Introduction

Spectral Separation is a technique that allows the user to separate images containing data from more than one fluorochrome into channels that contain single unmixed fluorochromes. In order to achieve this separation or un-mixing, sample images must be acquired at a minimum of two different wavelengths. Some technologies lend themselves naturally to this multiple wavelength sampling, such as colour cameras, which simultaneously sample at three different wavelengths (Red, Green and Blue). However, multiple wavelengths can just as effectively be acquired using monochrome cameras and wavelength changing devices (for sequential sampling).

The purpose of this study was to demonstrate the process of spectral separation in Volocity 4.0. Image sequences of beads stained with a mixture of two green dyes with very similar excitation and emission spectra were acquired using a monochrome camera. The data for each of the green dyes was then separated into individual, unmixed channels.

The samples used were Molecular Probes FocalCheck™ Double Green microsphere standards (catalogue number F-36905). These microspheres are composed of a ring stain of one green dye (Green 1), and are stained throughout with a second similar green dye (Green 2). The microspheres are supplied with a batch of spheres entirely labelled with the first Green 1, and a further batch entirely labelled with Green 2. The manufacturer states that "The dyes are spectrally similar, but sufficiently different to be resolved by the technique of linear unmixing in instruments such as the Zeiss LSM META fluorescent microscope"[1]. The excitation and emission wavelength maxima for the dyes are shown below.

Dye	Excitation Maximum (nm)	Emission Maximum (nm)
Green 1	500	512
Green 2	512	525

We attempted to separate the two dyes by acquiring images using a single excitation wavelength and just two emission wavelengths.

Method

All images were acquired using a Zeiss Axiovert 200M microscope with a 63x plan apochromat 1.4 NA lens. The microscope was fitted with a CSU22 Yokogawa spinning disk and an Orca ERG camera. Excitation light was supplied by a Spectral LMM5 laser unit and emission light was filtered using a Ludl filter wheel fitted with Chroma filters ET525/50M (maximal transmission 525 nm) and ET535/30M (maximal transmission 535 nm). A single excitation wavelength of 491 nm was used

throughout the study, with emission wavelengths of either 525 nm or 535 nm. All images were acquired using Volocity Acquisition installed on an Apple G5 computer.

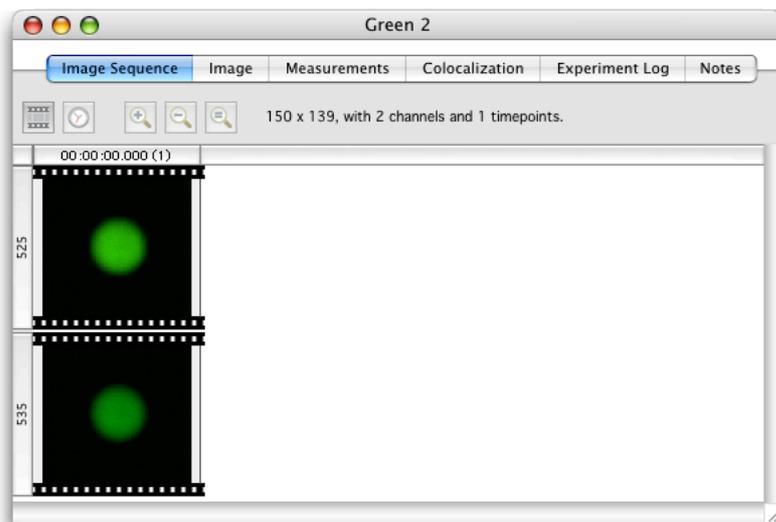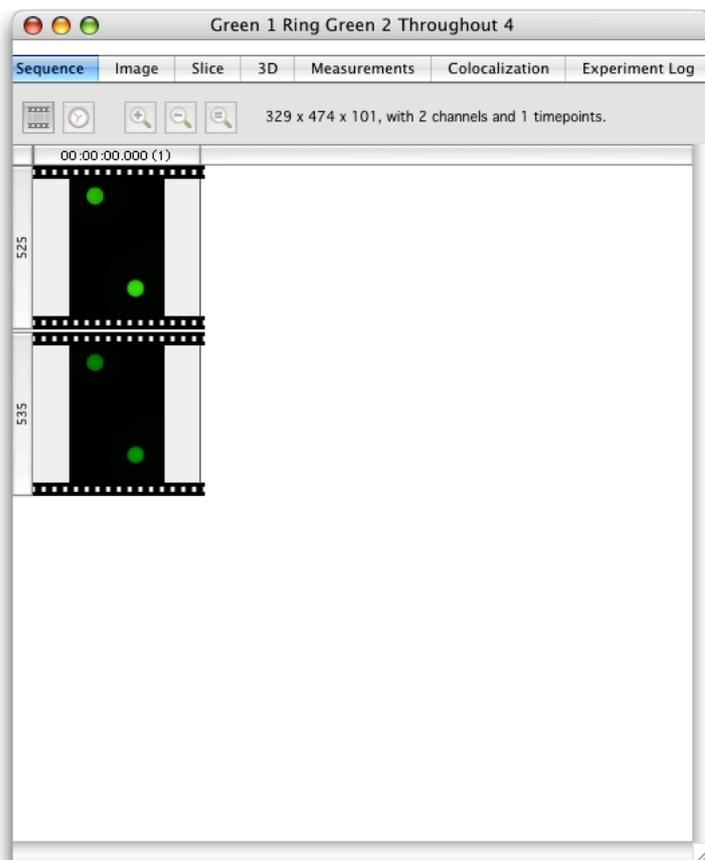**Creating a spectral signature for Green 1**

A 2D lambda stack of a Green 1 bead was acquired (i.e. a bead labeled only with Green 1). The stack consisted of two channels. The excitation wavelength for both images was 491 nm. One image was acquired using a filter with an emission maximum of 525 nm, and the second image was acquired using a filter with an emission maximum of 535 nm. The same exposure was used for both images.
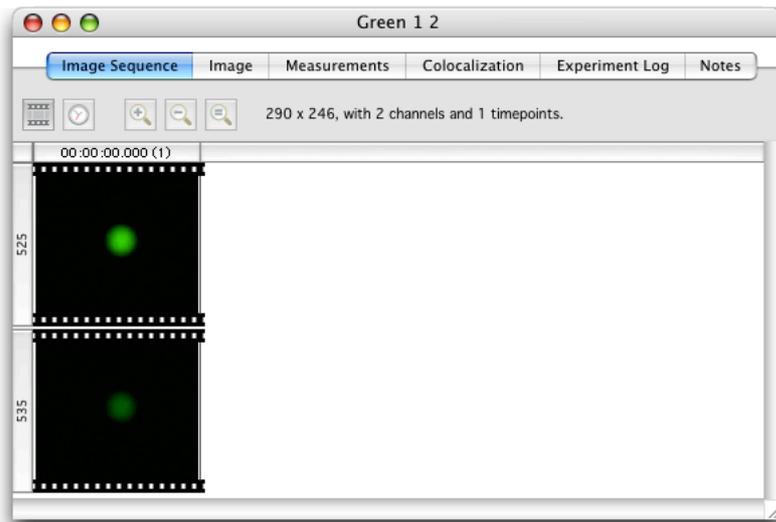
A bright area within the bead was selected in the Image view of the Image Sequence in Volocity and a spectral signature was created for Green 1.

Creating a spectral signature for Green 2

A 2D lambda stack of a Green 2 bead was acquired (i.e. a bead labeled only with Green 2). The stack consisted of two channels. The excitation wavelength for both images was 491 nm. One image was acquired using a filter with an emission maximum of 525 nm, and the second image was acquired using a filter with an emission maximum of 535 nm. The same exposure was used for both images.

A bright area within the bead was selected in the Image view of the Image Sequence and a spectral signature was created for Green 2.

Acquiring and unmixing a lamda stack for Double Green beads

Double Green beads are labeled with Green 1 as a ring stain and Green 2 throughout. A 3D two channel lambda stack of Double Green beads was acquired. The stack consisted of two channels, and the excitation and emission wavelengths of the channels were the same as those used to acquire the lambda stacks from which the Green 1 and Green 2 spectral signatures were derived. The same exposure was used for both channels. Each channel was 10 μm thick, with a step size for each plane of 0.1 μm.
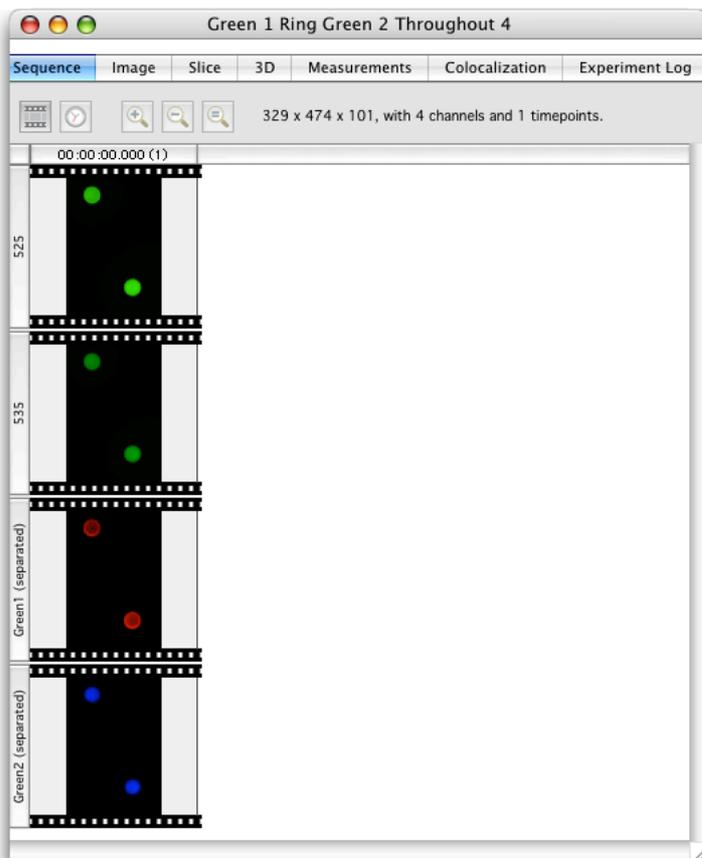

The spectral signatures for Green 1 and Green 2 were used to separate images of the two dyes into individual channels.
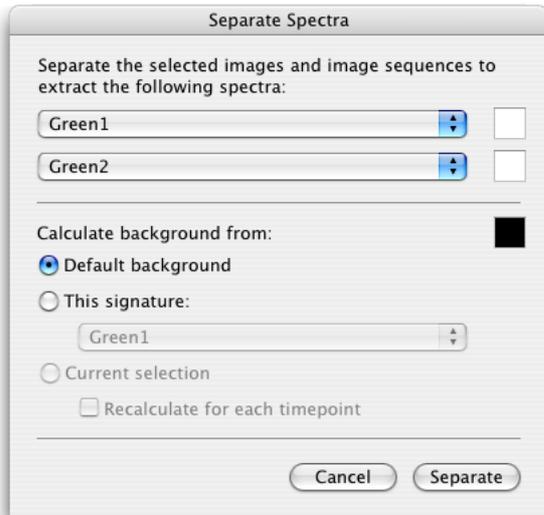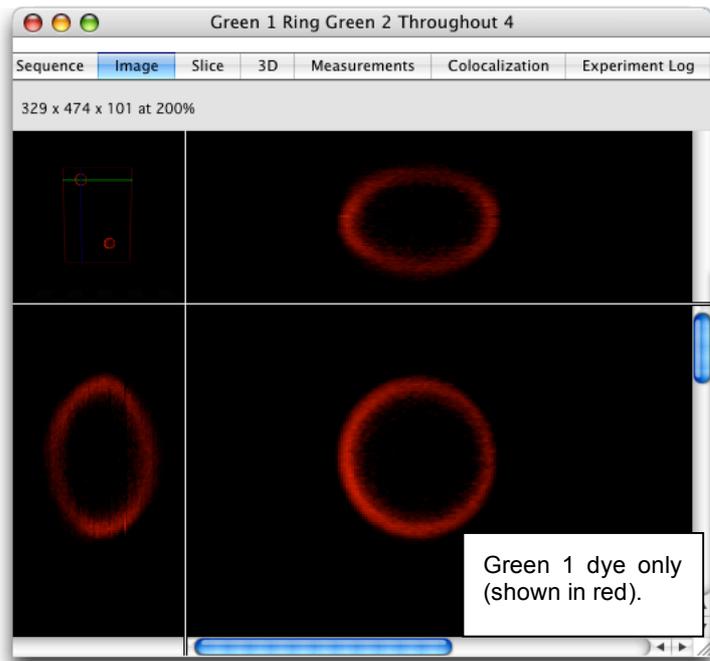
Results

The Green 1 and Green 2 dyes were correctly separated into single dye channels. The Green 1 channel was colored red and the Green 2 channel was colored blue for clarity.
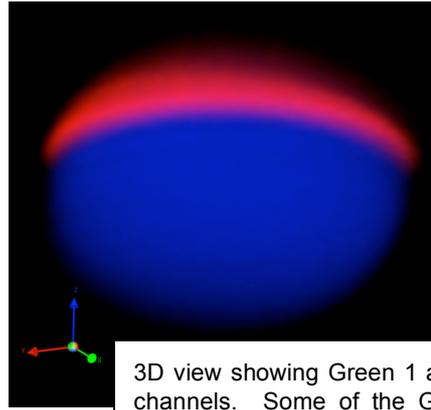

Inspection of the separated channels revealed that Green 1 was correctly located only in the ring stain, and Green 2 was located throughout the bead.

Green 1 dye only (shown in red).

Green 1 (shown in red) and Green 2 (shown in blue).
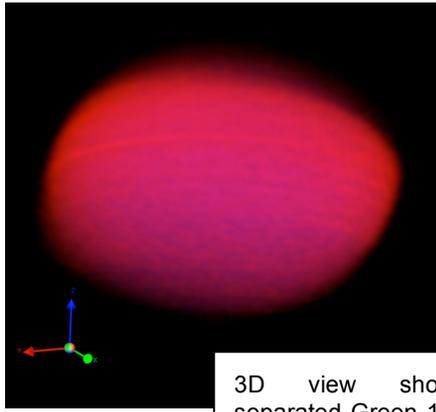
3D view showing separated Green 1 and Green 2 channels

3D view showing Green 1 and Green 2 channels. Some of the Green 1 ring stain (shown in red) was removed from this volume to reveal the Green 2 stain beneath

Conclusion

Double Green FocalCheck™ microspheres are designed to test spectral separation[1]. They are composed of two spectrally similar green dyes. This study showed that Volocity 4 can successfully separate images containing contributions from both green dyes into channels that each contain data from only one of the dyes using the spectral separation feature.

Discussion

Bibliography

1. FocalCheck™ Fluoresccent Microsphere Standards. MP 07234. Molecular Probes Product Information.