* Significantly different vs. other stimulus durations (P<0.05). ns, not significantly different. **D**) With spontaneous vasomotor tone, vasodilation at Site 1 increased with peak fluorescence across stimulus intensities (r^2= 0.69; P<0.01).

Figure 3. Fluorescence responses across levels of vasomotor tone. For these experiments, the ACh stimulus was constant (500 ms, 500 nA). **A**) Recorded at Site 1, representative traces of Ca^{2+} fluorescence (F/F_o) in response to ACh with spontaneous vasomotor tone and during maximal dilation with SNP (black). **B**) Summary data (n=5) for total fluorescence intensity across the width of an arteriole during spontaneous vasomotor tone (resting diameters, 20-30 μm) vs. during maximal vasodilation with SNP (diameters, 40-50 μm; n= 5). **C**) Peak Ca^{2+} fluorescence at Sites 1-5 (see Figure 1B) under varying levels of vasomotor tone. Peak F/F_o was not different across levels (up to 50%) of vasomotor tone (n=5 each). **D**) Vasodilation (Diameter Change, recorded at Site 1) was greatest at higher levels of vasomotor tone. ns, not significantly different. † Significantly different vs. 50% tone (P<0.05). ‡ Significantly different vs. other levels of tone (P<0.05).

Supplemental Video 1: Diameter and Fluorescence tracking using a custom analysis program. **Top)** Arteriolar segment upstream from stimulus with 10 line scans corresponding to the line scans in Figure 1B. Stimulus pipette was adjacent to the black line scan. Respective scan lines positioned ~25 μm apart, colors correspond with lower trace. Blood flow is left to right. **Middle)** Representative trace of internal diameter at sites 1 and 10. **Bottom)** Representative traces of fluorescence responses (F/F_o) from each of the 10 line scans shown in the video. Arteriolar diameter and fluorescence responses to ACh play back simultaneously in real time.

Supplemental Figure 1: Screen shot of User Interface for DiaFluor Software. The superimposed numbers are defined in the following Guide.

Guide to DiaFluor User Interface Diagram

1. Run button
2. Allows for adjustment of the number of ROIs.
3. Adjustment for the number of frames (beginning at Frame 1) used to calculate Fo.
4. Drop-down menu for selecting a column from the Calibration Look-Up table (see #18)
5. Recalls ROIs from the previous program run.
6. Once the first ROI has been set, subsequent ROIs will have the same height and width.
7. Saves a .png file of the ROIs and final tiff image of the image stack to the parent folder.
8. Saves a .txt file of the data output from the program which includes fluorescence intensities and calculated Fo values.
9. Once an ROI is placed and adjusted so the left edge is parallel to the vessel wall, the user can set the ROI.
10. Adjustment of left outer diameter (OD) contrast and pixel controls.
11. Adjustment of right OD controls.
12. Adjustment of the line scan length as a percentage of the ROI height. Line scan only becomes visible when the program begins to run. Multiple trial runs may need to be performed, adjusting this control each time. Ultimately the line scan length should be slightly larger than the maximum vessel diameter to maximize the detected change in F/Fo.
13. Once OD parameters adjustments are complete, press this button to set.
14. Plot of diameter versus time for each analyzed image.
15. Plot of Fluorescence (F/Fo) versus time.
16. Legend for diameter and fluorescence plots according to ROI number.
17. ROIs placed, numbered and set prior to a run of the program.
18. Magnification, grab and ROI tool for adjusting image. To make an ROI, the user must use the ROI tool (selected (white background) in the representative screen shot)
19. Image info including the image resolution in pixels as well as bit depth.
20. Calibration look-up table (microns per pixel). User can preset up to three settings. Calibrations are determined by inserting the number of pixels that are present in the image (see #19) and the number of microns across the width of the image (determined using a stage micrometer at the same magnification). The linearity of the calibration in the vertical and horizontal directions will be determined by the linearity of the camera used.
21. Distances between ROI in microns.