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CONFOCAL APPLICATION LETTER

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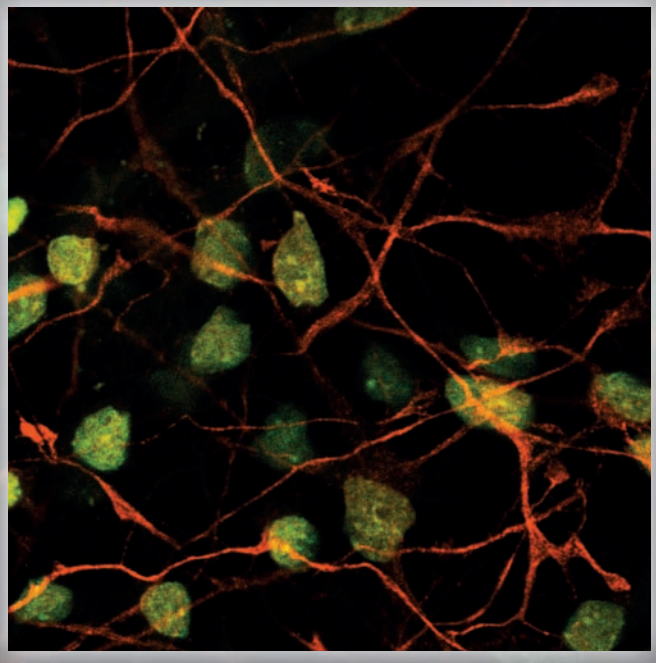
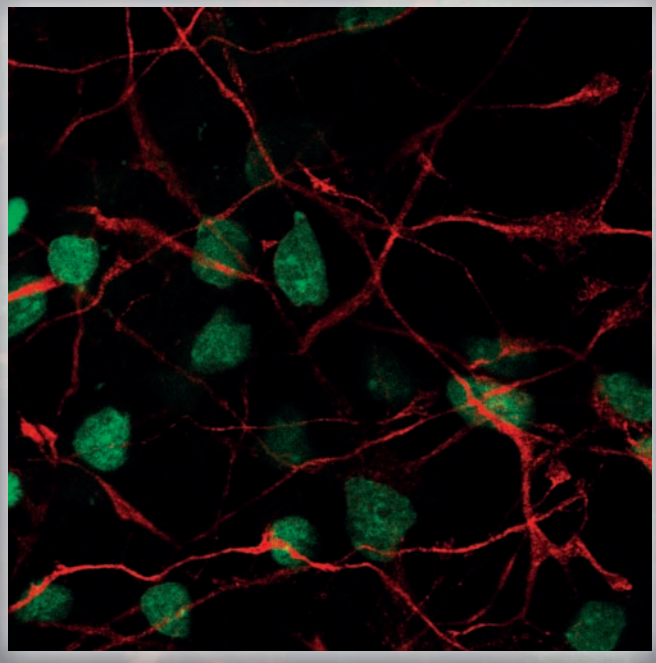
reSOLUTION

Sequential Image Recording with the Leica TCS SP8





This application letter describes how to improve image quality, e.g. avoid crosstalk, using sequential image recording. Guided work steps show you how to create and optimize instrument parameter settings (IPSs) and combine them for sequential image recording. The sequential image recording option allows the combination of any number of IPSs for any number of fluorescent dyes. This set is applicable for automatic recording of multi-labeled specimens within one scanning series.

Advantages at a Glance

Avoiding crosstalk

Frequently, fluorochromes with different excitation wavelengths have emission spectra that show a wide overlap, e.g. FITC and TRITC (Fig. 1). When two or more of such fluorochromes are simultaneously excited in a specimen, it is inevitable that several fluorescence signals are recorded in one detection channel and can no longer be separated in the image. This *crosstalk* or bleed-through can be avoided by using the sequential image recording method.

Scanning multi-stained specimens with a limited number of detection channels

Specimens labeled with multiple fluorochromes can be recorded within one scanning series, even if the microscope system is equipped with fewer detection channels than required. For example, it is possible to image a triple stained specimen using a microscope system with only two detection channels.

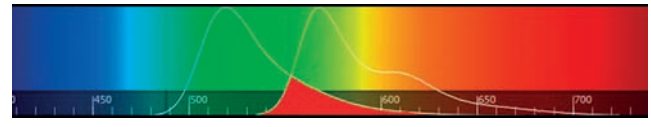


Figure 1: Emission spectra of FITC and TRITC. The emission spectra of FITC and TRITC have a wide overlap (red area).

Optional Sequential Scanning Modes

Depending on your system configuration, you can choose from three different modes of sequential image recording:

- **between lines**
- **between frames**
- **between stacks**

Between lines (Fig. 2)

In sequential mode, the detection channel and the excitation wavelength are changed with every single line scan until the full image is recorded. This mode only applies for microscope systems that are equipped with an acousto-optical tunable filter (AOTF). The **between lines** mode is particularly useful for minimizing movement artifacts that can occur during *in vivo* investigations.

Figure 2: Scanning pattern of the **between lines** mode.
Rectangles: optical sections. Black arrow: recording mode.

Cover: Parasagittal section of mouse cerebellum

Figure 3: Scanning pattern of the **between frames** mode.
Rectangles: optical sections. Black arrow: recording mode.

Between frames (Fig. 3)

With this sequential mode, a complete image is recorded with instrument parameter settings (IPSs) that are adapted to one fluorochrome before the system switches to a different IPS to record the same optical section. This pattern is repeated in the subsequent optical planes. **Between frames** provides sequential image recording even if lasers are used without AOTF, e.g. IR lasers for multiphoton excitation. In addition, triple stained specimens can be investigated using a two-channel microscope system, since the time between the recording of two images is sufficient to change certain time-sensitive hardware parameters, which is not the case in the **between lines** mode.

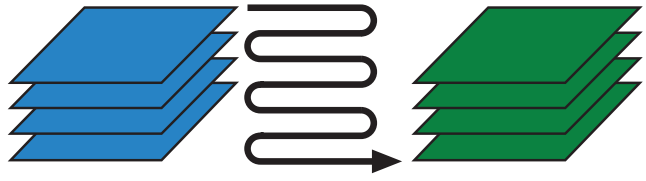


Figure 4: Scanning pattern of the **between stacks** mode.
Rectangles: optical sections. Black arrow: recording mode.

Between stacks (Fig. 4)

This sequential imaging mode records all optical sections that make up an image series before the system switches to the next sequential parameter setting to record another image series. This method is only suitable for imaging fixed, immobile specimens. Recording **between stacks** is less damaging to fluorescent dyes prone to bleaching. For example, if, in a double stained specimen, the excitation line of one fluorochrome simultaneously excites a second, more responsive fluorochrome, it is recommended to record the complete image series using the IPS that is adapted to the second, more responsive fluorescent dye.

How to Perform Sequential Image Recording Using the **Between Lines** Method, for Example with FITC/TRITC

Between lines is the fastest sequential scan recording mode. Here, the single lines of the sequential images are scanned in alternate order with the different AOTF, AOBS, Gain, and Offset settings of the respective sequences. This means that all sequential images are recorded simultaneously. This method is extremely beneficial for scanning moving specimens such as living cells. However, with overlapping detection ranges or filter changes it is not possible to use the **between lines** mode. Due to the extremely fast recording changes, the mechanical components, e.g. the detection bandwidth, the excitation beam splitter, the laser shutter and the diameter of the detection pinhole, have to be the same for all sequences.

1. Load the Leica IPS FITC/TRITC from the list of Leica Single Settings in order to load hardware settings preset by Leica (**see Fig. 5**):
- Laser, AOTF
- Excitation beam splitter or AOBS setting
- PMT (Photomultiplier/Detector)
- Detection bandwidth
- LUT (color look-up table) for color coding fluorescent dyes
- PMT Gain/Offset (signal amplification and threshold value of detectors)

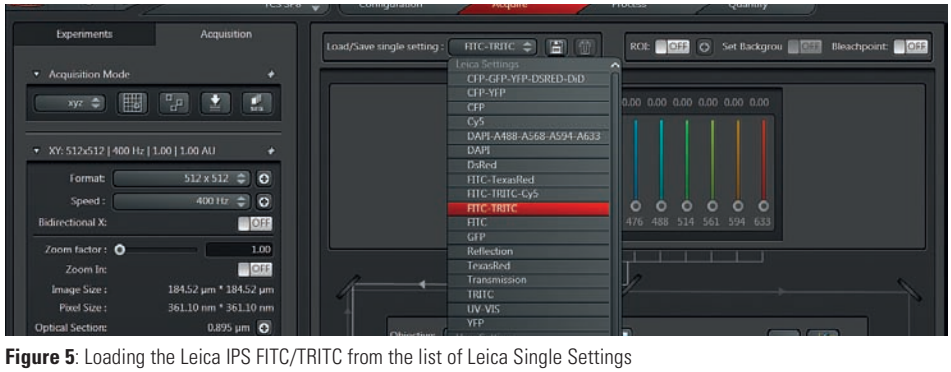


Figure 5: Loading the Leica IPS FITC/TRITC from the list of Leica Single Settings

2. Select the desired scan format, scan speed and zoom factor (**Fig. 6, 1**). In **Live** scanning mode (**Fig. 6, 2**), the detector gain and the offset values as well as the intensity of the excitation wavelengths (**Fig. 6, 3 and 4**) can be optimized.

3. Stop the **Live** scan.

4. Click the **SEQ** button in the **Acquisition Mode** (**Fig. 6, 5**) panel to open the **Sequential Scan** dialog. In the default setting, the first sequence is already active in the **Between Lines** mode (**Fig. 6, 6**).

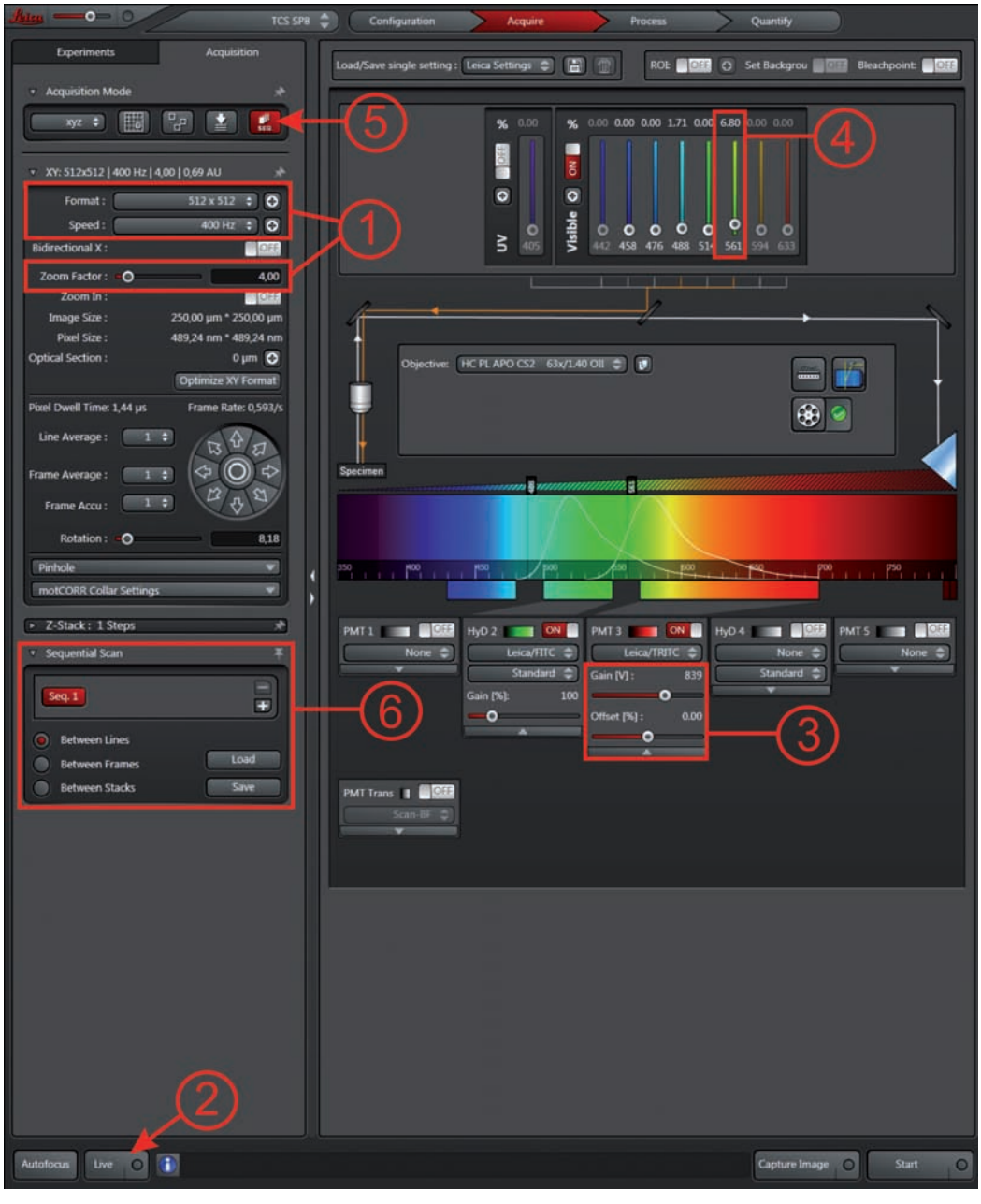


Figure 6: Defining the first sequential setting

5. Click + to open **Seq. 2** (**Fig. 7, 1**). This step copies **Seq. 1** to **Seq. 2**.

6. Activate **Seq. 1** again by clicking the corresponding button. The activated sequence is colored in red. Set the excitation line 561 to 0% and deactivate the detector for the TRITC detection.

7. Activate **Seq. 2** to keep the above (FITC) settings for **Seq.1** and to customize (TRITC) settings for **Seq. 2**.

8. Set the excitation line 488 to 0% and deactivate the detector for the FITC detection (**see Fig. 7, 2**).

Figure 7

9. Click **Start** to start the sequential scan.

10. With the buttons **Load** and **Save** in the **Sequential Scan** dialog, you can load a previously saved sequential scan setting or save the current setting

Note:
Changing hardware parameters such as the detection bandwidth, the excitation beam splitter, the laser shutter or the diameter of the detection pinhole between the single IPS implies that these parameters **cannot** be used in the **between lines** mode (see explanation above page 5)!

How to Perform a Sequential Image Recording Using the **Between Frames** and **Between Stacks** Methods, for Example with FITC/TRITC

Defining a sequential scan in the **between frames** or **between stacks** mode follows the same steps as defining sequential scans in the **between lines** mode (see instructions above). Instead of activating **Between Lines, Between Frames** or **Between Stacks** is chosen.

Alternatively, instead of using predefined Instrument Parameter Settings all Parameters for all sequential imaging methods can be manually adjusted.

Please note: The smaller the difference between the hardware settings of the single IPS, the faster the system can switch between the IPS and consequently, the faster the complete image is recorded. This is of importance when it comes to time-sensitive experiments.

1. Select the excitation wavelength for the first fluorochrome, activate the laser line and set its intensity.

2. Select an appropriate excitation beam splitter (preferably an excitation beam splitter that is suitable for all fluorochromes you use for the sequential recording). This step is redundant if the system is equipped with an AOBS.

3. Click one of the detector check boxes to select a detection channel and define the color look-up table as well as the detection bandwidth.

4. Select the desired scan format, scan speed and zoom factor (**Fig. 6, 1**). In **Live** scanning mode (**Fig. 6, 2**), the detector gain and the offset values as well as the intensity of the excitation wavelengths (**Fig. 6, 3 and 4**) can be optimized.

5. Open the **Sequential Scan** dialog by clicking the **SEQ** button in the **Acquisition Mode** panel. Click **+** to save the above settings for **Seq.1** and to open **Seq. 2** (**Fig.7, 1**).

6. In the following steps, the instrument parameters are set for the second sequential recording method and saved as instrument parameter setting. Select the next excitation wavelength, activate the excitation line and set its intensity. Remember to deactivate the excitation wavelength and detector that are not required.

7. Select an appropriate excitation beam splitter that preferably fits all fluorochromes used for the sequential recording. This step is redundant if the system is equipped with an AOBS.

8. Click one of the detector check boxes to select a detection channel and define the color look-up table plus the detection bandwidth.

9. In **Live** scanning mode (**Fig. 6, 2**), the detector gain and the offset values as well as the intensity of the excitation wavelengths (**Fig. 6, 3 and 4**) can be optimized.

10. In the **between stacks** mode, the dimensions of the spatial series have to be defined in the respective dialog, e.g. Time- or z-Series during live scan.

11. To save the settings for the two sequences, click on **Save** in the **Sequential Scan** dialog.

12. Once having started the between frames or between stacks sequential recording method, toggling between the different sequential settings, such as in the between lines mode, is not possible due to the reasons explained above (see page 5).

13. More information about the operation of the Leica Confocal Software is provided in the online tutorials. Click **Help** and **Tutorials** in the menu.

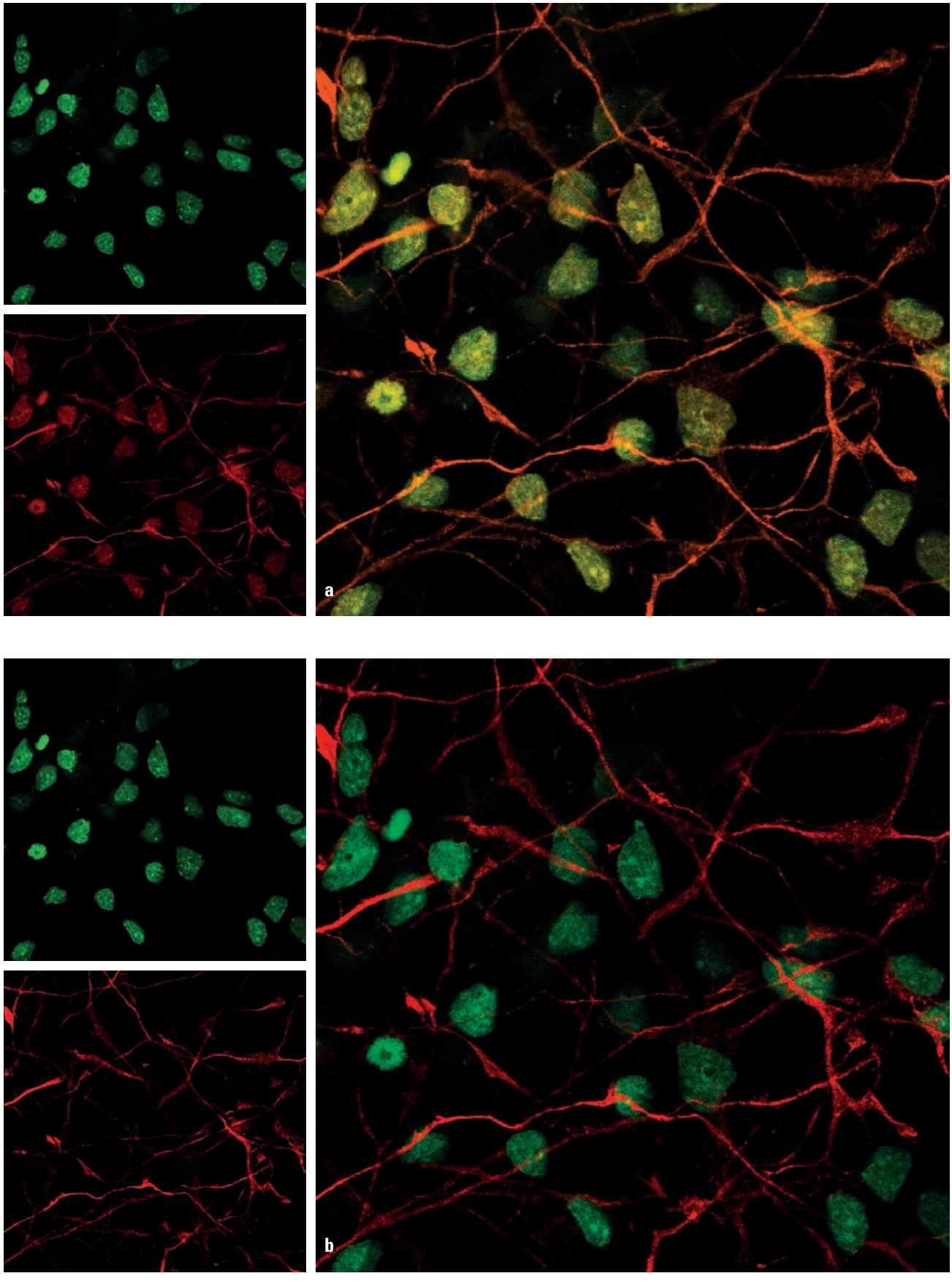


Figure 8a and b: Rat neuronal primary culture.
Cell nuclei, green. Nestin, red.
a) Mixture of red and green (crosstalk)
b) Separation of red and green (no crosstalk)

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