cellular differentiation within the mesenchymal lineage. For any given skeletal structure, a series of GFP reporters have been developed whose expression in bone and cartilage has been characterized by a cryohistological approach that preserves the GFP signals in adult animals and in mineralized structures [5]. Primary cultures derived from mice carrying the genetic combinations of the GFP colors that in vivo demonstrates a progenitor to mature cell progression provides the opportunity to isolate and characterized this

transition if the same progression can be replicated in the primary culture system [6].

The initial challenge in characterizing whether the primary culture system will prove to replicate the in vivo progression is based on direct visualization of the culture dish in a fluorescent tissue culture microscope. The observer obtains a qualitative impression of the onset of GFP expression as well as the increase in cellularity of the culture. Eventually, a user-dependent estimation as to when to harvest the tissue for FC analysis is required. The fluorescence microscopy method can be time-consuming, labor intensive, and lacks the ability for quantitative analysis of a large cell population, which makes it difficult to construct a comprehensive experiment to define the optimal conditions for expanding a certain cell population. It would be particularly helpful to characterize the optimal growth and differentiation conditions, and the time-course effects in 12–24 well tissue culture plates using FC analysis to obtain an observer independent assessment of cell growth and differentiation. Due to the fact that conventional flow cytometry systems are relatively expensive, complex, and require considerable amount of maintenance, they are only practical in core facilities that can often have availability limitations. In addition, although the use of high power lasers in conventional flow cytometers can enhance the fluorescence signals, the available excitation wavelengths can induce fluorescent cross-talk between various fluorescence channels, which generates the need for compensation.

Numerous fluorescence-based image cytometers and image analysis programs to assess a suspension of cells in a hemacytometer-like counting chamber have been developed [7, 8]. These image cytometers can be optimized for the combinations of GFP reporters that are utilized for tracking cell expressions. In particular, the widely used enhanced GFP (eGFP) fluorescence channel has the disadvantage of detecting the GFPcyan (Cyan), GFPtopaz (Topaz), GFPsapphire (Sapphire), and even red fluorescent protein (RFP) spectra, such as RFPtdtomato (tdTomato), thus limiting its use in combination with other fluorescent proteins. In the recent years, the Cellometer image cytometry system (Nexcelom Bioscience LLC, Lawrence, MA) has been shown to perform rapid fluorescence-based cellular analysis [9–12]. Furthermore, image cytometry method allows enumeration of cell concentration as well as the ability to quantify cell diameter simultaneous. The ability to utilize different light-emitting diode (LED) excitation wavelengths can allow optimization of fluorescence detection and eliminate the need for compensation.

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 population to flow cytometry. The fluorescent proteins expressing cell sample including Cyan, eGFP, Topaz, Sapphire, tdTomato, mCherry, and Crimson are measured in both image and 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 method is capable of measuring the fluorescent positive cell populations. Since image cytometry requires only a small volume of sample, cell concentration and cell size are measured in two-day intervals from sister wells within a single 24 well plate. In order to separate Cyan and eGFP fluorescence, a Yellow Fluorescent Protein (YFP) filter combination was used instead of eGFP filter to eliminate cross-talk of Cyan into the eGFP channel. The developed image cytometry method should be useful for a time-course 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 Vision instrumentation has been described previously [9, 10, 12]. The system utilized bright-field (BR) and dual-fluorescent (FL1 and FL2) imaging modes to quantitatively analyze and measure the fluorescence intensities of target cells. Bright-field imaging used a white LED and fluorescent imaging used a combination of various monochromatic LEDs (380, 420, 470, 505, 527, and 624 nm) as the excitation light sources. The monochromatic LEDs are integrated into specific fluorescence optics modules (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). The system used a magnification objective of 5X and the optical detection limit has been previously described [10].

Typically, the fluorescently labeled cell sample is pipetted into the Nexcelom disposable counting chambers with a fixed height of less than 100 μm, which held precisely 20 μl of volume. The concentration dynamic range of the system was 5×10^4–7×10^7 cells/ml, which is equivalent to 20–10,000 cells per counting chamber. The counting slide is held in position by a stage, which automatically moved to 4 locations on the chamber for cellular analysis by the Cellometer software. 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 (De Novo Software, Los Angeles, CA).

Preparation of Reporter Mice

Mice are selected from our GFP colony to represent the range of colors that are commonly used in an experimental setting. Traditional primary bone marrow stromal cultures that expressed the reporters are established and used to optimize the LED excitation and emission filter settings [3]. The transgenic mice used for this purpose include a Col3.6GFPcyan, Col3.6GFPtopaz, Dkk3eGFP, Col3.6GFPsapphire, Ai9 (tdTomato) [13], Col3.6RFPmcherry, and Col3.6RFPcrimson [14]. The cultures are performed in 6 well Costar tissue culture plates using aMEM containing 10 % fetal bovine serum (FBS) and 1 % penicillin/streptomycin. All of the cultures are harvested on the same day and used to compare the functionality of image and flow cytometric analysis.

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 that was obtained from the Mutant Mouse Regional Resource Centers (MMRRC:MGI:4846992) (http://www.mmrrc.org/). The second was a GFPcyan reporter driven by a fragment of the mouse Col2A1 gene [15, 16]. The animals are intercrossed and offspring expressing both transgenes are identified by fluorescence microscopy of tail snips. All mice used in the study followed protocols approved under the animal care number ACC2010-610.

Image Cytometric Detection of Fluorescent Proteins

In order to show the capability of measuring specific fluorescent protein signals using 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. VB-535-302, VB-480-402, VB-535-402, VB-535-403, VB-605-502, VB-625-502, and VB-695-602 were used to detect the fluorescent proteins Sapphire, Cyan, eGFP, Topaz, tdTomato, mCherry, and Crimson, respectively (filter bandwidths are described previously). The imaging exposure times for Sapphire, Cyan, eGFP, Topaz, tdTomato, mCherry, and Crimson were 3000, 10000, 8000, 15000, 2500, 10000, and 10000 ms, 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. Each fluorescent protein is examined using each fluorescence optics module to observe the fluorescence intensities as well as optical cross-talk between filters with emission wavelengths in close proximity. The measured fluorescent positive cell population results are compared to flow cytometry for validation.

Flow Cytometric Detection of Fluorescent Proteins

Single cell suspensions from sister wells of the bone marrow stromal cultures utilized for image cytometric analysis are prepared and independently read on a Beckton Dickinson Aria II cell sorter (Franklin Lakes, NJ). This instrument is equipped with five lasers exciting fluorophores at the following wavelengths: 488 nm (blue laser), 633 nm (red laser), 407 nm (violet laser), 355 nm (UV laser), and 561 nm (yellow/green laser). For the purpose of the analyses, samples are screened with each laser: the 488 nm laser is used to excite eGFP, Topaz, and Sapphire; the 633 nm laser is used to excite Crimson; the 407 nm laser is used to excite Cyan; and the 561 nm laser is used to excite mCherry and tdTomato. The detection of different fluorochromes is performed on dedicated detectors whose photo-multiplying tube (PMT) voltages were independently calibrated to a base line using non-fluorescent cultured cells. For the detection of fluorescence, the best available filters are used to selectively detect the emission signals defined for each fluorescent protein. For eGFP, Topaz, and Sapphire detection, a 505LP filter is used followed by a 530/30 filter. For Crimson detection, a 660/20 filter is used. For Cyan detection, a 450/50 filter is used. For mCherry detection, a 600LP filter is used followed by a 610/20 filter. For tdTomato, a 570LP filter is used followed by a 582/15 filter. Samples are flown through a 100 μm nozzle, at a flow rate between 5,000 to 10,000 events per second, and 100,000 events of ungated cell populations are collected.

Histology

The expression pattern of the two reporters is assessed in articular cartilage using a cryohistological technique that preserves the GFP signal in adult tissues [5, 17]. The animals are given a single IP injection of alizarin complexone (30 mg/kg, Sigma-Aldrich A-3882) 1 day prior to sacrifice to identify active mineralizing surfaces. Briefly, formaldehyde fixed non-decalcified tissues are equilibrated with 30 % glycerol overnight and embedded in Cryomatrix (Thermo Fisher Scientific, Waltham, MA) for sectioning. The sections are cut at 5–7 μm using tape stabilization (Section-Lab, Hiroshima, Japan), and then the section is transferred to a glass slide for imaging. The sections are examined with a Zeiss Axiovision 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. This was done by adjusting the left slider to the base of the histogram, and moving the right slider to position the mid-range pointer at

the other base of the histogram. Done in this manner the midrange pointer remains at the midpoint between the left and right sliders (1.0). The adjusted files were superimposed and merged using the screen mode making the blue and green only cells visually distinguishable from the merged color of the double positive cells. Animals at 1–3 months of age are used to characterize the expression pattern in articular cartilage tissue.

Articular Chondrocyte Cell Culture Preparation

The mice are sacrificed at 3–4 weeks of age and the intact tibia and femur dissect free of adventitial tissues. Using a dissecting microscope, the periosteal and ligamentous tissue surrounding the diaphyseal and metaphyseal bone is scraped from the bone surface leaving the articular cartilage of the distal femur, femur head and proximal tibia as the only viable tissue on the bone surface. The intact bone is placed in phosphate buffered saline (PBS) on ice. Once all the dissected bones are collected, they are placed in a digestion solution containing 3 mg/ml collagenase D (Roche, Indianapolis, IN) and 2 mg/ml dispase (Gibco, Grand Island, NY) dissolved in PBS. The sample is incubated at 37 % with shaking for 3 sequential 40 min digestion cycles. The isolated cells from each cycle are transferred to a 15 ml tube containing equal volume of culture medium and placed on ice until the other aliquots are collected in the same tube. The suspension of isolated cells is passed through a 40 μm cell strainer, harvested by centrifugation, resuspended in 6 ml of culture medium and distributed to 12 wells of a 24 well culture plate at a density of $25–30\times10^3$ cells/cm^2 (0.3×10^6 cells/well). This number of cells can usually be obtained from a single mouse.

Because articular cell cultures maintained their cartilage phenotype in a low O_2 (5 %) atmosphere better than ambient atmospheric culture conditions [18–22], all of the plates were maintained in a Sanyo O_2/CO_2 incubator at 90 % N, 5 % O_2, 5 % CO_2 at 37ºC. However, the cell manipulation steps are performed in a tissue culture hood at ambient atmospheric conditions. Prior to harvesting, the selected wells are photographed with an inverted Olympus fluorescent microscope to demonstrate the distribution of GFP colors within the well. To dislodge the cells, the well is washed with PBS followed by addition of 0.25 % trypsin/EDTA (Gibco). The detached cells are placed in a 1.5 ml eppendorf tube and diluted with an equal volume of culture medium. The cells are pelleted by centrifugation and resuspended in PBS (between 60 and 180 μl depending on the pellet size) from which 20 μl is pipetted into a Nexcelom disposable counting chamber for analysis in the image cytometer.

Time-Course Study of Cyan and eGFP Expression Using Image Cytometry

Two separate experiments are performed. The first is 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) are established in alpha MEM (Gibco 12571-063) containing 10 % fetal calf serum and harvested between day 0 to day 20 in 2-day intervals. The second experiment assessed the impact of adjusting the medium to contain 4 mM ß-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 are employed. The cells are grown for 6 days in standard medium and then switched to the supplemented medium or maintained in control medium. Wells are photographed using the Olympus fluorescent microscope and collected at two-day intervals from day 8 to day 20. The Col3.6GFPcyan and Dkk3eGFP were imaged separately and processed as described in the histological sections.

It has been known that the emission wavelengths of Cyan would cross-talk into a typical eGFP emission channel, thus large compensation is required in FC analysis [23]. This Cyan fluorescence cross-talk signal is also observed in the eGFP detection FOM VB-535-402 in Fig. 1. In order to eliminate the need for fluorescence compensation, we utilized the Topaz FOM VB-535-403 to completely remove the optical cross-talk signals of Cyan. The time-course detection of Cyan and eGFP fluorescence is performed by using VB-480-402 and VB-535-403, respectively. Both FOMs are used for each prepared cell sample (eGFP Negative, Dkk3eGFP, Col2A1GFPcyan, Dkk3eGFP/Col2A1GFPcyan), as well as the sample in differentiation medium. The samples are analyzed at exposure times 15000 and 7000 ms for Cyan and eGFP, respectively. Bright-field and fluorescent images are captured at each time point to determine the changes in Cyan and eGFP fluorescence in the cell populations. Each sample is measured in duplicate.

Time-Course Analysis of Concentration, Cell Size, and Fluorescence Intensity

At each time point during the 20 days proliferative period, the cells are collected from each well and analyzed in the image cytometer. Under bright-field image analysis, the concentration and cell diameter are automatically generated. The cell size data is exported to FCS Express 4 to compute the changes in mean cell diameters over 20 days. Similarly, at each time point, the concentration each cell sample is measured using the image analysis software, which is then multiplied by the

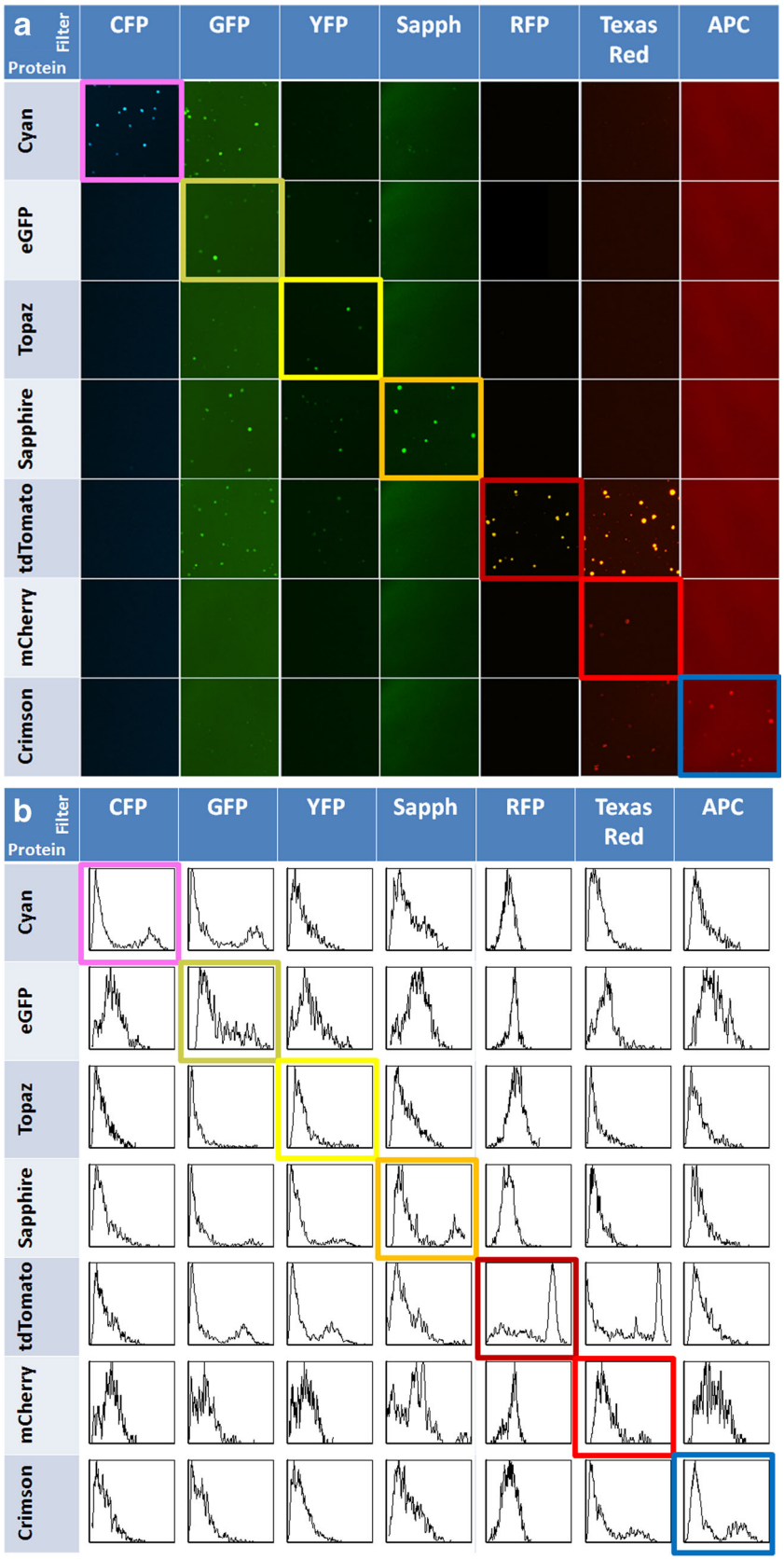

Fig. 1 Establishing excitation/emission conditions of the image cytometer for a spectrum of GFPs colors. **a** Fluorescent images of Sapphire, Cyan, eGFP, Topaz, tdTomato, mCherry, and Crimson in FOMs VB-535-302 (Sapphire), VB-480-402 (CFP), VB-535-402 (GFP), VB-535-403 (YFP), VB-605-502 (RFP), VB-625-502 (Texas Red), and VB-695-602 (APC). For the GFP reporters, Cyan, Topaz, Sapphire, and tdTomato showed optical cross-talk into the GFP channel. For the RFP reporters, tdTomato and Crimson showed optical cross-talk into the Texas Red channel. **b** Fluorescence histograms generated in FCS Express 4. Each fluorescent protein was measured in each channel, which can be observed in the cell population displayed in the histograms. The background in eGFP filter was higher than other filters, which was due to the plastic auto-fluorescence from 470 nm excitation

correct dilution factor to obtain accurate concentration values over 20 days. Under fluorescence image analysis, the fluorescence intensities of every cell are measured and exported to FCS Express 4 for population analysis. The gating

of each cell population is set by using the control sample as reference in order to properly gate the fluorescent protein positive populations.

Results

Establishment of the Filter Conditions

To demonstrate the comparability of image to flow cytometry, seven FOMs are used to detect seven types of fluorescent proteins. Each fluorescent protein is examined in each FOM to determine signal strength, exposure time, and potential cross-talk signals (Fig. 1). Fluorescent images of Sapphire, Cyan, eGFP, Topaz, tdTomato, mCherry, and Crimson in FOMs VB-535-302, VB-480-402, VB-535-402, VB-535-403, VB-605-502, VB-625-502, and VB-695-602 are shown in Fig. 1a. Under fluorescence imaging, the majority of fluorescent proteins are only detected in its respective FOM with a few exceptions. The Cyan, Topaz, Sapphire, and tdTomato fluorescent signals showed optical cross-talk into the GFP fluorescence channel, while the Texas Red fluorescent channel can detect tdTomato and Crimson, which can be observed in both fluorescent images (Fig. 1a) and fluorescence histograms (Fig. 1b).

Figure 2 showed the fluorescence histogram comparison between image and flow cytometry. The cell population results showed 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 cytometer could have similar performance 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.

Histological Characterization of the Dkk3eGFP and Col2A1GFPcyan in Articular Cartilage

Figure 3 is obtained from a coronal section through the knee joint showing the thin rim of articular chondrocytes on the tibial plateau and femoral condyles. Because the section is non-decalcified, the mineral of bone and cartilage can be image by dark field optics and site of active mineralization (tide mark) can be seen as a red line that is formed by the alizarin complexone injection given one day prior to sacrifice. Dkk3eGFP is strongly expressed in the ligaments and the meniscus of the knee [24, 25], both of which are removed by dissection prior to initiating the primary articular cartilage. 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. It was from the two GFP transgenic lines that the articular cartilage cultures are obtained.

Separation of the Cyan and eGFP Signal in Articular Chondrocyte Culture

Separating the Cyan from eGFP fluorescence using standard epifluoresence microscopy commonly used in tissue culture can be problematic because Cyan fluorescence can cross-talk into the eGFP channel. The eGFP could also cross-talking into the Cyan if the emission filter bandwidth is not properly

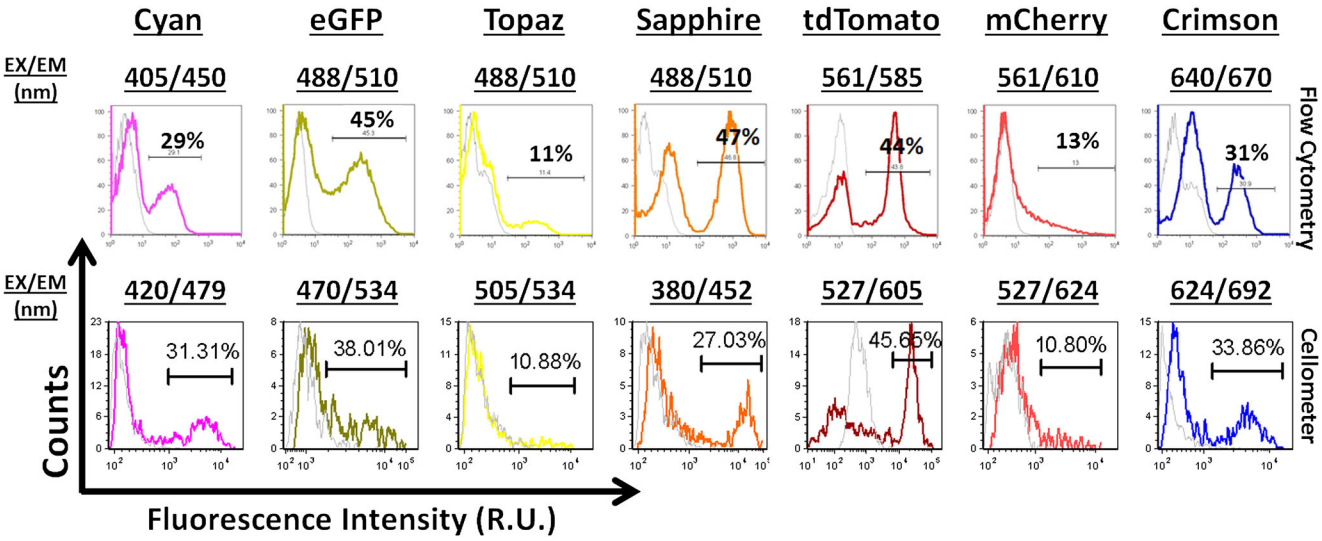

Fig. 2 Comparison of image and flow cytometry fluorescent protein positive cell population analysis. Cyan, eGFP, Topaz, Sapphire, tdTomato, mCherry, and Crimson expressing cells were detected in both image and flow cytometry, which showed comparable results. The linear marker ranges were selected from the right edge of the control in *gray*. Note that the Sapphire excitation and emission filter were different between both systems, which may have resulted in the different population percentages

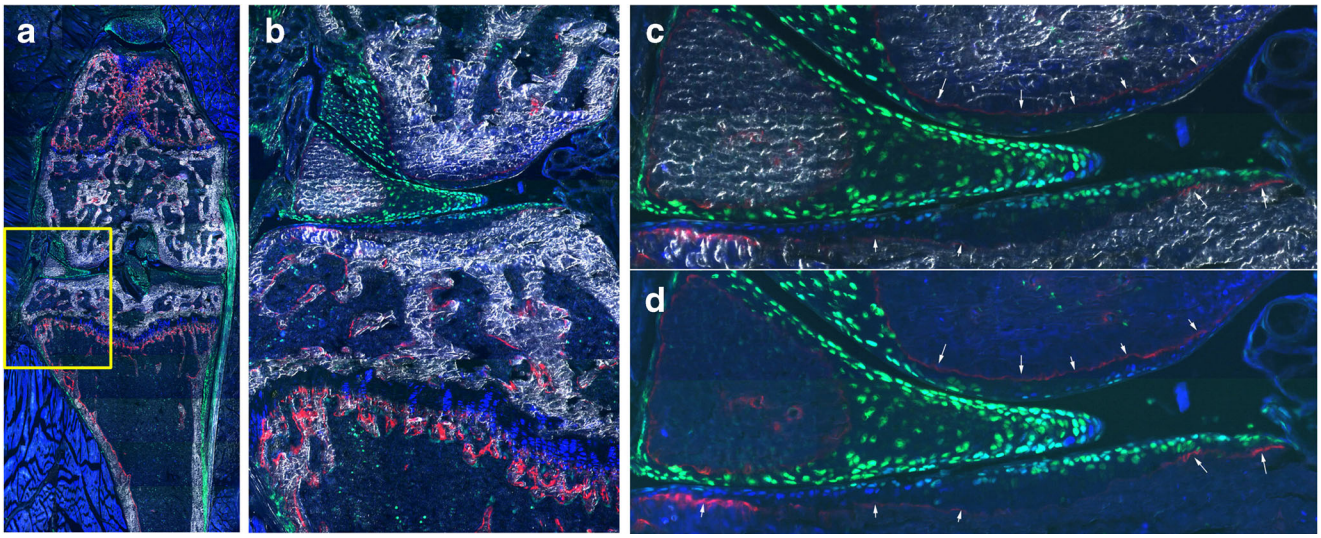

Fig. 3 Epifluorescent image of a non-decalcified section taken as a coronal section through the knee of a Col2A1-GFPcayn/Dkk3-eGFP reporter mouse. **a** Full view showed the strong Dkk3-eGFP signal in adjacent ligaments and the meniscus. The *yellow* square was shown in the enlarge section in (**b**). The silver color was 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. None of these GFP expressing contaminants should have seeded the primary cultures. Panel (**c**) and (**d**) showed 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

selected. Therefore, when examining in the eGFP channel, it was difficult to know if a Cyan positive cell was Cyan only, eGFP only, or double positive. It was only by a cell by cell assessment in both the blue and green channel that made it possible to make this distinction. When the eGFP signal was very strong, it could be imaged with a Topaz optimized filter, which eliminated the Cyan signal, but this step underestimated the number of eGFP cells. These imaging problems are eliminated with the narrow laser excitation bands used in FACS analysis and the spectrum conditions developed with the excitation LEDs using the test reporter cells (see above) suggested that a similar separation was possible using image cytometry.

To test this possibility, 12-well tissue culture plates from primary chondrocyte cultures are seeded with cells from 4 separate reporter lines, and the cells are harvested at 2 day intervals. Figure 4a illustrated the ability of the CFP and YFP filter set to visually distinguish the blue and green colors of the Dkk3eGFP and Col2A1GFPcyan reporters with the epifluroescent microscope, while Fig. 4b showed the onset and progression cell fluorescence as the culture wells become more confluent. Although deep blue, light blue and green cells can be appreciated, the complexity of the cell pattern was beyond quantification. Figure 5 demonstrated the ability of the image cytometry optics to resolve the three color possibilities in an aliquot of a cell suspension taken from a culture well. Figure 6 showed the representative images at the different time points as recorded by the image cytometer for eGFP Negative (CD1), Dkk3eGFP, Col2A1GFPcyan and Dkk3eGFP/ Col2A1GFPcyan double transgenic lines. From Fig. 6, it was clear that for single fluorescent proteins of eGFP and Cyan, the optical cross-talk was eliminated through the selection of optical filters, thus the images showed only the respective fluorescent signals for each protein. In addition, the captured fluorescent images are analyzed in FCS Express to properly gate and measure the fluorescent positive cell population percentages (Fig. 7).

The data from the articular chondrocyte culture experiment grown under basal conditions is summarized in Fig. 8, panels a–d. As expected, no background fluorescence was detected throughout the culture period (Fig. 8a). From the histology, it was not surprising that the Col2A1GFPcyan cell was the more dominant population at the early time points (Days 2–4), and the percentage decreased to a steady level of 15–20 % of the cell at the later time points. In contrast, the Dkk3eGFP culture showed a gradual increase from 2 to 29 % of cell in the culture by day 14, after which the percentage began to decrease (Fig. 8b). In the double reporter culture, 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 % (Fig. 8d). There may be a discrepancy in the Col2A1GFPcyan cells between the single and double reporter combinations. In the single reporter culture, Col2A1GFPcyan remained constant at about 15 % from day 2 forward (Fig. 8c), while in the double reporter, a high

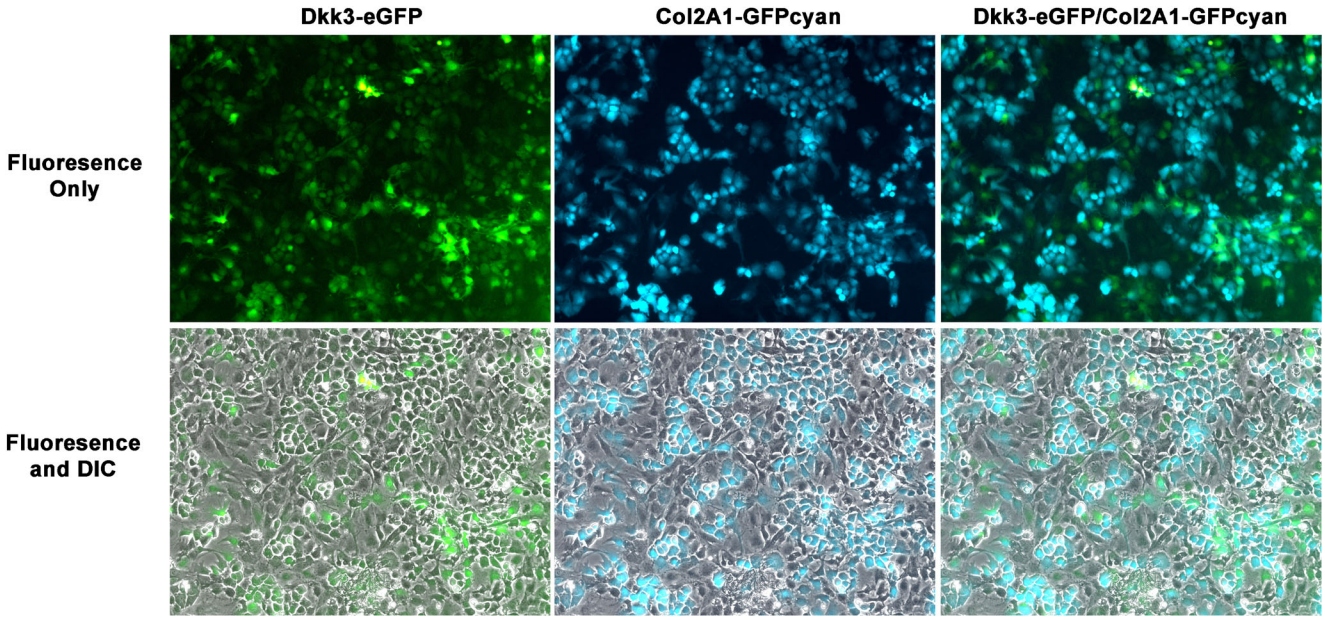

Fig. 4 Discrimination of Dkk3-eGFP and Col2A1-GFPcyan in primary culture of articular chondrocytes. The Dkk3-eGFP was imaged with a YFP filter (emission 535/30) which excluded the majority of the GFPcyan channel (middle panel). The composite of the two colors as well as the underlying morphology of the cells were revealed by the DIC image demonstrates the visual complexity of the culture. Resolving the individual components of the culture requires a cell by cell analysis by either FACS or image cytometry

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 panel f. The color from this excessive population is gradually lost either due to apoptosis or progression to the hypertrophic level of differentiation.

At day 8 of culture, the medium is supplemented with dexamethasone, ascorbic acid and ß-glycerol phosphate to promote chondrogenic progression to terminal differentiation (Fig. 8e). This change resulted in an increase of the double positive Dkk3eGFP/Col2A1GFPcyan cells from 15 to 30 % with 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 [26].

The magnitude of the expansion of cells at each level of differentiation under the differentiation conditions is illustrated in Fig. 8f. The Dkk3eGFP/Col2A1GFPcyan population increases almost 6 fold (3.5 population doublings) while the single color and non-GFP population increase about 3 fold in number. After day 16 of culture, the number begins to fall suggesting the culture is losing viability. The software also computed cell volume (Supplementary Figure 1) and did not identify a significant difference between cells expressing different colors. The cell volume started out at approximately 6–8 μm for the first 2 days of the culture, and then the diameter increases by 2.5 fold to approximately 14–18 μm, which stabilized from day 6–15. Finally, the cell diameter decreased to approximately 12 μm after 15 days.

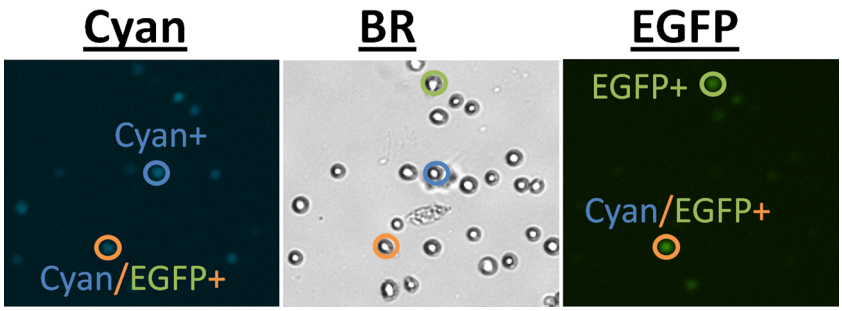

Fig. 5 Fluorescent images of single and double colored articular chondrocyte cells using the Cyan and eGFP FOMs. Cyan only and eGFP only were observed only in their respective channels, but the double colored cells are seen in both channels

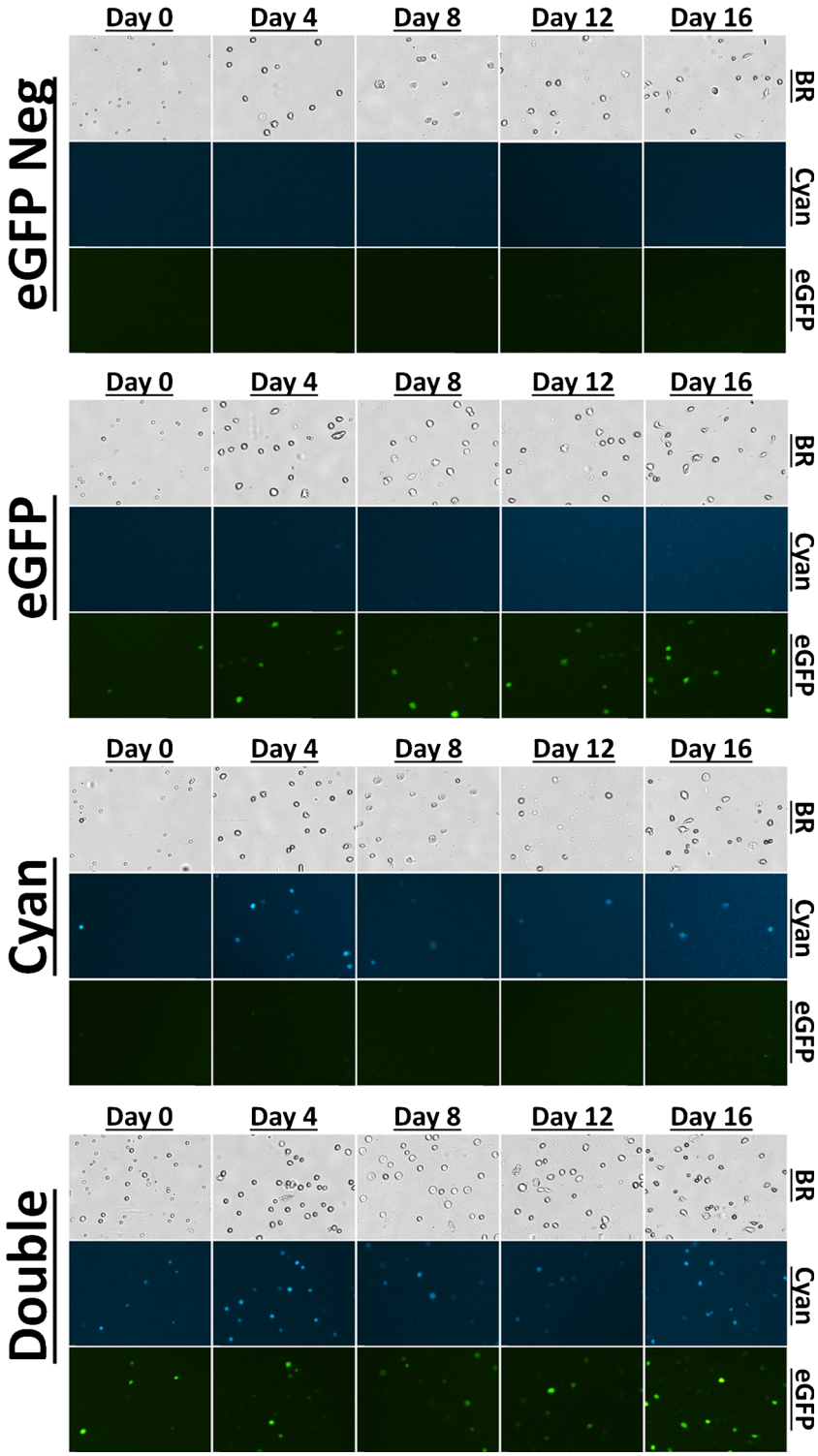

Fig. 6 Bright-field and fluorescent images captured in the image cytometry system. The CD1 sample showed no fluorescence, while the double sample showed strong fluorescence in both Cyan and eGFP channels. Both eGFP and Cyan only samples showed fluorescence in its respective fluorescence channel

Discussion

The wide spectrum of GFP colors now available provides the opportunity to multiplex reporters that reflect a progression of differentiation in primary cultures derived from a tissue of interest. The best tissue sources for establishing the primary culture are young mice in which the amount of tissue is limited. Therefore, the ability to accurately document the number of cells expressing one or more colors over time in a multi-well culture plate had many technical advantages over the other alternative of FACS or high content image analysis. The lower limit of cell number for an accurate FACS is

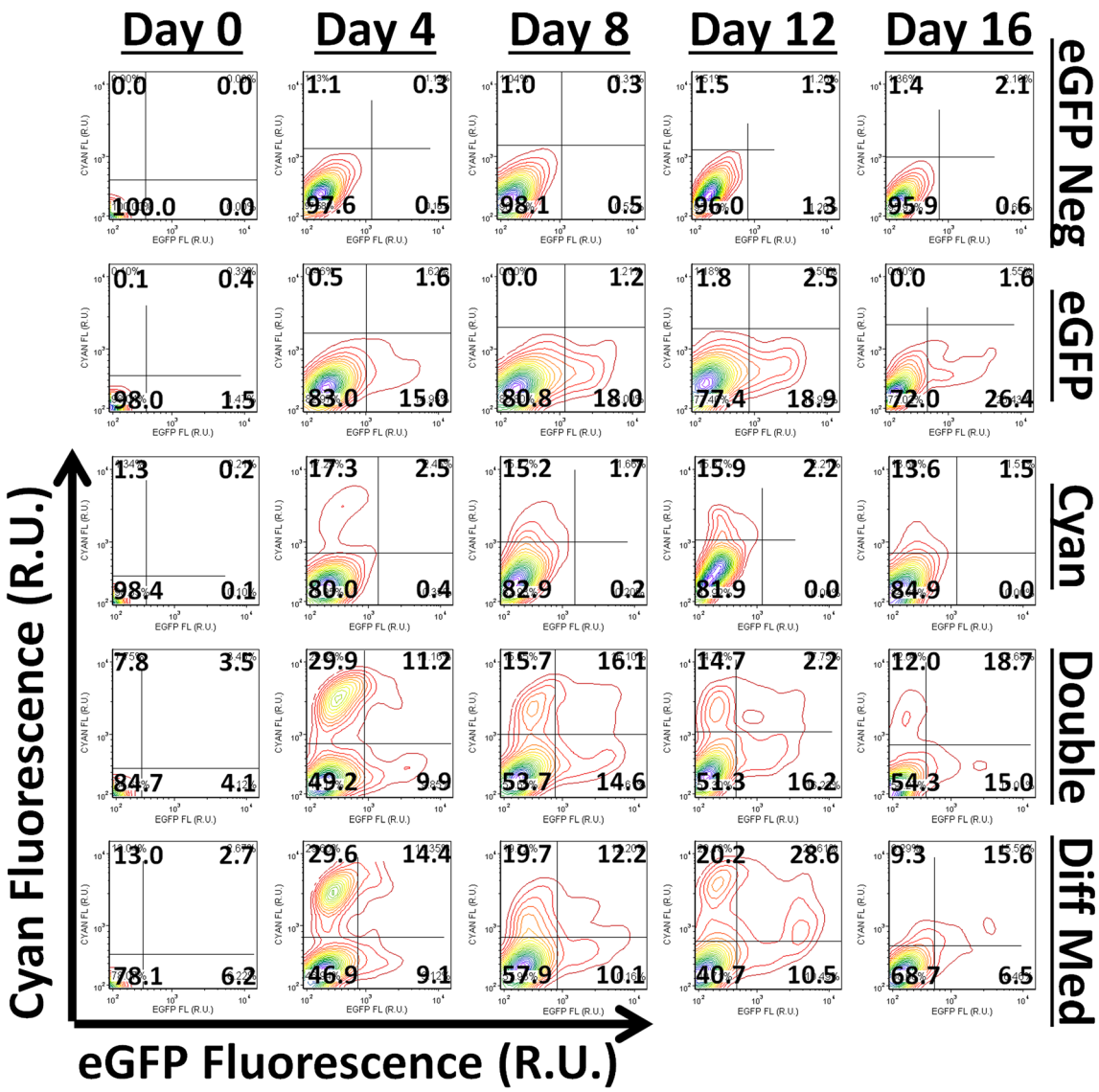

Fig. 7 Fluorescence histograms of Cyan fluorescence in respect to eGFP generated in FCS Express 4. On day 0, no fluorescent populations were measured as expected. As the proliferative period progressed, CD1 sample remained non-fluorescent, eGFP and Cyan only sample increased in its respective populations, and double sample showed high increase in eGFP, Cyan, and double fluorescent cell populations. The sample induced in differential medium showed similar increase as the double sample, with higher increase in the double fluorescent cell population

approximately 10^4 cells, while gaining access to the instrument and running multiple samples at a specific time window can be difficult with a FACS core facility. The fluorescence-detection 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. On the other hand, high content image analysis will not be able to discriminate cultures that require high cell density, especially cell lineages that require a three dimensional architecture for full differentiation. Therefore, for screening and characterizing a wide variety cell culture and differentiation conditions, using a multi-well format and a rapid cell counting instrument capable of detecting individual colors of GFP enables an experimental approach that otherwise would be difficult to obtain.

The majority of GFP reporters have used eGFP in part because its spectrum is readily detected with fluorescein isothiocyanate (FITC) filter sets, which is commonly available in most laboratories. However, eGFP has a relatively wide emission band that can prevent its use with reporters in the blue to orange spectrum. This problem also exists with the RFPcrimson reporter, which is particularly valuable for in vivo imaging, but limiting its use in an experiment that requires reporter multiplexing. Separation of eGFP from overlapping colors using epifluoresence requires filters with narrow emission windows, which diminishes the eGFP signal intensity and becomes self-defeating. In this work, the original

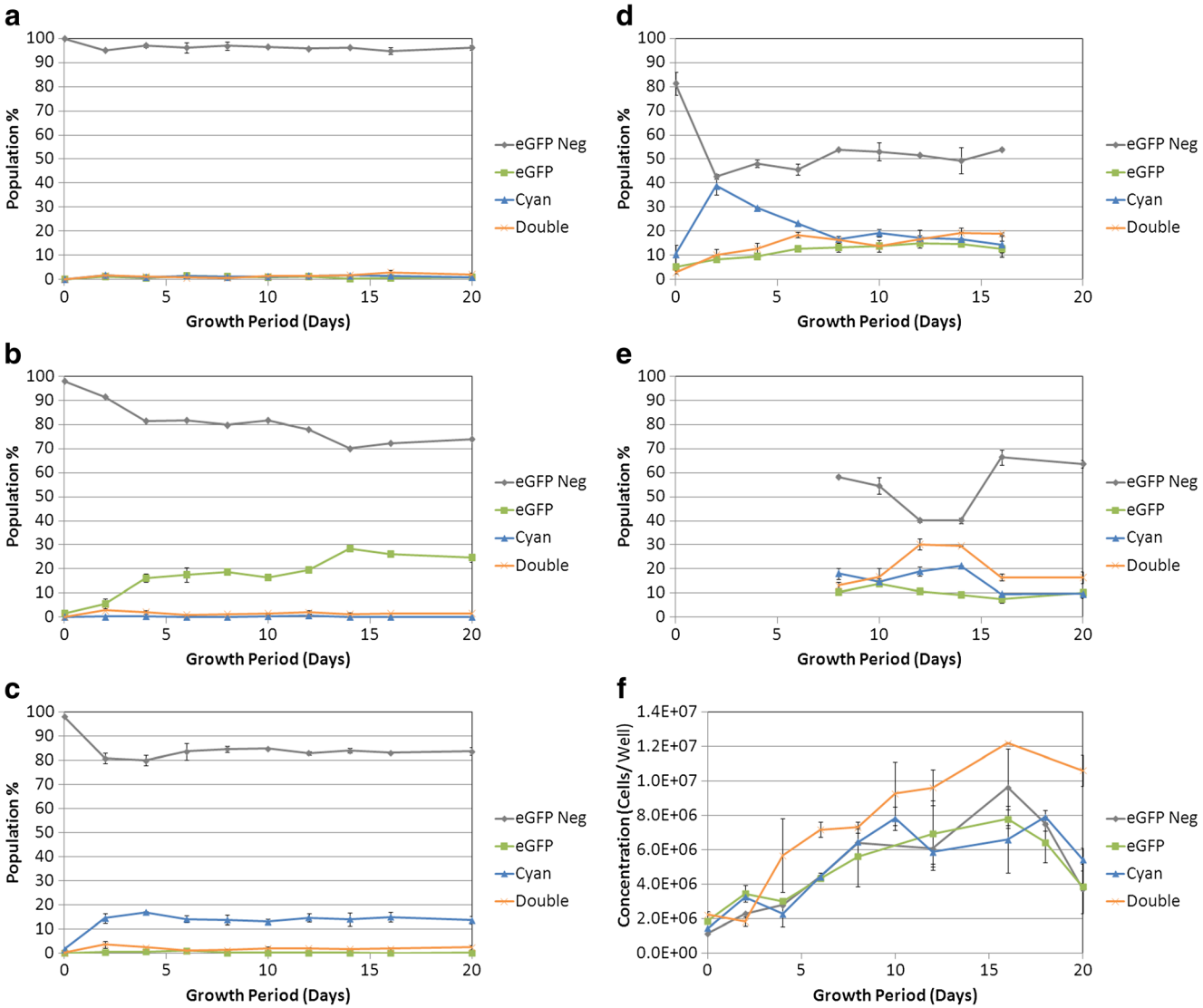

Fig. 8 Data generated in the FCS Express software from the articular chondrocyte culture experiment. Panels **a**–**d** were cultures grown under control conditions with **a** eGFP Negative (CD1), **b** Dkk3eGFP only, **c** Col2A1GFPcyan only, and **d** Dkk3eGFP/Col2A1GFPcyan double transgenic mice. Panel **e** was the double transgenic line that was switched to differentiation medium at day 6 of culture. Panel **f** illustrates 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. Note the reversal in cell number from day 0–2 between the Dkk3eGFP/Col2A1GFPcyan double and the Col2A1GFPcyan only population as one explanation for the high percentage of Col2A1GFPcyan only cells seen in panel **d**. In addition, Two-sample T-Test was performed to show statistical significant separation between Dkk3eGFP and Col2A1GFPcyan for panels **b**, **c**, **d**, **e**, and **f**. For panel **b**, eGFP showed significant increase in cell population in comparison to Cyan (p-value <0.05). For panel **c**, Cyan showed significant increase in cell population in comparison to eGFP (p-value <0.05). For panel **c** and **d**, Double cell type showed similar increase in cell population in comparison to eGFP and Cyan (p-value >0.05). For panel **f**, the Double cell type concentration showed a significant increase in comparison to eGFP and Cyan (p-value <0.05)

eGFP filters were replaced with YFP filters to minimize or potentially eliminate optical cross-talk of Cyan fluorescence into eGFP channel. The eGFP fluorescence may also potentially cross-talk into Cyan filters, however, we have narrowed the bandwidth of the Cyan filters, thus eliminating the unwanted fluorescence signals. In addition, laser-excited images as used in FACS instruments can overcome this problem, and the excitation diodes utilized in the image cytometer were formulated to approximate the specificity of a FACS instrument.

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 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, can be significantly augmented by applying flow cytometric principles to cell proliferation and differentiation, which are the hallmarks for describing lineage regulation. Establishing stable and reproducible culture models are an essential first step before growth factors, scaffolds, 3D hydrogels or other manipulation are explored as potential agents to promote lineage expansion and differentiation, or to study the impact of a mutation that compromises lineage health. The modifications to image cytometry system described here can provide an efficient and simple method for the cytometry community and can conform within the daily workflow of a cell culture based laboratory.

Conflict of Interest LLC declares competing financial interests, and the work performed in this manuscript is for novel assay development using instrument provided by Nexcelom Bioscience LLC. The performance of the system has been compared to standard method currently used in the biomedical research institutions.

References

1. Tsien RY (2010) The 2009 Lindau Nobel Laureate Meeting: Roger Y. Tsien, Chemistry 2008. J Vis Exp 35:e1575
2. Chen R, Greene EL, Collinsworth G, Grewal JS, Houghton O, Zeng H, Garnovskaya M, Paul RV, Raymond JR (1999) Enrichment of transiently transfected mesangial cells by cell sorting after cotransfection with GFP. Am J Physiol 276(5 Pt 2):F777–F785
3. Kalajzic I, Kalajzic Z, Kaliterna M, Gronowicz G, Clark SH, Lichtler AC, Rowe D (2002) Use of type I collagen green fluorescent protein transgenes to identify subpopulations of cells at different stages of the osteoblast lineage. J Bone Miner Res 17(1):15–25
4. Kalajzic I, Staal A, Yang WP, Wu Y, Johnson SE, Feyen JH, Krueger W, Maye P, Yu F, Zhao Y et al (2005) Expression profile of osteoblast lineage at defined stages of differentiation. J Biol Chem 280(26): 24618–24626
5. Jiang X, Kalajzic Z, Maye P, Braut A, Bellizzi J, Mina M, Rowe DW (2005) Histological analysis of GFP expression in murine bone. J Histochem Cytochem 53(5):593–602
6. Bilic-Curcic I, Kronenberg M, Jiang X, Bellizzi J, Mina M, Marijanovic I, Gardiner EM, Rowe DW (2005) Visualizing levels of osteoblast differentiation by a two-color promoter-GFP strategy: type I collagen-GFPcyan and osteocalcin-GFPtpz. Genesis 43(2):87–98
7. Louis KS, Siegel AC (2011) Cell viability analysis using trypan blue: manual and automated methods. Methods Mol Biol 740:7–12
8. Ongena K, Das C, Smith JL, Gil S, Johnston G (2010) Determining cell number during cell culture using the Scepter cell counter. J Vis Exp 45:2204
9. Chan LL, Lai N, Wang E, Smith T, Yang X, Lin B (2011) A rapid detection method for apoptosis and necrosis measurement using the Cellometer imaging cytometry. Apoptosis Int J Program Cell Death 16(12):1295–1303
10. Chan LL, Zhong X, Qiu J, Li PY, Lin B (2011) Cellometer vision as an alternative to flow cytometry for cell cycle analysis, mitochondrial potential, and immunophenotyping. Cytom A J Int Soc Anal Cytol 79(7):507–517
11. Robey RW, Lin B, Qiu J, Chan LL, Bates SE (2011) Rapid detection of ABC transporter interaction: potential utility in pharmacology. J Pharmacol Toxicol Methods 63(3):217–222
12. Chan LL, Zhong X, Pirani A, Lin B (2012) A novel method for kinetic measurements of rare cell proliferation using Cellometer image-based cytometry. J Immunol Methods 377(1–2):8–14
13. Madisen L, Zwingman TA, Sunkin SM, Oh SW, Zariwala HA, Gu H, Ng LL, Palmiter RD, Hawrylycz MJ, Jones AR et al (2010) A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. Nat Neurosci 13(1):133–140
14. Strack RL, Hein B, Bhattacharyya D, Hell SW, Keenan RJ, Glick BS (2009) A rapidly maturing far-red derivative of DsRed-Express2 for whole-cell labeling. Biochemistry 48(35):8279–8281
15. Chokalingam K, Hunter S, Gooch C, Frede C, Florer J, Wenstrup R, Butler D (2009) Three-dimensional in vitro effects of compression and time in culture on aggregate modulus and on gene expression and protein content of collagen type II in murine chondrocytes. Tissue Eng A 15(10):2807–2816
16. Chokalingam K, Juncosa-Melvin N, Hunter SA, Gooch C, Frede C, Florert J, Bradica G, Wenstrup R, Butler DL (2009) Tensile stimulation of murine stem cell-collagen sponge constructs increases collagen type I gene expression and linear stiffness. Tissue Eng A 15(9): 2561–2570
17. Ushiku C, Adams DJ, Jiang X, Wang L, Rowe DW (2010) Long bone fracture repair in mice harboring GFP reporters for cells within the osteoblastic lineage. J Orthop Res 28(10):1338–1347
18. Egli RJ, Bastian JD, Ganz R, Hofstetter W, Leunig M (2008) Hypoxic expansion promotes the chondrogenic potential of articular chondrocytes. J Orthop Res Off Publ Orthop Res Soc 26(7):977–985
19. Meyer EG, Buckley CT, Thorpe SD, Kelly DJ (2010) Low oxygen tension is a more potent promoter of chondrogenic differentiation than dynamic compression. J Biomech 43(13):2516–2523
20. Schrobback K, Klein TJ, Crawford R, Upton Z, Malda J, Leavesley DI (2012) Effects of oxygen and culture system on in vitro propagation and redifferentiation of osteoarthritic human articular chondrocytes. Cell Tissue Res 347(3):649–663
21. Markway BD, Tan GK, Brooke G, Hudson JE, Cooper-White JJ, Doran MR (2010) Enhanced chondrogenic differentiation of human bone marrow-derived mesenchymal stem cells in low oxygen environment micropellet cultures. Cell Transplant 19(1):29–42
22. Foldager CB, Nielsen AB, Munir S, Ulrich-Vinther M, Soballe K, Bunger C, Lind M (2011) Combined 3D and hypoxic culture improves cartilage-specific gene expression in human chondrocytes. Acta Orthop 82(2):234–240

23. Hawley TS, Herbert DJ, Eaker SS, Hawley RG (2004) Multiparameter flow cytometry of fluorescent protein reporters. Methods Mol Biol 263:219–238
24. Mori Y, Jiang X, Adams DJ, Rowe DW (2011) A Dkk3 reporter identifies progenitors of the fibrocartilage and periosteum of the fracture callus
25. Utreja A, Yadav S, Jiang X, Nanda R, Rowe DW (2012) Response of GFP reporters expressed in the TMJ condylar cartilage to mechanical loading. Minneapolis, MN
26. Yadav S, Utreja A, Jiang X, Huang J, Maye P, Kalajzic I, Rowe DW (2012) GFP reporters for assessing lineage progression in the condylar cartilage. Pittsburgh, PA