

128 x 128 pixels  
or  
500 x 500 pixels

# Trlmager

500 x 500 pixels

*conventional  
frame scanning*

*interlaced  
frame scanning*

generate sequence of x-mirror command voltage values  
(128 or 500 lines with 128 or 500 steps each - bidirectional)

generate sequence of x-mirror command voltage values  
(50 lines with 500 steps each - bidirectional)

generate sequence of y-mirror command voltage values  
(128 or 500 steps)

generate 10 sequences of y-mirror command voltage values  
(50 steps each, each sequence shifted by 1/10 step size)

retrieve most recently used Pockels cell command value from ScanImage \*  
and generate sequence of Pockels cell command values  
(minimal - ScanImage value - minimal)

## I/O channel initialization

setup photomultiplier tube (PMT) analog input channel  
(start synchronized to start of Pockels cell command)

setup 10 photomultiplier tube (PMT) analog input channels  
(start synchronized to start of Pockels cell command)

setup scan mirror control analog output channels  
(start synchronized to start of Pockels cell command)

setup Pockels cell control analog output channel

### setup digital input channel to monitor ECG for triggering

setup digital input channel to monitor breathing

setup digital output channel to control shutter

generate index map used to assign binned data to individual image pixels

## start imaging

open shutter

FOR ... number of time points in time series

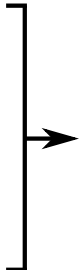
move to starting z-position

FOR ... number of z-sections (if z-stack)

Activate analog input channel and mirror control output channels  
(will start together with Pockels cell command)

**stay in WHILE loop while digital ECG input is "low"  
(OR digital breathing input is "high" - optional)**

start Pockels cell analog output  
(simultaneously starts all analog channels -  
scanning and acquisition takes place)



10 cycles  
(one for each subframe)

## data processing

IF z-stack: new z-position

data binning and assignment to pixels  
(accelerated through compiled MATLAB Extension (MEX) files written in C)

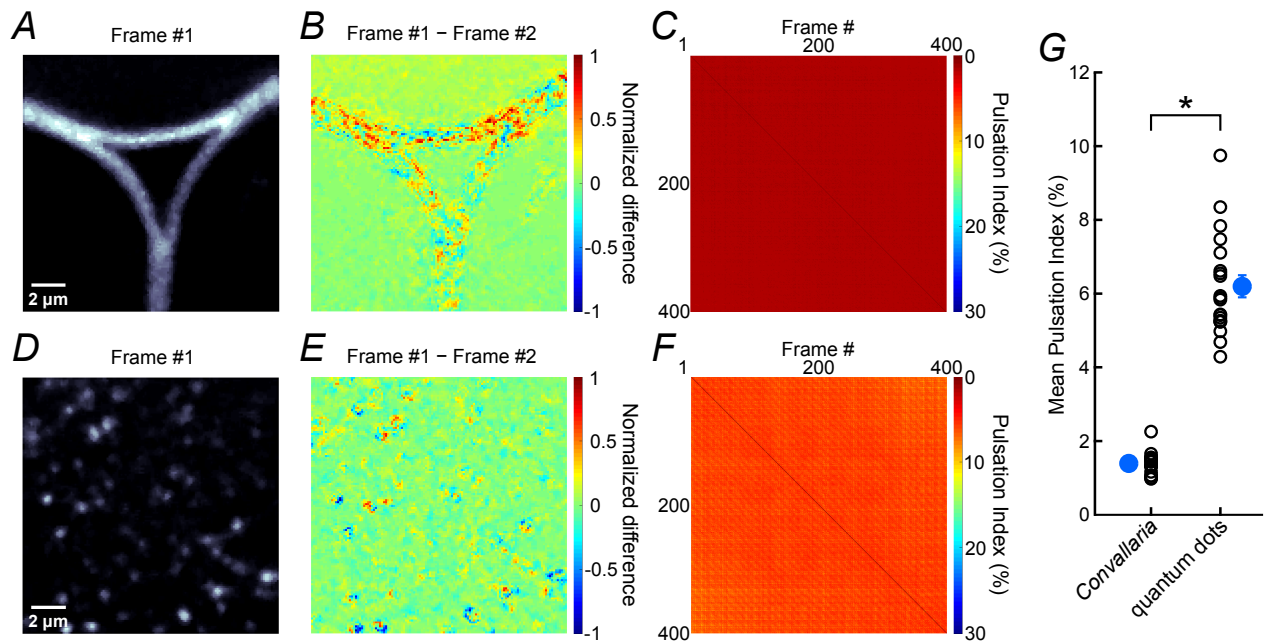
END ... FOR loop (z-stack)

END ... FOR loop (time series)

clean-up I/O channels

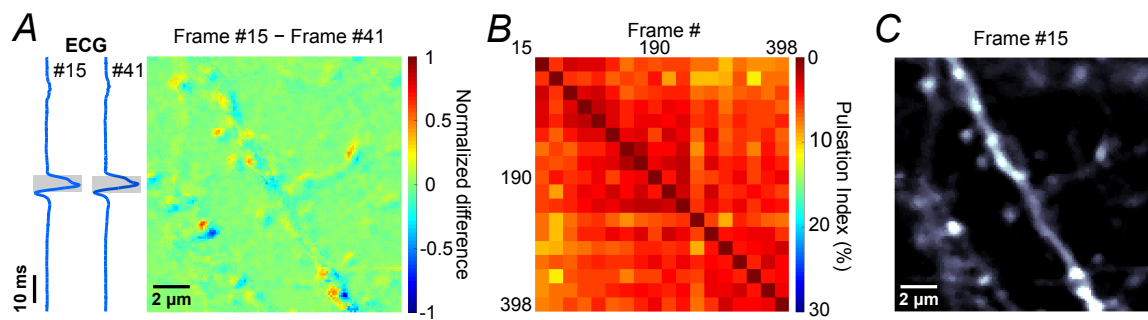
save data as .mat file (imaging data in variable "GUI.data.Channel1" and information about chosen protocol)

\* This is the only step that requires having run ScanImage before using Trlmager

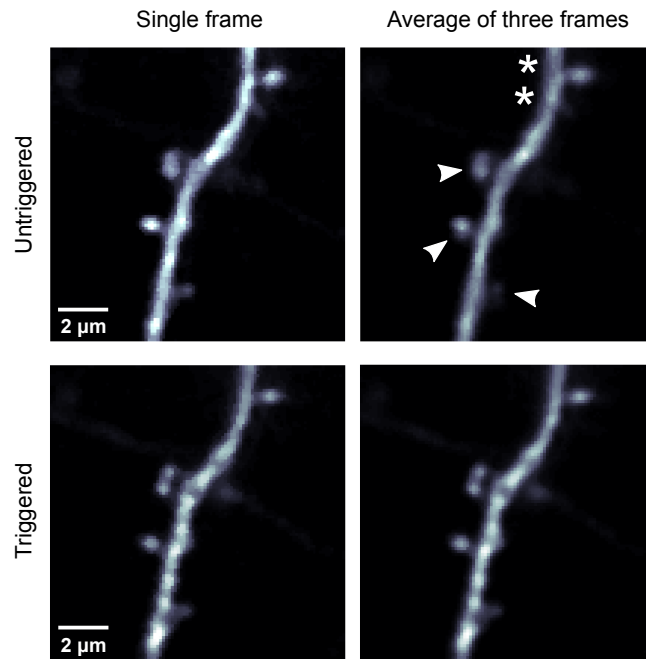


**Supplementary Figure 2. Impact of image complexity on the calculated correlation coefficient**

A, Fluorescence image of a portion of *Convallaria majalis*. B, Pseudocoloured difference image obtained by subtracting two consecutive images. Values were normalized to the largest absolute difference. C, Plot of PIs of image pairs for 400 consecutive images. (D-F), Same analysis as described for (A-C); performed using quantum dots. G, Plot of the average PI measured for *Convallaria majalis* and quantum dots. Blue symbols, mean  $\pm$  SEM; \*,  $P = 1 \times 10^{-16}$  (Student's  $t$  test).



**Supplementary Figure 3. Synchronizing scanning to the cardiac cycle is sufficient to reduce motion artifacts**  
 A, Pseudocoloured difference image obtained by subtracting two frames collected during the same phase of the cardiac R-wave, normalized to largest absolute difference determined in Figure 1A. Blue traces at left show the simultaneously recorded ECG signal. Grey boxes highlight the search window for the cardiac R-wave. B, Plot of PIs for image pairs in which acquisition serendipitously occurred when the R-wave occurred during the middle of the scan. This condition was met by 18 of 400 frames. C, Representative fluorescence image (#15) from the 400 frames collected.



**Supplementary Figure 4. ECG-triggered scanning reduces distortions caused by frame averaging**

Comparison of single frame images of a single dendritic shaft to averages of three consecutive frames from the same FOV. Averaging images without ECG-triggered acquisition resulted in blurring of several dendritic spines (highlighted by arrowheads) and a portion of the shaft (highlighted by asterisks). Note that from the averaged image alone it is not possible to determine which components were distorted.