Chapter 6

Instrumentation and Detection

Presented is a description of how fluorescently labelled DNA molecules are detected by the optical system of the MegaBACE 1000.

The Instrument Optics: The Excitation Pathway

Laser, Shutter, Beam-combiner and First Turning Mirror
Second and Third Turning Mirrors, Pirmary Beam-splitter and Scan Head

The Instrument Optics: The Emission Pathway

Scan Head, Primary Beam-splitter and Confocal Optics
Secondary Beam-splitter, Filtering and Photomultiplier Tubes
Data Collection:Scanning

The Instrument Optics: The Capillary Detection System

Fluorescence Separation

Emission Beam-splitters and Emission Filters
The Forward Scan Filtering System, Channels One and Two
The Return Scan Filtering System, Channels Three and Four
The Instrument Response to Emitted Light
Colour Separation Matrix
Data Collection

Capillary Focus

Appendix A. The Effect of Filter and Beam-splitter Orientation on MegaBACE Data

Appendix B. The Effect of Stage Position on Signal Strength and the Ability to Find Capillaries

The Instrument Optics: The Excitation Pathway

The Excitation Light Path: Laser, Shutter, Beam Combiner, and First Turning Mirror

The MegaBACE™ 1000 DNA Sequencing System uses patented confocal optics to identify fluorescently labelled sequencing products as they migrate past the detection window during electrophoresis (16,17). The system uses epi-illumination in which excitation light is focused on the capillaries, and emitted light is collected by the same objective lens. **Figure 6.1** is a line drawing of the entire optical path. The optical path begins with one of the two lasers: an argon ion laser (blue) that emits light of 488 nm or a solid-state He-Ne laser (green) that emits light of 532 nm. Directly in front of each laser is an interference filter with a very narrow band pass centered at the laser's wavelength (**Figure 6.1 and Figure 6.2A**). The band-pass filter makes sure that light transmitted through the filter is pure 488 nm or 532 nm. A shutter is used to select either laser. After the shutter is the first of three turning mirrors. The turning mirrors are used to move and align the laser beam. When the mirrors are properly adjusted, they deliver laser light to the scan head with accuracy. Each mirror can be adjusted in three axes. Two outside adjustment screws are used to point the beam, and the middle screw is used to position the beam laterally (**Figure 6.2B**). Therefore, not only is the end location of the beam controlled, but the initial point of reflection is also controlled. Three-axis optical adjustment is a difficult task and should only be attempted by properly trained personnel.

A beam combiner is positioned immediately after the first turning mirror. The beam combiner is a special filter that is used to overlay the green laser onto the path of the blue laser. The filter is designed to pass light of 488 nm and reflect light of 532 nm. When used in conjunction with the first turning mirror, the beam combiner places the green and blue laser beams on the same path so that all the downstream optics treat the two laser sources equivalently.

Figure 6.1 Instrument Optics and Light Path. The figure shows the optical path of the MegaBACE instrument. Each optical subsystem is discussed in later figures.

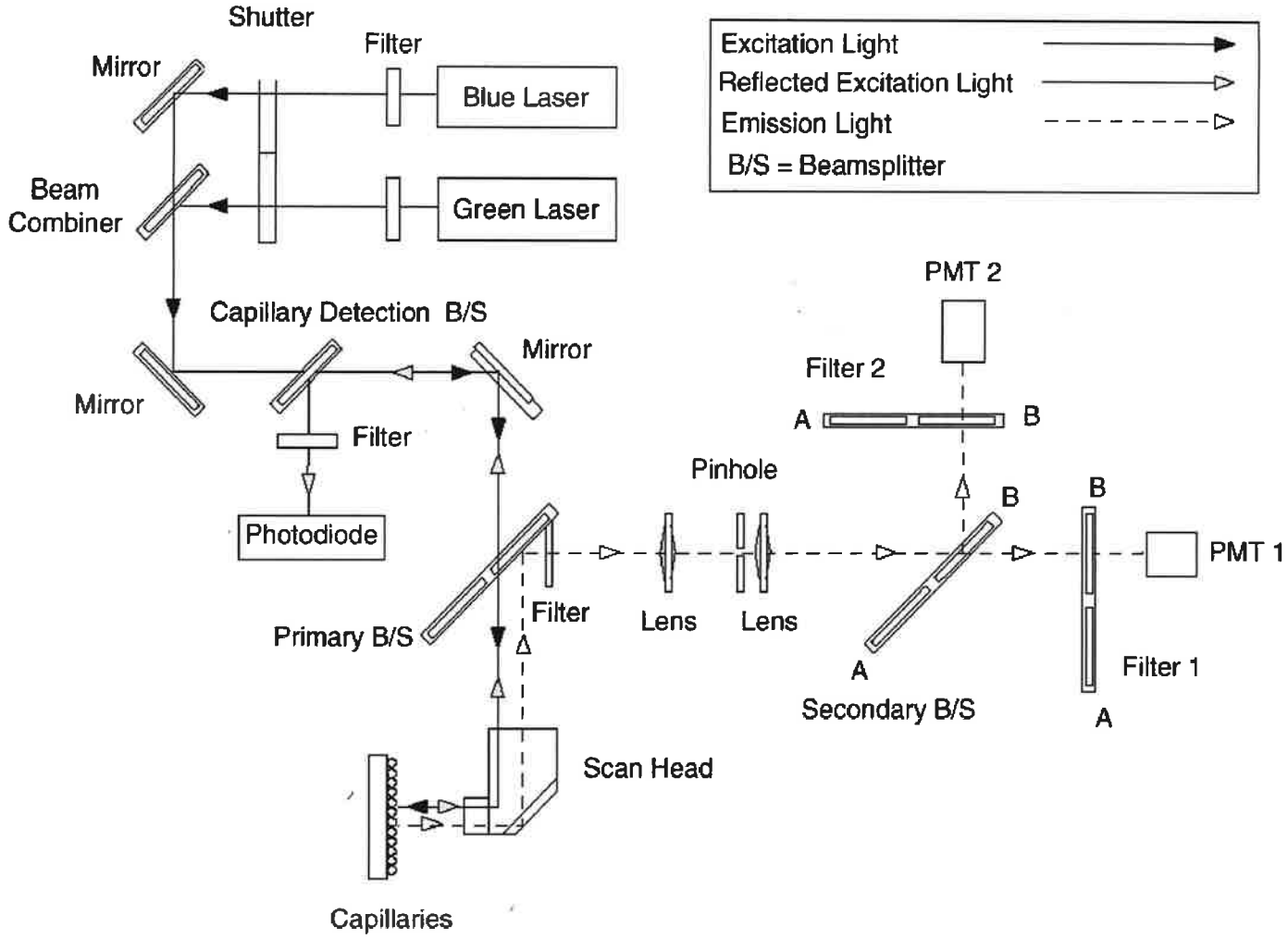

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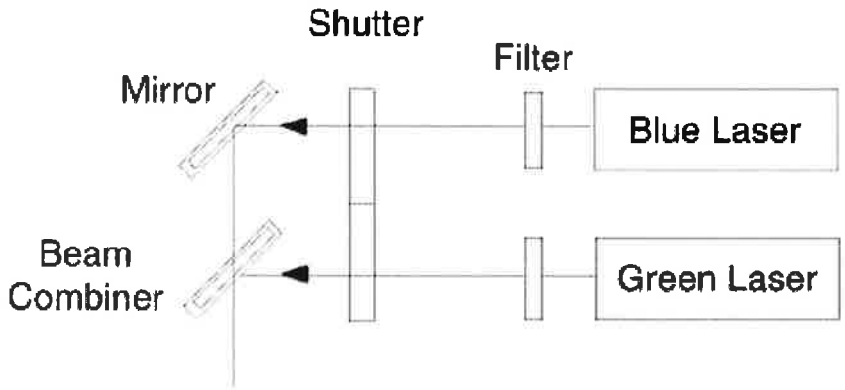
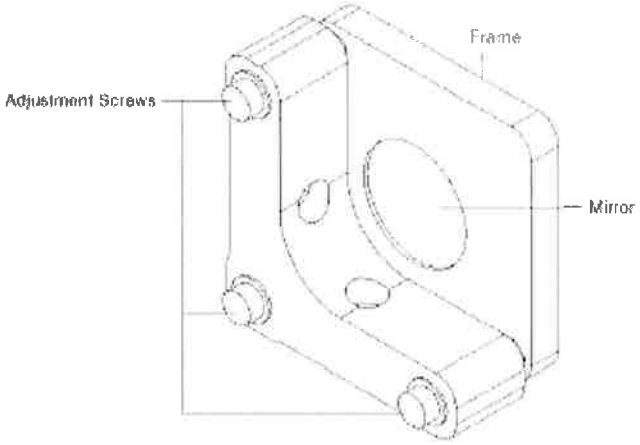
Figure 6.2. The Excitation Light Path. A. Laser to Beam Combiner. The figure shows the excitation light path from the laser to the beam combiner. The system has two lasers. Directly in front of each laser is an interference filter that allows the transmission of a single wavelength of light. The next element is the shutter that can select either laser or turn to block both lasers. After the shutter is the first turning mirror and the beam combiner. **B. Turning Mirrors.** Each turning mirror has have a set of adjustment screws that are used to position the beam correctly.

A. Laser to Beam Combiner



B. Turning Mirror

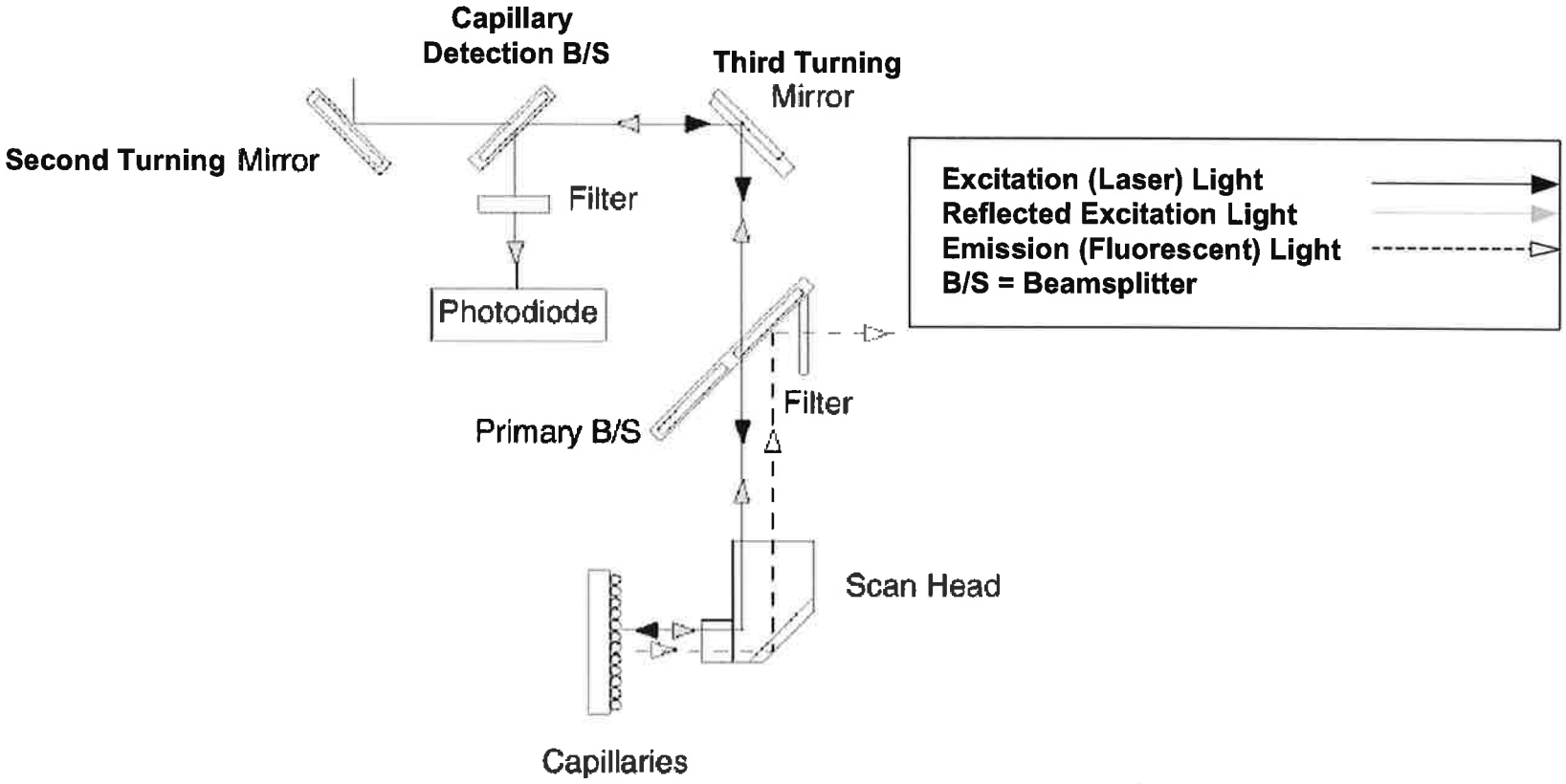


The Excitation Light Path: Second and Third Turning Mirrors, Primary Beamsplitter, and Scan Head

The laser beam is directed from the beam combiner to the second turning mirror, and then through a capillary detection beamsplitter. Subsequently, the beam reflects off the third turning mirror and passes through the primary beamsplitter and into the scan head (**Figure 6.1 and Figure 6.3**). When the blue laser is selected, the primary beamsplitter is a 510-nm dichroic reflective short-pass filter (510drsp). The filter is designed to pass the excitation light (488 nm) while reflecting the fluorescence of the labelled DNA fragments emitted above 510 nm. The filter is the key element in the separation of the excitation light path from the emission light path.

The laser light coming from the third turning mirror is reflected off the turning mirror in the scan head and is focused by the objective lens to a point within the bore of the capillaries. The objective lens is the last element the light passes through before entering the capillaries. The lens is optimized to yield the best combination of spot size, power density, collection efficiency, and resolution. The end result is a maximized fluorescent signal coming from the fluorescing dyes.

Figure 6.3. The Excitation Path: Second and Third Turning Mirrors, Primary Beamsplitter, and Scan Head. The figure shows the excitation light path from the second turning mirror to the capillaries. The laser beam passes from the beam combiner to the second turning mirror, and then passes through the capillary detection beamsplitter. Next the laser beam is reflected off the third turning mirror and passes through the primary beamsplitter and into the scan head. The objective lens focuses the laser light to a point within the bore of the capillaries.



The Instrument Optics: The Emission Pathway

The Emission Path: Scan Head, Primary Beamsplitter, and Confocal Optics

Fluorescently labelled DNA fragments emit photons in all directions when excited by the laser light delivered to the capillaries. This laser light is collected by the objective lens, collimated, and directed out of the scan head to the primary beamsplitter (**Figure 6.4**). The primary beamsplitter passes the excitation light (488 nm) and reflects the light emitted by the fluorescing dyes. This emitted light travels through several lenses that focus the light through a pinhole, which eliminates most of the undesirable optical noise in the instrument. The objective lens, the first focusing lens, the pinhole and the re-collimating lens combine to mimic a confocal microscope (**Figure 6.4**). This patented design is the heart of the MegaBACE optical path (16,17).

The Emission Path: Secondary Beamsplitter, Filters, and Photomultiplier Tubes

The light coming out of the pinhole travels to a secondary beamsplitter (**Figure 6.5**). Similar to the primary beamsplitter, the secondary beamsplitter is designed to separate light at a specific wavelength by passing light of longer wavelengths and reflecting light of shorter wavelengths. Band-pass filters further refine the light, which passes to photomultiplier tubes (PMTs) where the photons are converted into electrical current. For more information about dyes, filters, and colour separation, see the **Fluorescence Separation** section later in this chapter.

Figure 6.4. The Emission Path: Scan Head, Primary Beamsplitter, and Confocal Optics. The figure shows the emission light path from the capillaries to the pinhole. The fluorescent emission of the excited dyes is collected by the objective lens and collimated. The emission light travels out of the scan head and reflects off the primary beamsplitter. This emitted light then travels through a pinhole and is re-collimated.

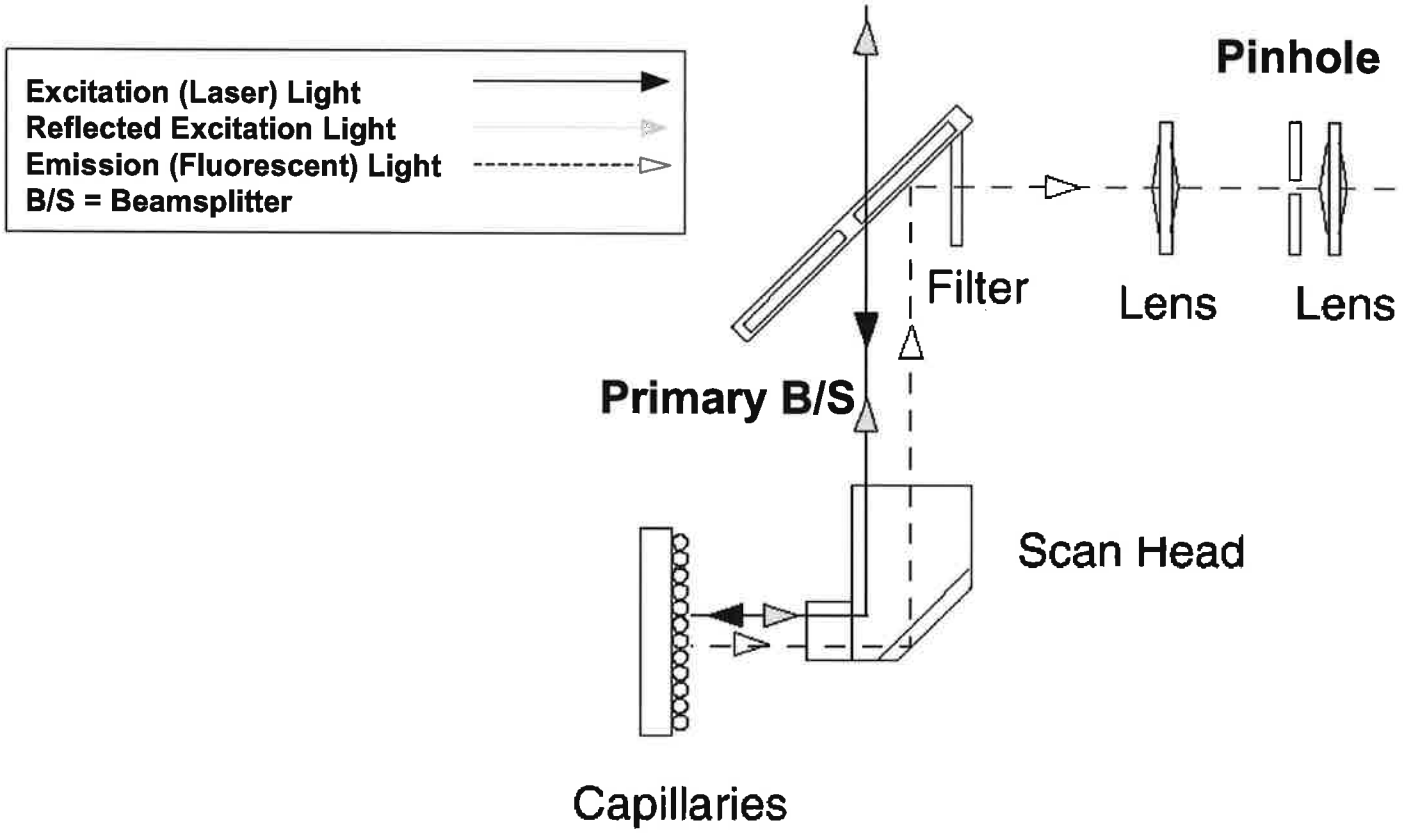
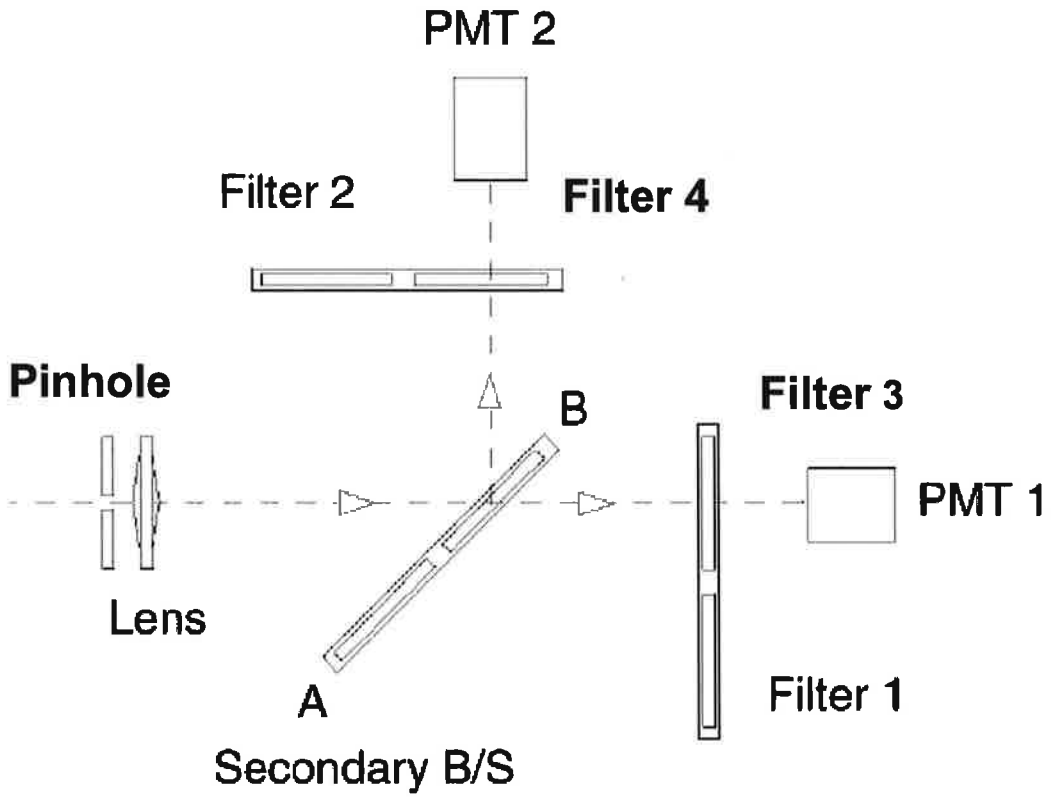


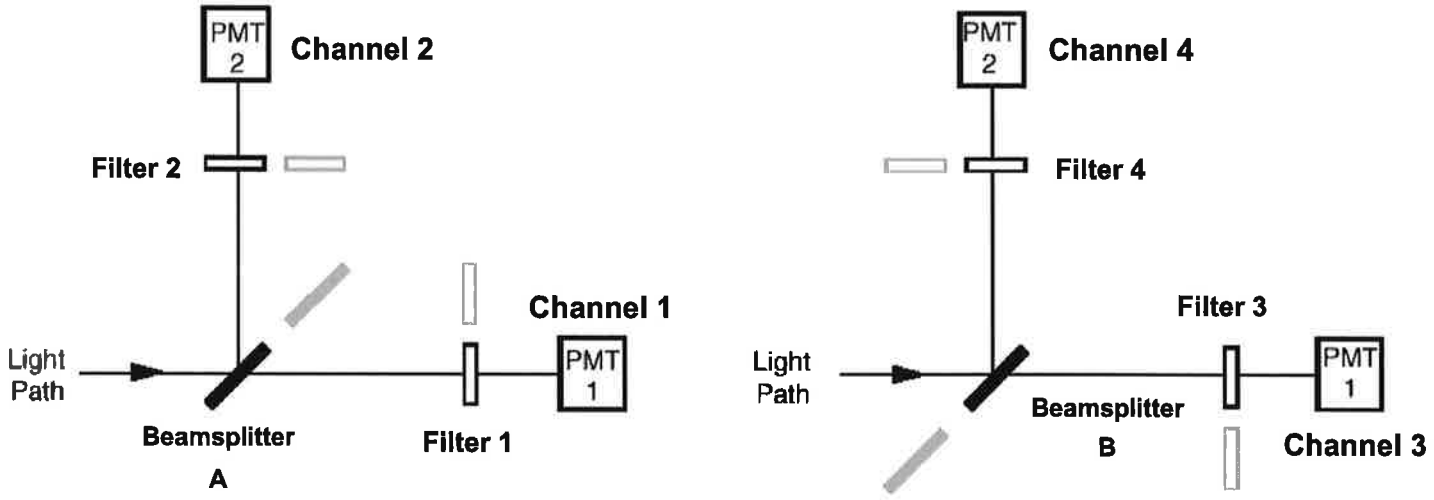
Figure 6.5. The Emission Path: Secondary Beamsplitter, Filters, and Photomultiplier Tubes. The figure shows the emission light path from the pinhole to the photomultiplier tubes (PMTs). The light traveling through the pinhole is separated at the secondary beamsplitter and is directed through a band-pass filter that further refines the light. The PMTs convert the photons into an electrical current.



Data Collection: Scanning

The MegaBACE instrument contains a detection system that uses two PMTs to collects the filtered light. The detection system uses two optical filter sets to record the data from the four fluorescent dyes. Each optical filter set consists of a beamsplitter and two emission filters. The beamsplitter changer and the two filter changers move synchronously with the scan head to place one optical set at a time in the light path, which allows the system to record the fluorescence of the dyes in four separate channels. **Figure 6.6** shows the position of the optics during both the forward and return scans. During the forward scan, the instrument records channels 1 and 2 using beamsplitter A and filters 1 and 2. During the return scan, the instrument records channels 3 and 4 using beamsplitter B and filters 3 and 4. The data from both directions are gathered and displayed together.

Figure 6.6. Data Collection: Scanning. The figure shows the positions of the optics during both the forward and return scans. **A. Forward Scan**. During the forward scan, the instrument records channels 1 and 2 using beamsplitter A and filters 1 and 2. **B. Return Scan**. During the return scan, the instrument records channels 3 and 4 using beamsplitter B and filters 3 and 4. The data from both directions are gathered and displayed together.



a) Forward Scan

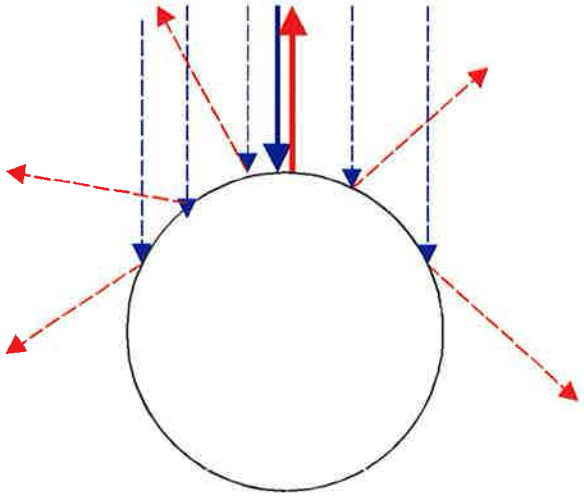
b) Return Scan

The Instrument Optics: The Capillary Detection System

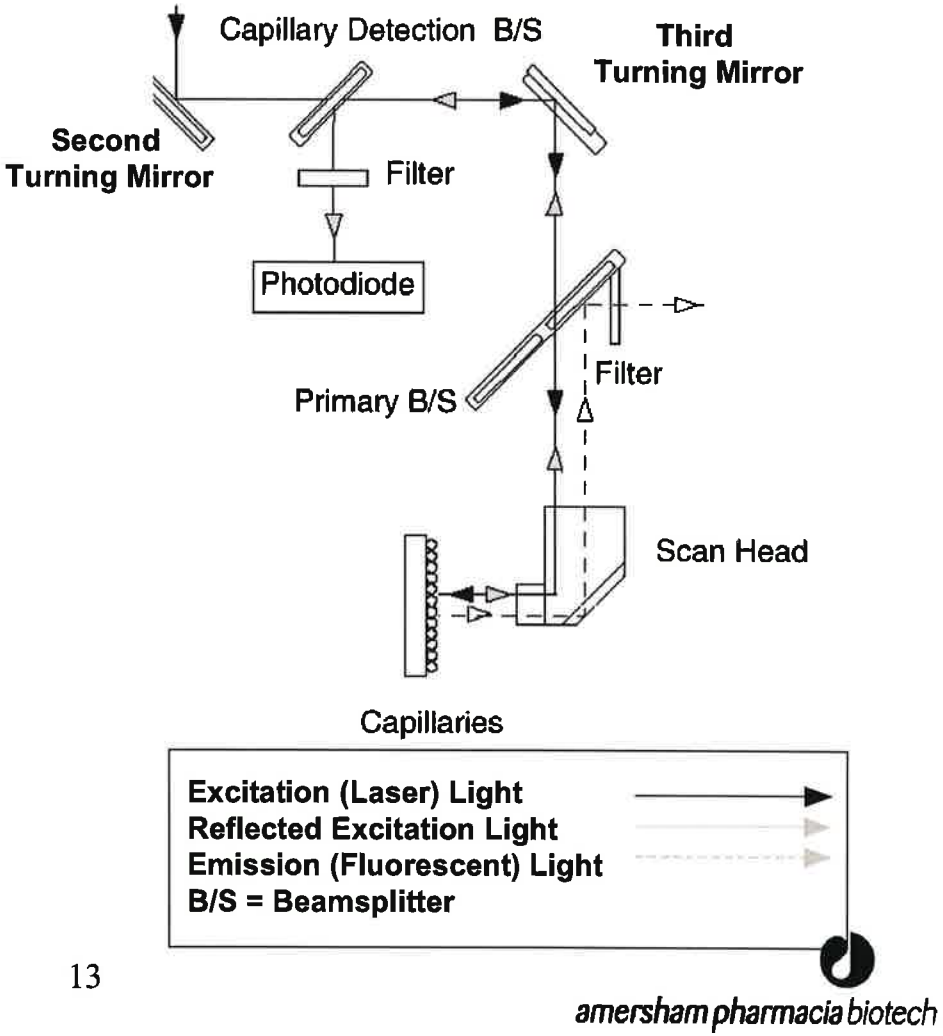
The MegaBACE instrument has a capillary detection system that locates the centers of the capillaries so that only the data of interest are collected. The system takes advantage of the fact that, when laser light strikes the curved surface of a capillary, some of the light is reflected. (**Figure 6.7A**). As shown in **Figure 6.7B**, the reflected light from the front surface of the capillary travels back on its original path through the primary beamsplitter. Next, the light is reflected off the third turning mirror and onto the reversed, non-chromatic capillary detection beamsplitter. This 45° beamsplitter is a thick piece of glass placed between the second and third turning mirrors. A small amount of the reflected laser light is directed by this beamsplitter through a filter and is collected by a photodiode (**Figure 6.7B**). Whenever the photodiode transmits a strong signal, the excitation beam is passing directly over the top of a capillary.

Figure 6.7. The Capillary Detection System. The figure shows how laser light is reflected off a capillary (A) and the light path of the capillary detection system (B).

A. Reflection of Laser Light Off a Capillary (Blue is Excitation and Red is Reflection)



B. Line Drawing of the Capillary Detection Light Path



Fluorescence Separation

Emission Beamsplitters and Emission Filters

The MegaBACE fluorescence separation system is made up of high-quality interference filters and beamsplitters. Each of the five filters and three beamsplitters is custom made and designed specifically for the MegaBACE instrument. The two secondary beamsplitters and four emission filters can be exchanged for other beamsplitters and filters so that alternative chemistries can be used.

Emission Beamsplitters

The secondary dichroic beamsplitters separate fluorescent light at specific wavelengths that allow light of longer wavelengths to pass through and reflect light of shorter wavelength. For example, a 540drlp (dichroic reflective long pass) beamsplitter passes light longer than 540 nm while reflecting light of a shorter wavelength (**Figure 6.8**).

Emission Filters

The MegaBACE instrument uses two types of emission filters:

Band-pass (df) filters pass fluorescent light of a discrete range and reject most of the light with wavelengths shorter or longer than a specified wavelength cut-off. For instance, the 520df20 band-pass filter passes light ranging from 510 nm to 530 nm and rejects most light outside this range. See **Figure 6.8** for a spectral representation of the data collected using a band-pass filter.

Long-pass (lp) filters pass fluorescent light of a specified wavelength and reject light of a shorter wavelength. For instance, the 610lp filter passes light of 610 nm or greater and rejects light of a shorter wavelength. See **Figure 6.9** for a spectral representation of the data collected using a long-pass filter.

In the MegaBACE system, each forward and return pass uses one optical set of filters and collects data in two channels giving a total of four channels, one for each dye. For the blue laser, the relationship between the channels and the dyes is shown in Table 6.1.

Table 6.1 Emission Beam-Splitters and Emission Filters

Scan Direction	Channel	Beam-Splitter	Emission Filter	Dye
Forward	1	540drlp	555df20	R6G
Forward	2	540drlp	520df20	R110
Return	3	595drlp	610lp	ROX
Return	4	595drlp	585df20	TMR

Channel	1	2	3	4
Dye	R6G	R110	ROX	TMR
ET Terminators	T	G	C	A
TS II Terminators	A	G	C	T
ET Primers	A	C	T	G

The Forward Scan Filtering System, Channels One and Two

In the forward scan direction, the first set of optical filters moves into place **(Table 6.1)**. The secondary beamsplitter is a 540drlp that separates the fluorescent dye emission at 540 nm **(Figure 6.8)**. The reflected fluorescent light passes through a 520df20 band-pass filter before being amplified by PMT 2. This path is designated channel 2 and is used to detect the emission from the R110-labelled DNA fragments. Light that passes through the beamsplitter continues through a 555df20 filter before being amplified by PMT 1. This filter detects the emission from the R6G-labelled DNA fragments and is designated channel 1. Note that the dye emissions extend into both channels, which demonstrates the cross-talk between channels. See **Fluorescence Separation and Spectral Cross-talk** for a discussion of this phenomenon.

The Return Scan Filtering System, Channels Three and Four

In the return scan, the second set of optical filters moves into place (**Table 5.1**). In this set, the 595drlp beamsplitter separates the fluorescent dye emission at 595 nm **(Figure 6.9)**. The reflected fluorescent light passes through a 585df20 band-pass filter before being amplified by PMT 2. This filter detects the emission from TMR-labelled DNA fragments and is designated channel 4. The light that passes through the beamsplitter continues through a 610lp filter before being amplified by PMT 1. This filter detects the fluorescence for ROX-labelled DNA fragments and is designated channel 3.

Figure 6.8. The Forward Scan Filtering System, Channels One and Two. The figure shows the relationship between the fluorescent spectra of the dyes (R110 and R6G), the secondary beamsplitter (540drlp), and the light collected by the emission filters (520df20 and 555df20) during the forward scan.

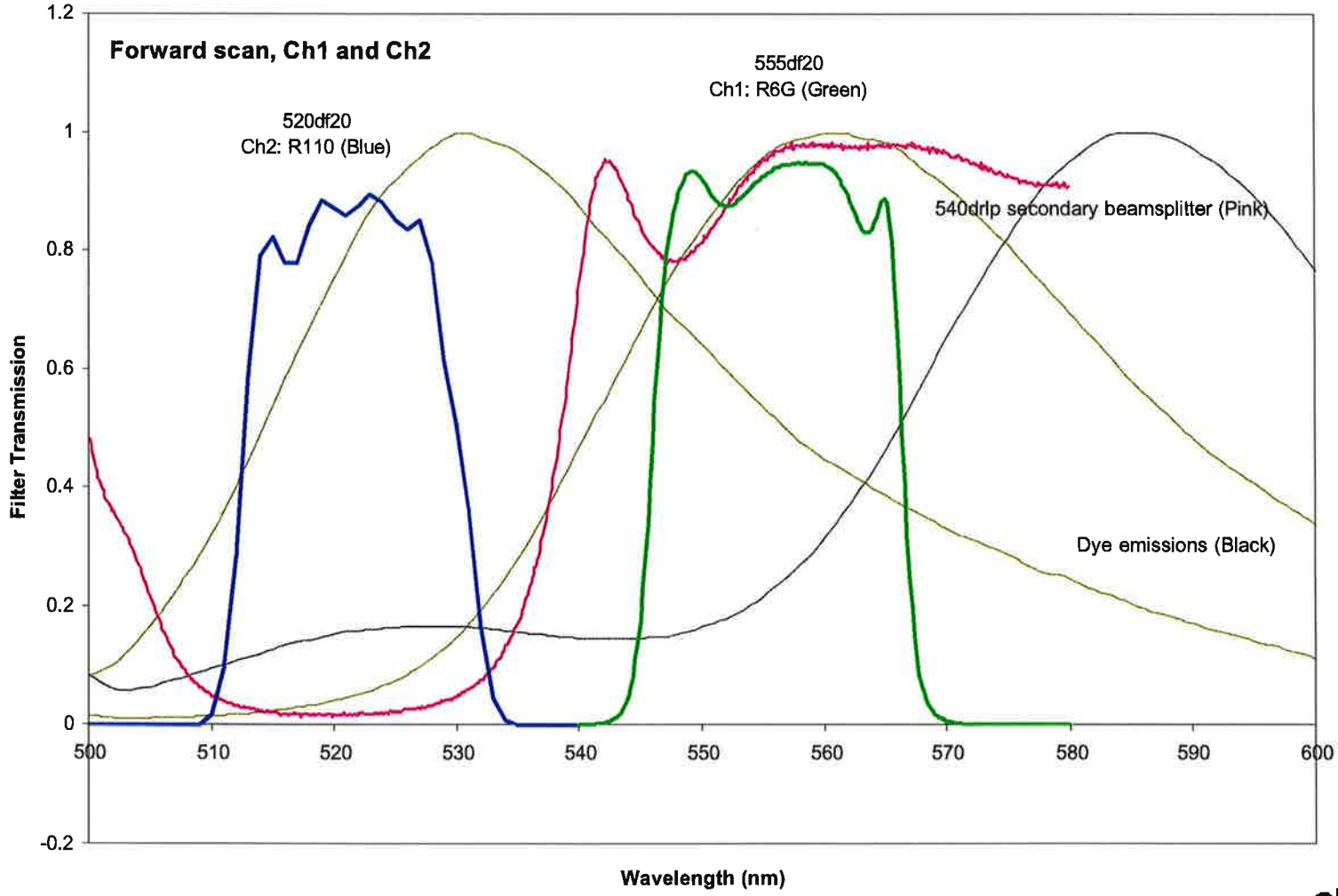


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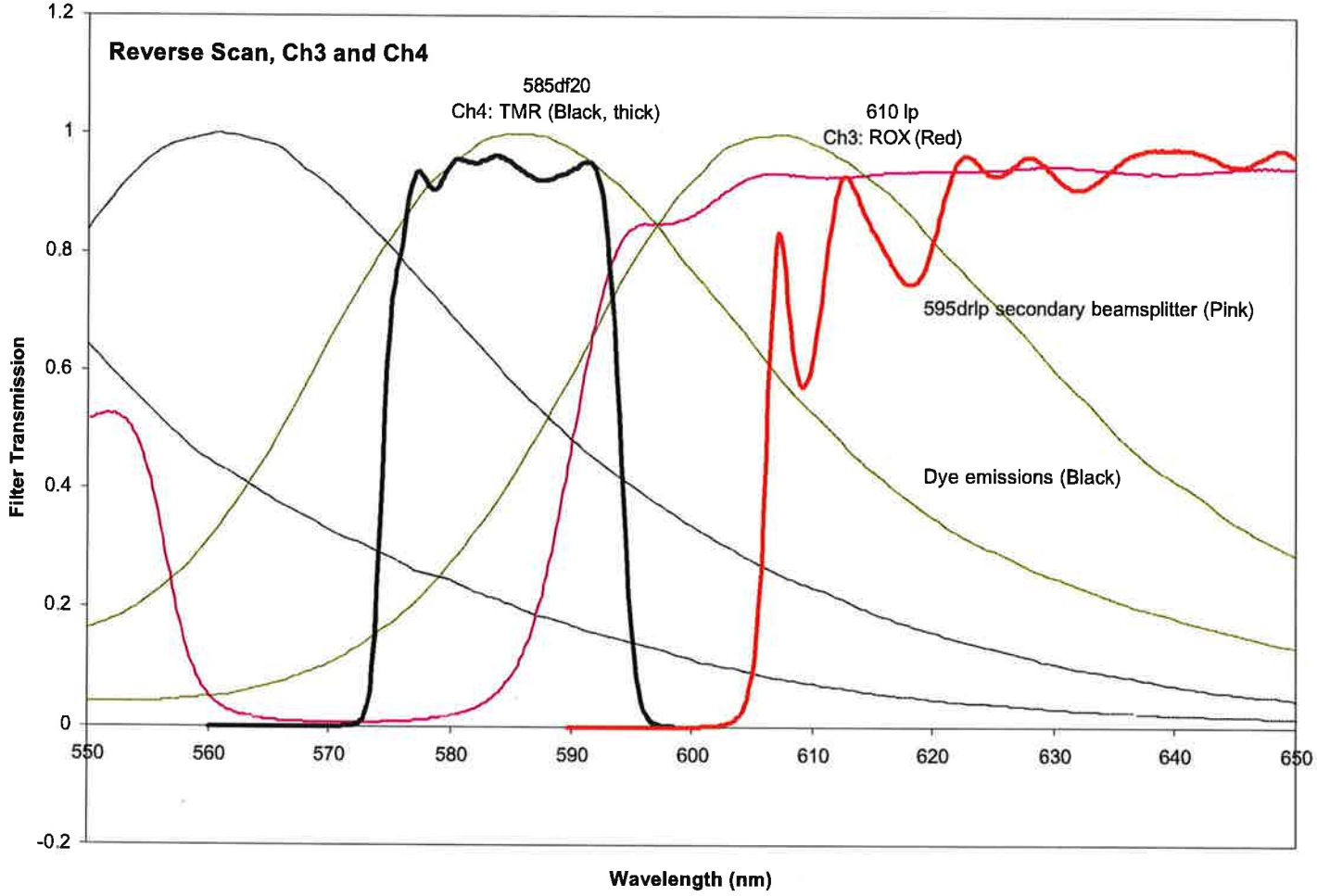
Figure 6.9. The Return Scan Filtering System, Channels Three and Four. The figure shows the relationship between the fluorescent spectra of the dyes (TMR and ROX), the secondary beamsplitter (595drlp), and the light collected by the emission filters (585df20 and 610lp) during the return scan.



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The Instrument Response to Emitted Light

The MegaBACE detection system uses emission filters and beamsplitters to separate the emitted light from the four fluorescent dyes and record them in four separate channels. However, because the spectra overlap, some emitted light from each dye will be detected in all four channels. This spectral cross-talk is depicted in four figures that show the relationship between the emission spectra of the dyes and the four emission filters (**Figure 6.10, 6.11, 6.12 and 6.13**). In these figures, all the dye spectra have been normalized for intensity. Note that while the emission spectrum from each dye is detected in all four channels, the greatest amount of fluorescence is collected in the channel specific for that dye. **Figure 6.10** and **Figure 6.11** show the instrument response to the fluorescence of R110 and R6G, respectively. **Figure 6.12** shows the instrument response to TMR fluorescence. Finally, the instrument response to the fluorescence of ROX is shown in **Figure 6.13**. Note that the bulk of the ROX emitted light is collected by the channel three filter, and the effect of the long-pass filter is to extend the amount of light collected from the dye.

In reviewing these four figures, observe again that each channel is specific for a particular dye and that each dye exhibits a distinct pattern of intensities with contribution from all four channels. This pattern is unique to each dye and is recorded by the instrument software as raw data (**Figure 6.14A**). Compare the profiles of each raw data peak with the emitted light collected across the four channels for each dye (Figures **6.10, 6.11**, **6.12** and **6.13**).

Figure 6.10. The Instrument Response to R110. The figure shows the relationship between the light transmitted by the filters and the emission spectrum of R110. The solid black line depicts the normalized fluorescence spectrum of R110. The coloured areas depicted under this curve represent the amount of R110 fluorescence transmitted by the four channel filters. The fluorescence of an R110-labelled DNA fragment is recorded by the system proportional to the blue area in channel two, the green area in channel one, the black area in channel four, and the red area in channel three.

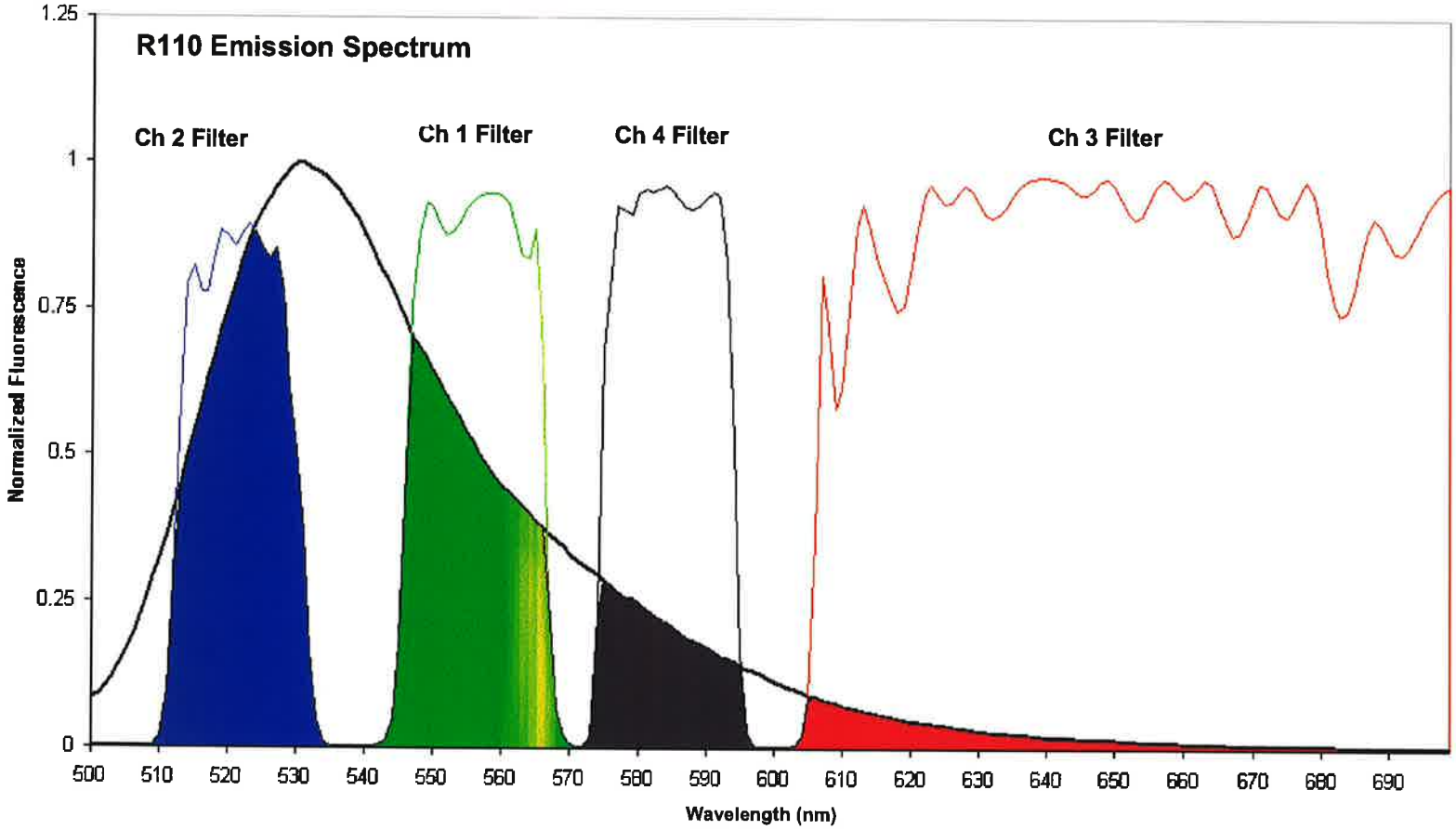

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Figure 6.11. The Instrument Response to R6G. The figure shows the relationship between the light transmitted by the filters and the emission spectrum of R6G. The solid black line depicts the normalized fluorescence spectrum of R6G. The coloured areas depicted under this curve represent the amount of R6G fluorescence transmitted by the four channel filters. The fluorescence of an R6G-labelled DNA fragment is recorded by the system proportional to the blue area in channel two, the green area in channel one, the black area in channel four, and the red area in channel three.

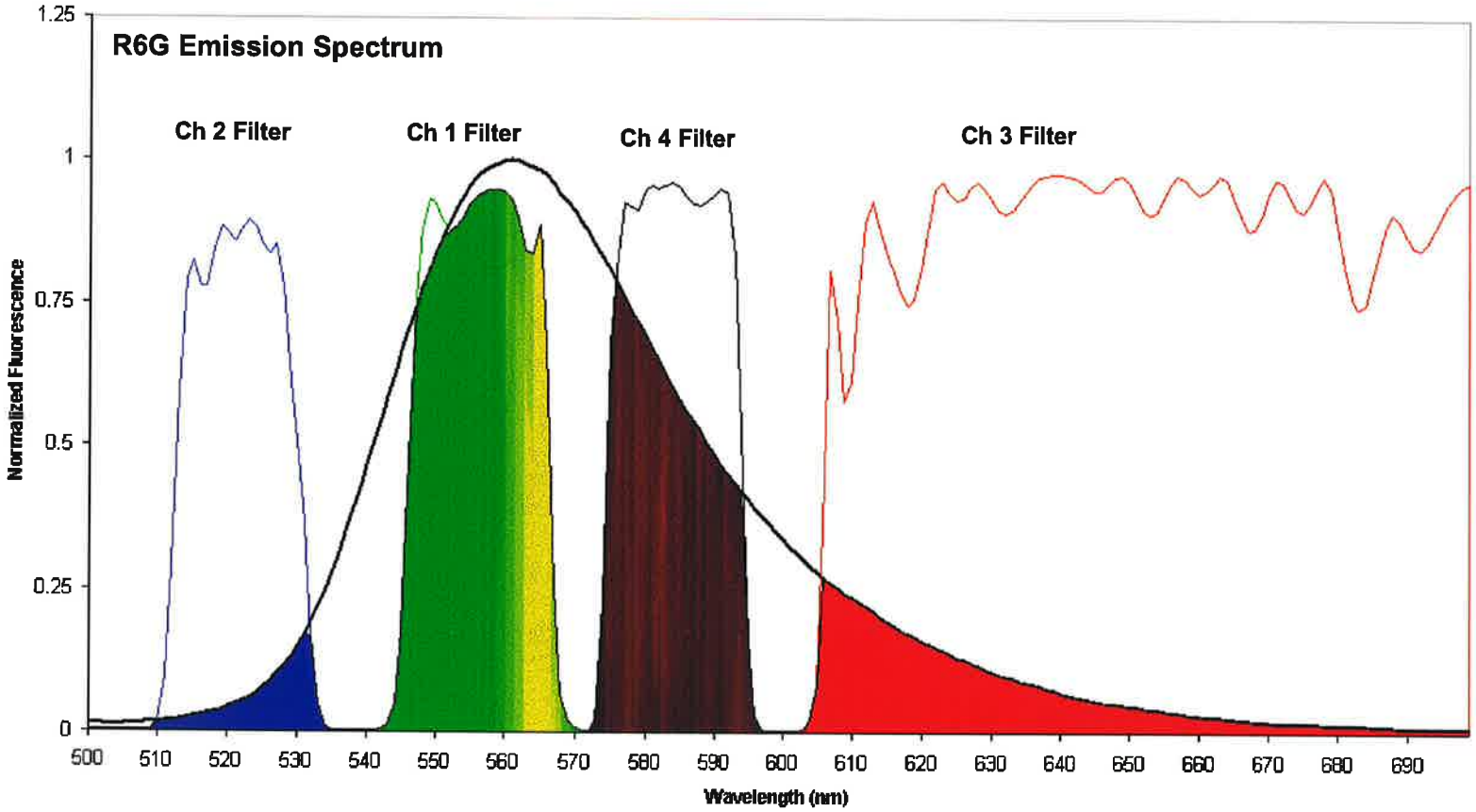


Figure 6.12. The Instrument Response to TMR. The figure shows the relationship between the light transmitted by the filters and the emission spectrum of TMR. The solid black line depicts the normalized fluorescence spectrum of TMR. The coloured areas depicted under this curve represent the amount of TMR fluorescence transmitted by the four channel filters. The fluorescence of a TMR-labelled DNA fragment is recorded by the system proportional to the blue area in channel two, the green area in channel one, the black area in channel four, and the red area in channel three.

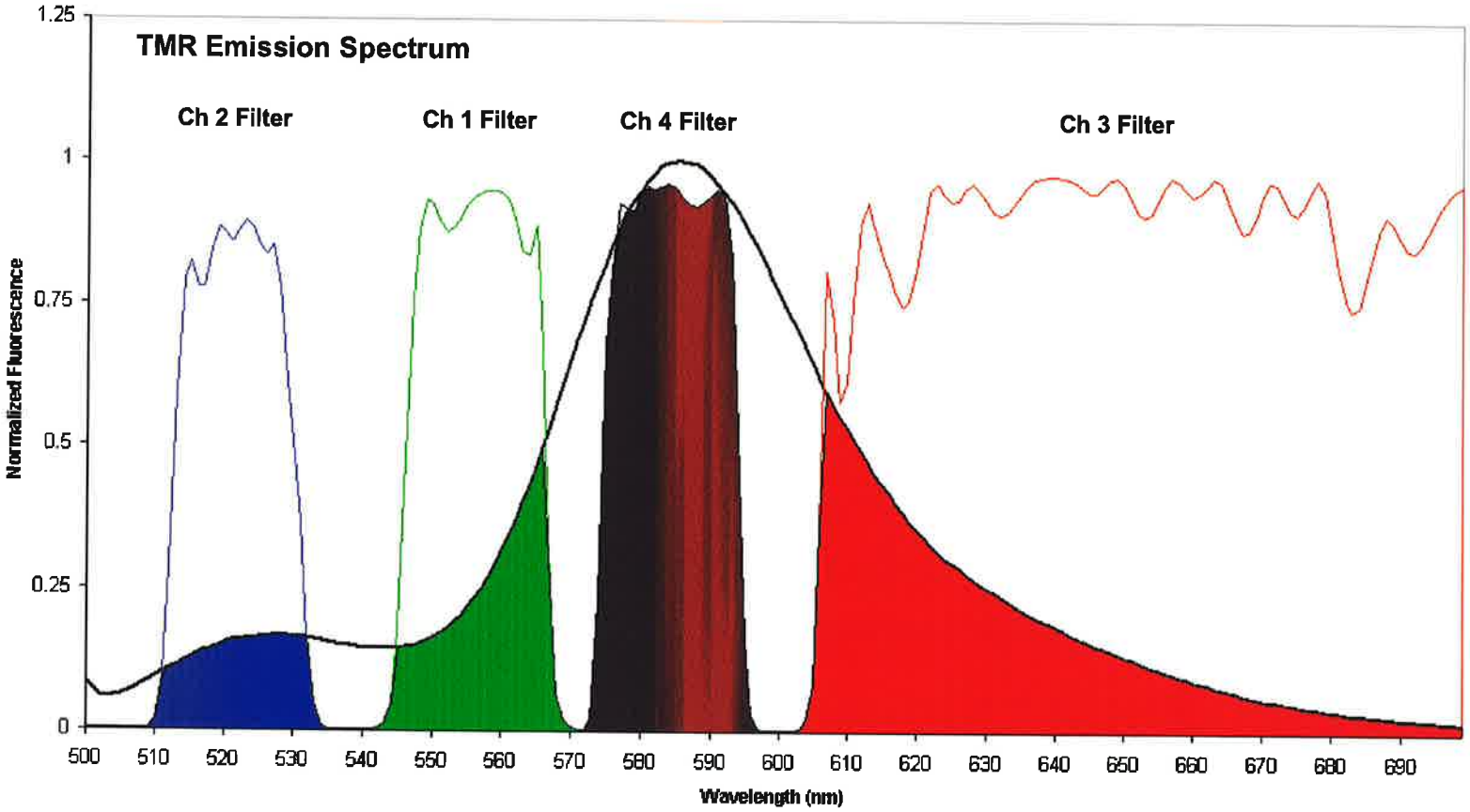


Figure 6.13. The Instrument Response to ROX. The figure shows the relationship between the light transmitted by the filters and the emission spectrum of ROX. The solid black line depicts the normalized fluorescence spectrum of ROX. The coloured areas depicted under this curve represent the amount of ROX fluorescence transmitted by the four channel filters. The fluorescence of a ROX-labelled DNA fragment is recorded by the system proportional to the blue area in channel two, the green area in channel one, the black area in channel four, and the red area in channel three.

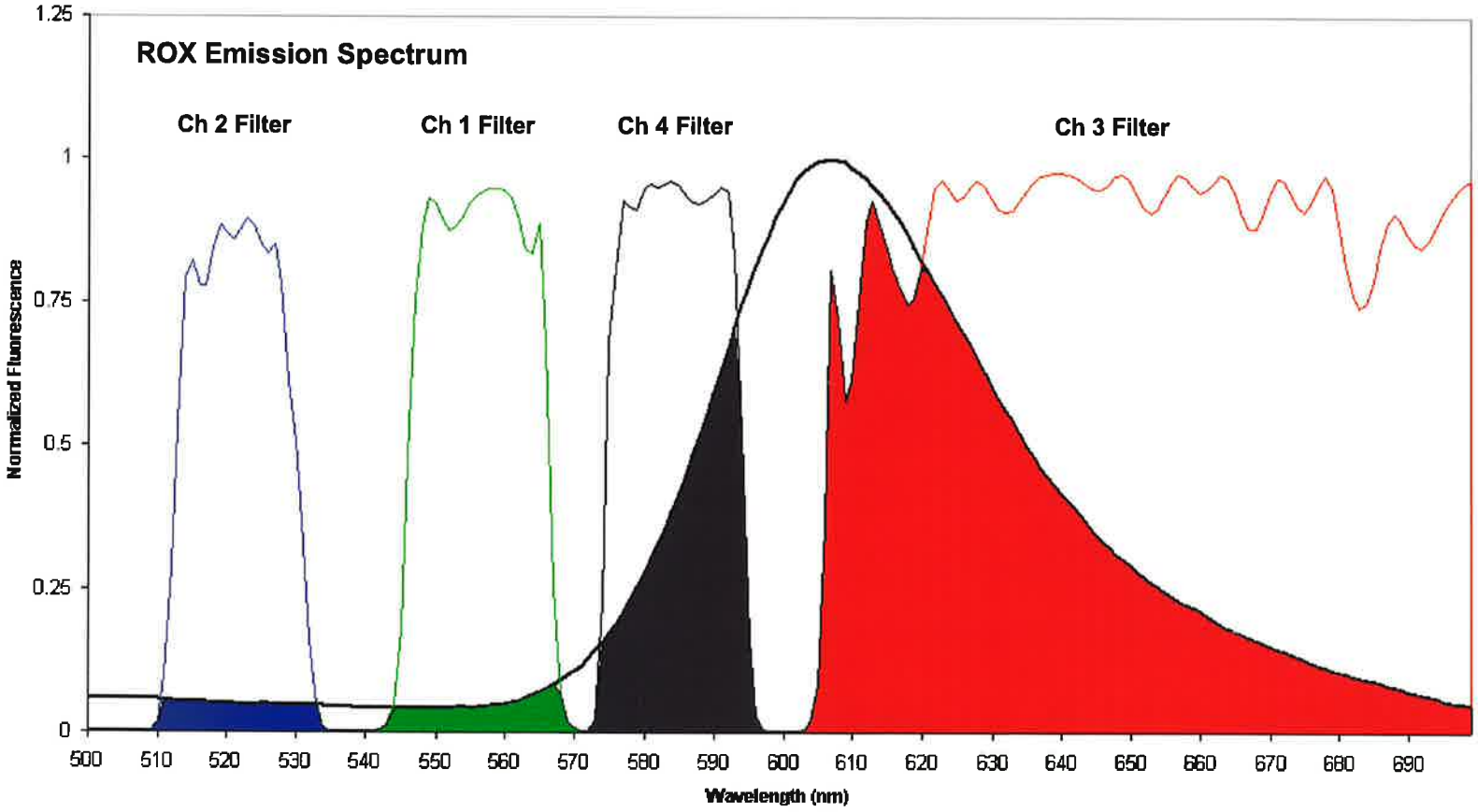
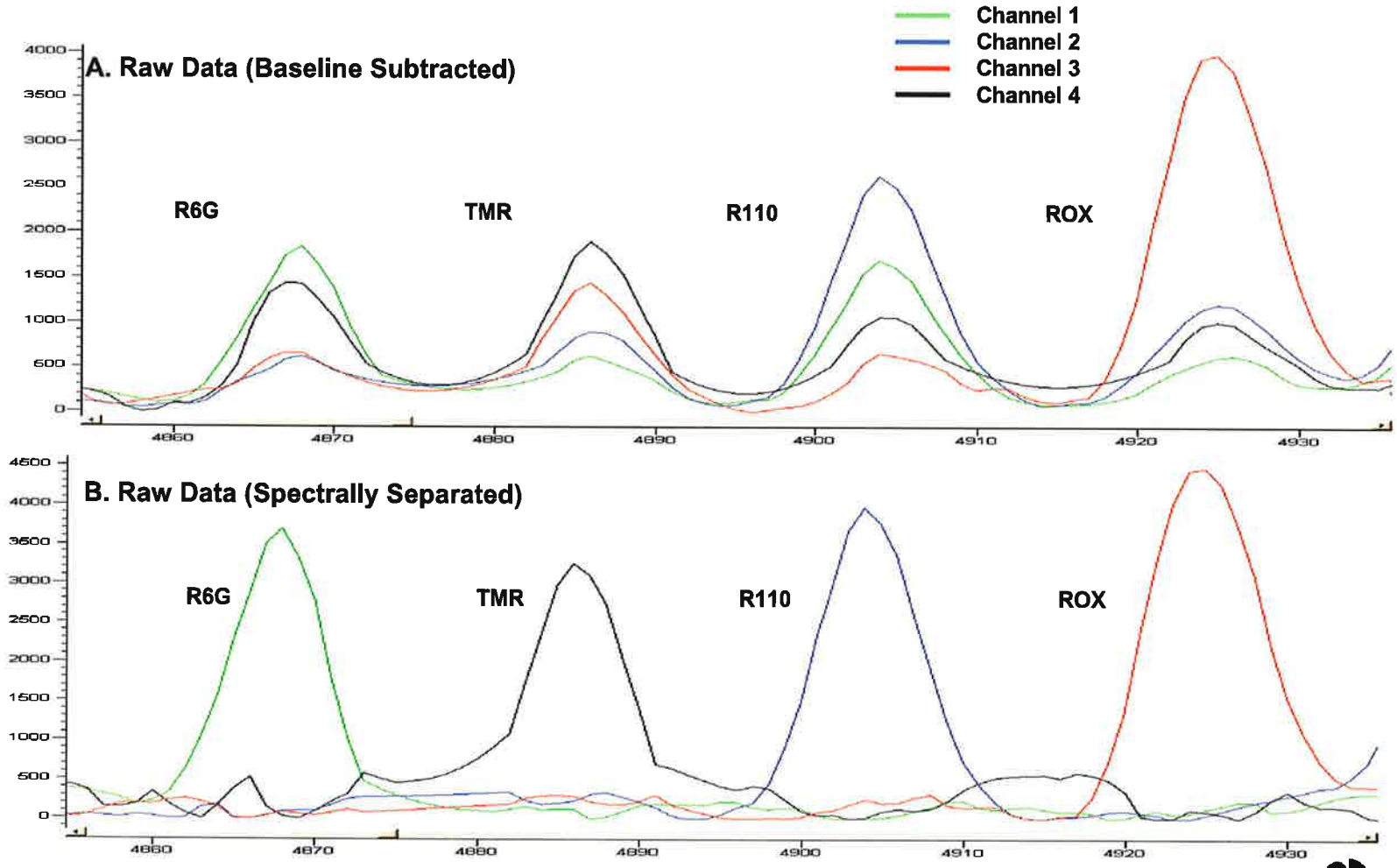


Figure 6.14. Raw Data. Each peak represents the fluorescence of a particular dye-labelled DNA fragment as seen by all four channels. Each dye yields fluorescence that is detected in all four channels with characteristic ratios.



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Colour Separation Matrix

The fluorescence of the four dyes in each channel (**Figure 6.14A**) is used to generate a colour separation matrix (**Table 6.2**). The numbers in this matrix are used to convert the raw data into colour-corrected data (**Figure 6.14B**). In **Figure 6.14B,** the height of each line represents the concentration of the specific dye.

Table 6.2 Example of a Colour Separation Matrix

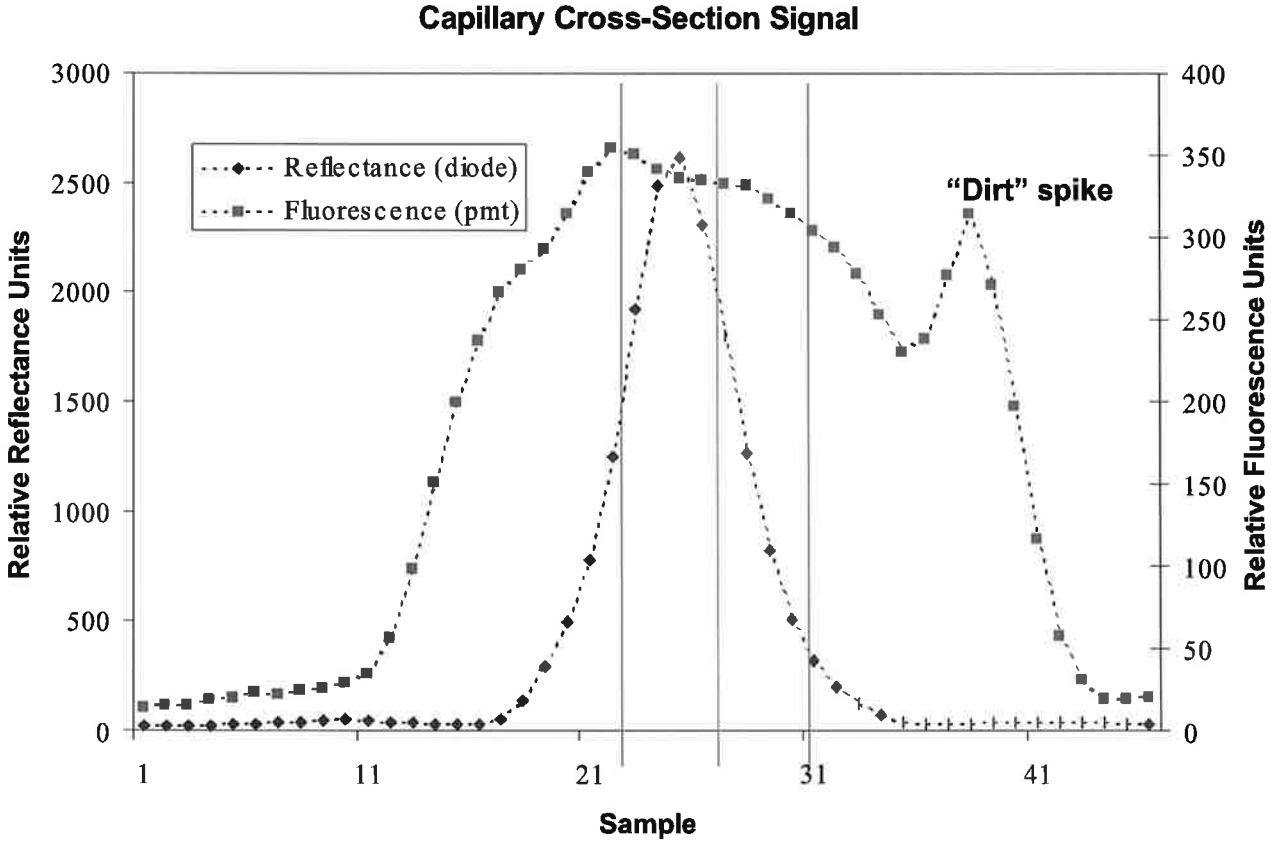
	Dye	Emission Filter	R110	R6G	TMR	ROX
Channel 2	R110	520df20	1.00	0.08	0.18	0.03
Channel 1	R6G	555df20	0.84	1.00	0.34	0.04
Channel 4	TMR	585df20	0.34	0.67	1.00	0.26
Channel 3	ROX	610lp	0.16	0.37	0.90	1.00

The key to spectral separation of the four dyes is the individual pattern created by their emissions within the four channels. The MegaBACE system software requires four unique peaks to form a spectral separation matrix and establish correct base identification. Some of the variables that can affect the colour separation matrix are PMT voltage, differences between filter and beamsplitter lots, differences in the optical alignment, and focus differences in the capillaries combined with non-achromatic system behavior.

Data Collection

As the instrument scans, filtered light is collected by the PMTs, and the filtered light is transformed from an analog signal to a digital signal. These discrete digital values are each referred to as pixels. A single scan across all 96 capillaries consists of over 7500 pixels. To reduce these pixels to 96 points that represent the 96 capillaries, the instrument takes advantage of the reflected laser light off the surface of the capillary to determine its exact location (**Figure 6.7**). In **Figure 6.15** the reflected light collected for a single capillary is represented by the blue pixels. Using the peak of reflected light, the software determines the location of the exact center of the capillary, which is shown as a blue vertical line. The corresponding fluorescent emission signal from one of the PMTs is represented by the red pixels. Based on the determined location of the capillary, the software collects a range of fluorescent signals represented by the two red vertical lines and averages these data to define a single scan line in the electropherogram (the *.ESD file).

Figure 6.15. Data Collection. The reflected light collected for a single capillary is represented by the blue pixels. Using the peak of the reflected light, the software determines the location of the exact center of the capillary (blue vertical line). The corresponding fluorescent emission signal from one of the PMTs is represented by the red pixels. Based on the determined location of the capillary, the software collects a range of fluorescent signals (two red vertical lines) and averages these data to define a single scan line in the electropherogram (the *.ESD file).



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Capillary Focus

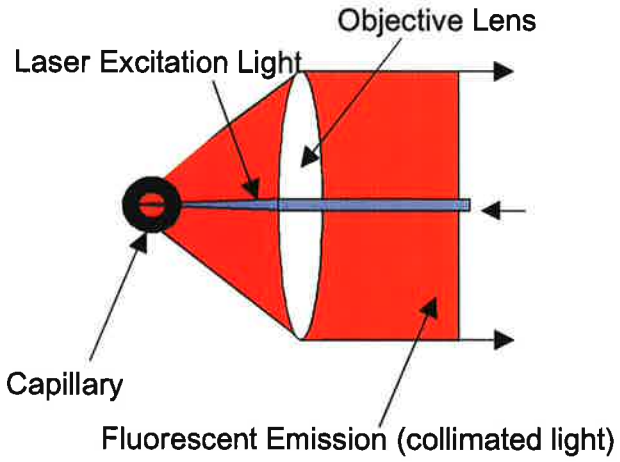
Proper capillary focus is a key element in the performance of the MegaBACE system. With proper focus, the instrument can obtain optimal excitation and emission detection of fluorescently labeled DNA during electrophoresis. Focusing the MegaBACE instrument requires a fine adjustment of the capillary position. Using an objective lens, the laser light is concentrated on a point within the bore of the capillaries. With a capillary internal diameter (I.D.) of 75 µm, the margin for error in the optimal focus position is quite small. To find the proper focal position, capillary focus is performed using a technique called the "step-through-focus" procedure. This procedure consists of the following steps:

1. The capillaries are filled with a dilute solution of fluorescent dye.
2. The instrument is set to scan the capillaries and collect data, which is displayed as an image.
3. Two of the four channels are set to collect capillary detection diode data (**Figure 6.7B** and **Figure 6.15**) whereas the other two channels collect fluorescent data.
4. The capillaries are moved away from the objective lens as the instrument scans (**Figure 6.16 A and B**).
5. During each scan cycle, the capillary stage is moved away from the objective lens in discrete 2-µm steps until the focal point past the center of the capillaries (**Figure 6.16B**).
6. The resulting image is analysed and the position of the average maximum fluorescent peak for all 96 capillaries is determined (**Figure 6.17**).
7. The capillaries are returned to the starting point, and then moved back to this optimal focus position.

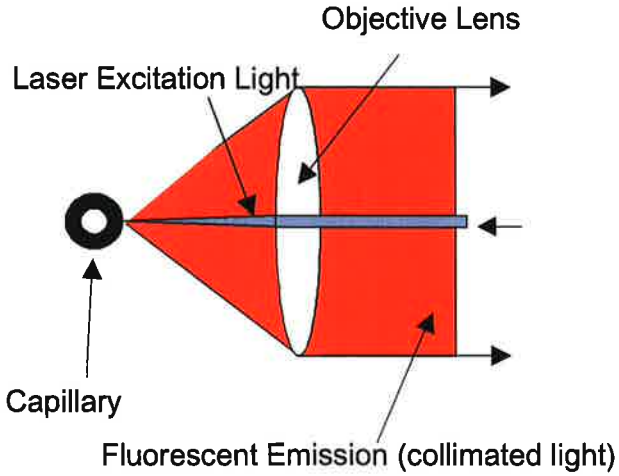
Figure 6.17 shows the relationship between the position of the focal point and the maximum fluorescence of the emitting dye. As the capillaries move away from the scanning objective lens, the collected fluorescence of the dye increases to a peak and then decreases as the capillaries pass through the optimum focus position (**Figure 6.17A**). By drawing a line in the step-through-focus image, a graphical representation of the data is obtained and the optimal focus position is determined by converting pixels to steps (**Figure 6.17B).**

Figure 6.16. Capillary Focus. The figure shows the movement of the capillaries during a step-through-focus procedure. During the step-through-focus procedure, the capillaries, which are filled with a dye solution, are moved away from the objective lens in 2-µm steps as the instrument scans the capillaries. The instrument is configured to collect both capillary diode data and fluorescent emission data and to display these data as an image (**Figure 6.17**).

A. Step-Through-Focus Starting Position



B. Step-Through-Focus Ending Position



Direction of Capillary/Array Holder Movement

Figure 6.17. Capillary Focus. The figure shows an image and a graphical representation of data collected during a step-through-focus procedure. **A. Image from Step-Through-Focus.** The image shows the relationship between the position of the focal point and the maximum fluorescence of the emitting dye. **B. Line Graph from Image.** Using the red line drawn in Figure 6.17A, a graphical representation of the data can be displayed.