GeoMx DSP Segmentation Guidelines

Tumor/stroma segmenting: the simplest path

For this example, we use a scan of colon tumor tissue treated with the following:

- Pan-CK (tumor) – Cy3 – appears as **yellow**
- CD45 (immune) – Texas Red – appears as **red**
- KI-67 (nuclear, proliferation) – Cy5 – appears as **green**

1. Create two segments using settings below and click **Generate Segments**.
   - **Tumor**: set Cy3 to + and choose a segment color (**green** for this example)
     - This includes Cy3 positive pixels in the mask.
   - **Stroma**: set Cy3 to − and choose segment color (**red** for this example)
     - This excludes Cy3 positive pixels from the mask.
     - Alternatively, this channel could be set to **not exclude** this channel from consideration in the mask using Ø.

![Segment Definitions](image1.png)

Figure 1: ROI on a colon tumor tissue scan

Figure 2: Segment definitions and resulting segments on scan image.
2. Assess the selection. You may find that some nuclei are not selected (see arrows in Figure 3). This may happen if using membrane stains which don’t stain nuclei. There are several parameters of the auto segmentation algorithm that can be altered to include all nuclei of interest.

Note: in Figure 3, the stroma segment has been hidden by clicking the eye icon.

- Potential solution: increase the hole size in the Tumor segment. The system will fill in holes in segments less than this parameter.

![Figure 4: Tumor segment definition and resulting segment with hole size setting increased. Arrows refer to nuclei previously unselected.](image)

- Potential solution: decrease the threshold setting for the channel exhibiting the holes. In this example, we decrease the Cy3 channel threshold.

![Figure 5: Channel Thresholds and resulting segments; decreasing threshold in Cy3 channel. Arrows refer to nuclei previously unselected.](image)
3. Assess the selection. You may find that an area at the edge of the segment is not selected (see circled area in Figure 6).

- Potential fix: increase the minimum particle size in the Stroma segment. The system will only select areas greater than or equal to this parameter. In this example, this change did not have an effect (image not shown).

![Figure 6: Area at edge of segment not selected.](image)

![Figure 7: Stroma segment definition; increasing particle size.](image)

- Potential fix: increase the erode setting in the segment with too much selected. The erode function increases the boundary between segments and smooths the edges of a segment.

![Figure 8: Stroma segment definition and resulting segment; increasing the erode function in the Stroma segment. Circle indicates area previously unselected.](image)

**Note:** to refresh the scan and segments on it, change a threshold setting and watch for the screen to refresh.
Tumor/stroma segmenting: removing dead space and autofluorescence.

For this example, we use a scan of colon tumor tissue (same scan as used in the example above) treated with the following:

- Pan-CK (tumor) – Cy3 – appears as yellow
- CD45 (immune) – Texas Red – appears as red
- KI-67 (nuclear proliferation) – Cy5 – appears as green

1. As above, create two segments. Use the settings below and click Generate Segments.

   - **Tumor:** set CY3 to + and choose a mask color (green for this example)
   - **Stroma:** set CY3 to - and FITC to + and choose mask color (red for this example)

2. Assess the selection. You may wish to deselect dead space and correct for autofluorescence (see arrows in Figure 10).

   - Potential fix: increase the N-dilate setting in the Stroma segment. This increases the maximum space allowed around nuclei to qualify them for selection. To use this, FITC must be set to + in the segment you wish to edit.
• Potential fix: increase the FITC threshold.

![Channel thresholds and resulting segments; increasing the FITC channel. Arrow indicates area previously unselected.](image)

• Potential fix: increase the minimum particle size in the Tumor segment.

![Tumor segment definition and resulting segment; increasing the minimum particle size. Arrow indicates area previously unselected.](image)
Co-localization segmenting

For this example, we use a scan of a healthy colon tissue treated with the following:

- Pan-CK (Tumor) – CY3 – appears as purple
- CD45 (Immune) – Texas Red – appears as green
- CD3 (T-cells) – CY5 – appears as red

Note: when studying markers that are co-expressed, you may choose to change the render settings to resolve the color mapping, as the combination of two co-expressed markers may appear as a third.

1. Create three segments using settings below and click Generate Segments.
   - T-cells: set Cy5 to + and Texas Red to + and choose a segment color (light blue for this example)
   - Immune: set Cy5 to - and Texas Red to + and choose a mask color (yellow for this example)
   - Tumor: set Cy5 to -, Cy3 to +, and Texas Red to - and choose a mask color (green for this example)
2. Assess the selection. You may find that one segment is not selected. In this example, there is no tumor selected.

- Potential fix: decrease tumor channel (Cy3) threshold. In this multi-channel case, this did not help.

![Figure 16: Channel thresholds and resulting segments on scan; decreasing Cy3 channel.](image)

- Potential fix: review the generation order of the segments. A dominant segment generated earlier in the sequence may be the issue. Consider changing the generation order or decreasing the threshold(s) selected in one of these earlier segments.

- Potential fix: assess all thresholds using this method:
  - Set all channels to 250 – this means nothing is selected
    ![Figure 17: Channel thresholds and resulting segments on scan; setting all channels to 250.](image)
  - Start with tumor channel – set to 30 and adjust this until desired selection.
    ![Figure 18: Channel thresholds and resulting segments on scan; setting Cy3 to 30.](image)
Next, set another channel (choose one that acts as a single-channel for one of the segments) to 30 and adjust this until desired selection.

![Channel Thresholds Table]

**Figure 19:** Channel thresholds and resulting segments on scan; setting Texas Red to 30.

- Decrease the erode function on one or more segments.

![Segment Definitions Diagram]

**Figure 20:** Segment definitions and resulting segments on scan; decreasing the erode function.

**Note:** Shape filters such as erode, hole size, and particle size may make it difficult to adjust the thresholds accurately. If thresholds are not behaving as you expect, disable these and adjust the thresholds. You can reenable them afterwards.

- Set the third channel to 30 and adjust this until desired selection.

![Channel Thresholds Table]

**Figure 21:** Channel thresholds and resulting segments on scan; setting Cy5 to 30.
3. Create a fourth segment, **Other**, and set it to all channels. Click **Generate Segments**. You may need to adjust particle size or other settings to adjust it.

![Image of segmentation settings and resulting segments on scan.](image)

*Figure 22: Fourth segment definition and resulting segments on scan.*