SUPPORTING INFORMATION

# Optical Switching Between Long-lived States of Opsin Transmembrane Voltage Sensors

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**Figure S1.** Absorption spectrum #7 of QuasAr2 measured in the cycle sequence shown in Figure S2 with no base-line corrections applied.

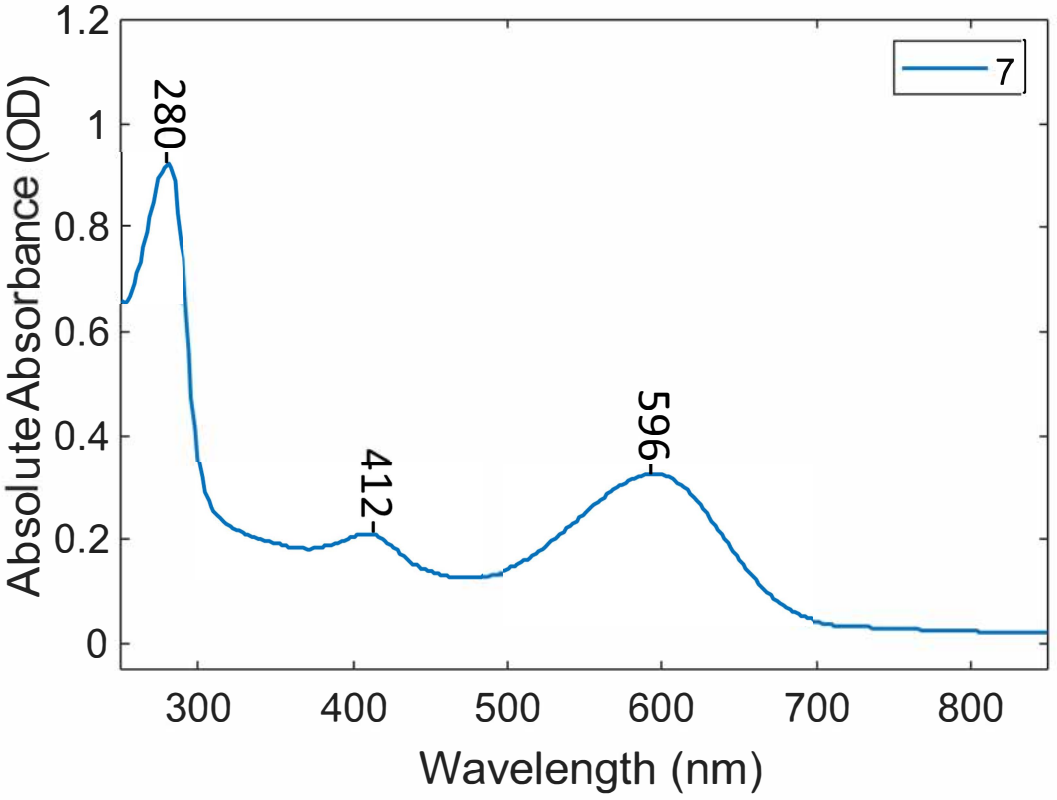
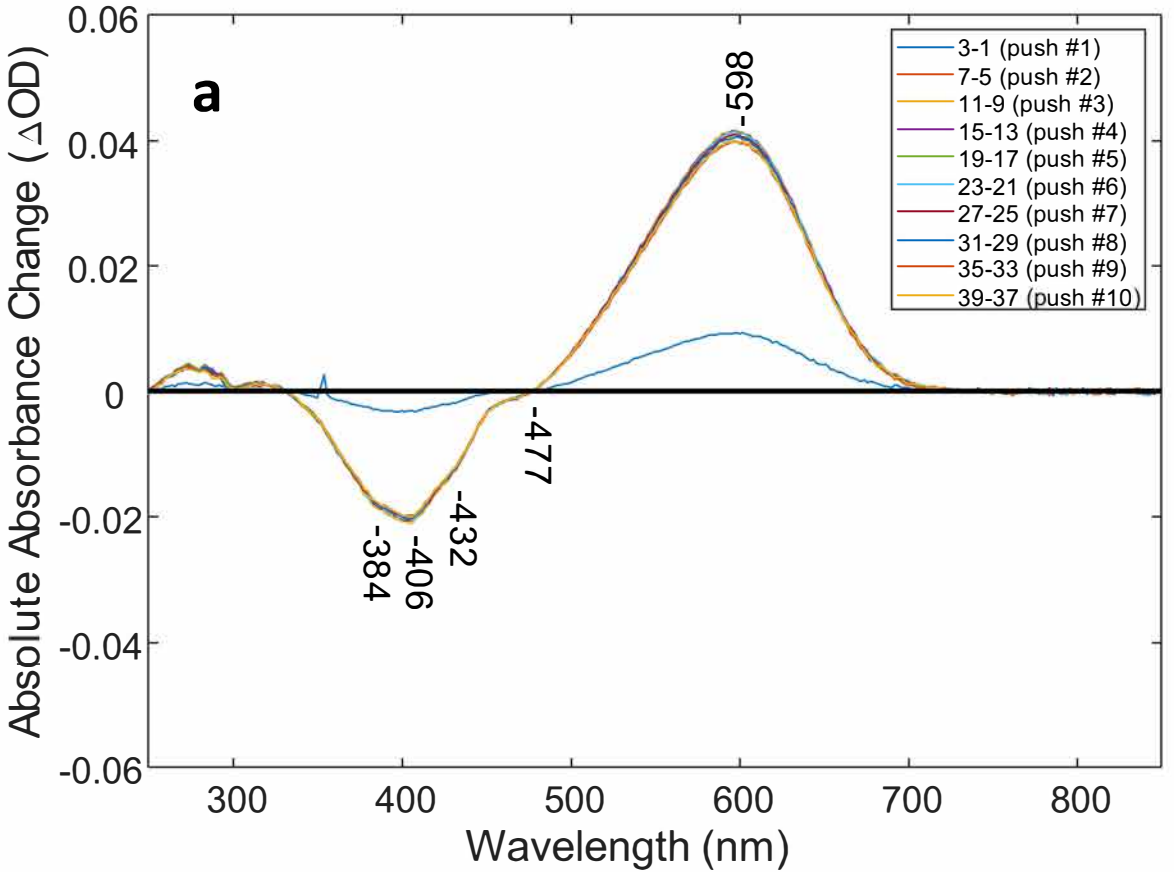


**Figure S2. Schematic showing the sequence of LED illumination used for switching between M-like and O-like states of QuasAr2 followed by 3 hours in dark.** The duration of the time for each measurement is listed in row 2 and illumination condition in row 3. After spectrum #12 was recorded the sample remained in dark for 3 hours (from scans #13 to #48). The columns show the number of the measured spectrum in the sequence.

| QuasAr2 Illumination Sequence | | | |
|---|---|---|---|
| 5min | 5min | 5min | 5min |
| **dark** | **405 LED** | **dark** | **660 LED** |
| 1 | 2 | 3 | 4 |
| 5 | 6 | 7 | 8 |
| 9 | 10 | 11 | 12 |
| **Dark for 3 hours from spectrum #13 to #48** | | | |

**Figure S3. Difference spectra of QuasAr2 cycled 10 times between O-like and M-like states.** Timing of individual steps in the cycle was similar to those shown in Figure S2 but extended to 10 cycles instead of 3. A) Base-line corrected differences obtained using 405 nm light for each of 10 cycles. Differences spectra shown are differences between spectra recorded in dark before and after 405 nm illumination (e.g. #3-#1, #7-#5 ….. #39-#37). The blue trace (#3-#1) reflects the lower level of M-like intermediate present when the sample was placed in the spectrometer compared to subsequent cycles. B) Same as A showing differences recorded in dark before and after 660 nm illumination (e.g. #5-3, #9-#7 …….#37-#35).



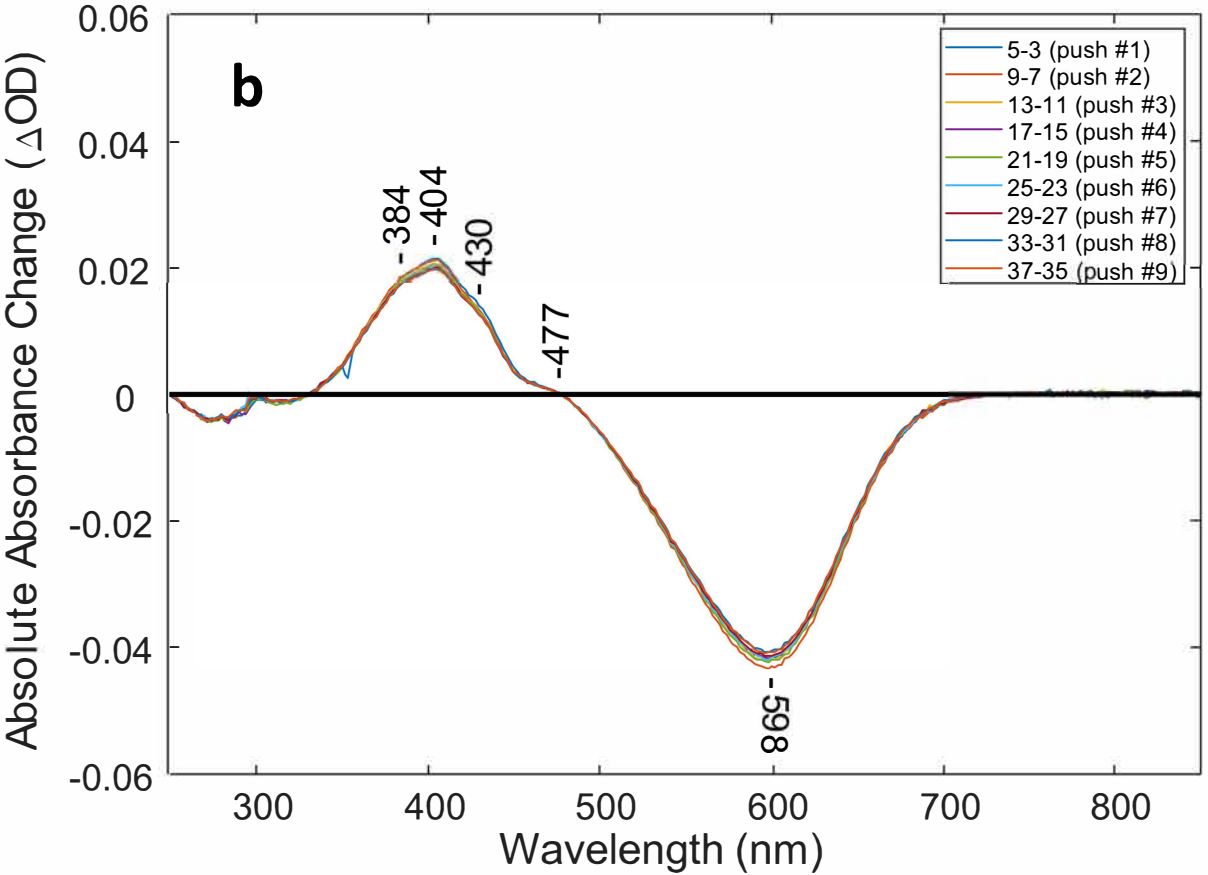
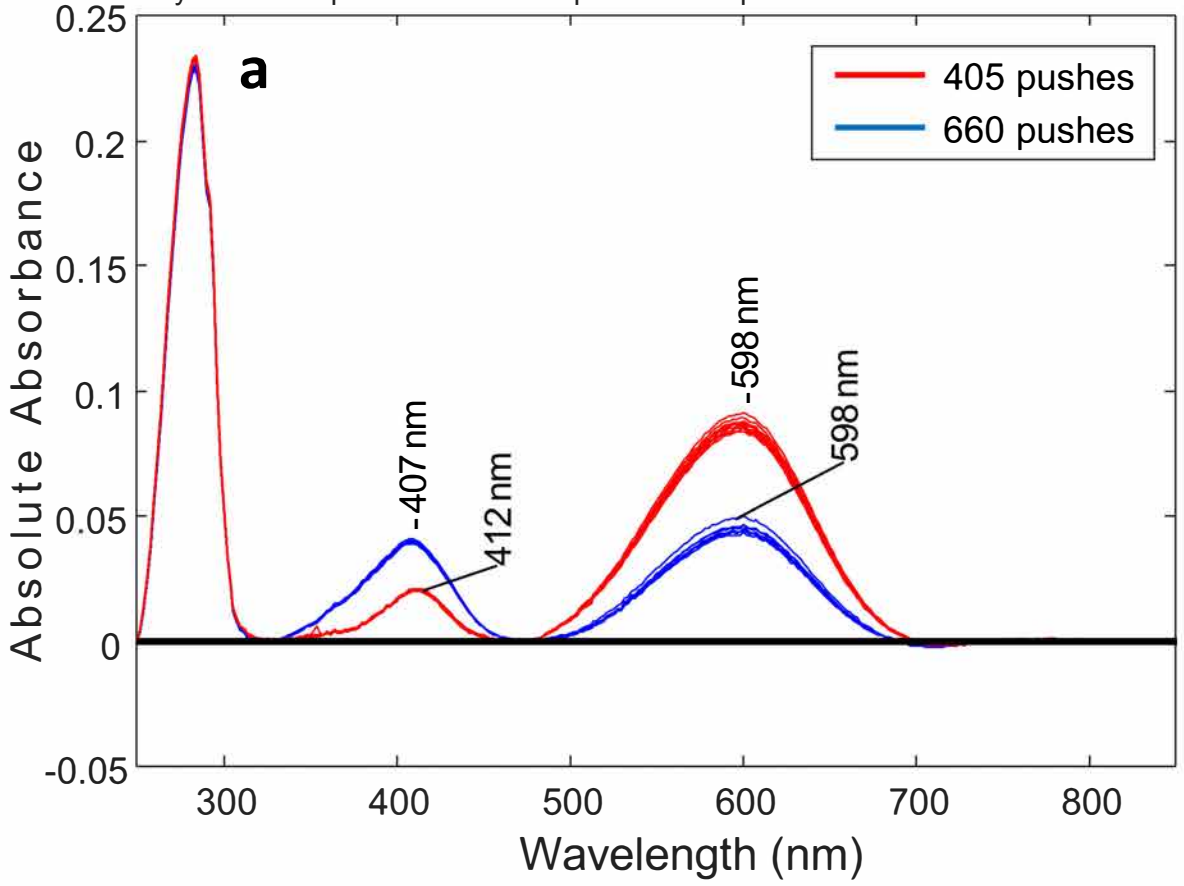



**Figure S4. Optical Switching of QuasAr2 during 10 cycles:** (A) Base-line corrected absolute absorption spectra recorded after 405 nm illumination (red traces) and 660 nm illumination (blue traces). (B) Cycling fraction (CF) of QuasAr2 for each individual LED illumination in 10 cycles. Each point is calculated as ratio of change of peak OD (ΔOD) between spectrum measured before and after illumination to the absolute OD measured before illumination at wavelength of maximum amplitude of difference spectra (404 and 598 nm (see Figures S3A,B)). Points shown correspond to CF calculated for 405 nm illumination (blue square) or 660 nm illumination (red square) for a particular cycle (x-axis). Note that in the first and last cycle the CF points that are not part of the equilibrium are not included.



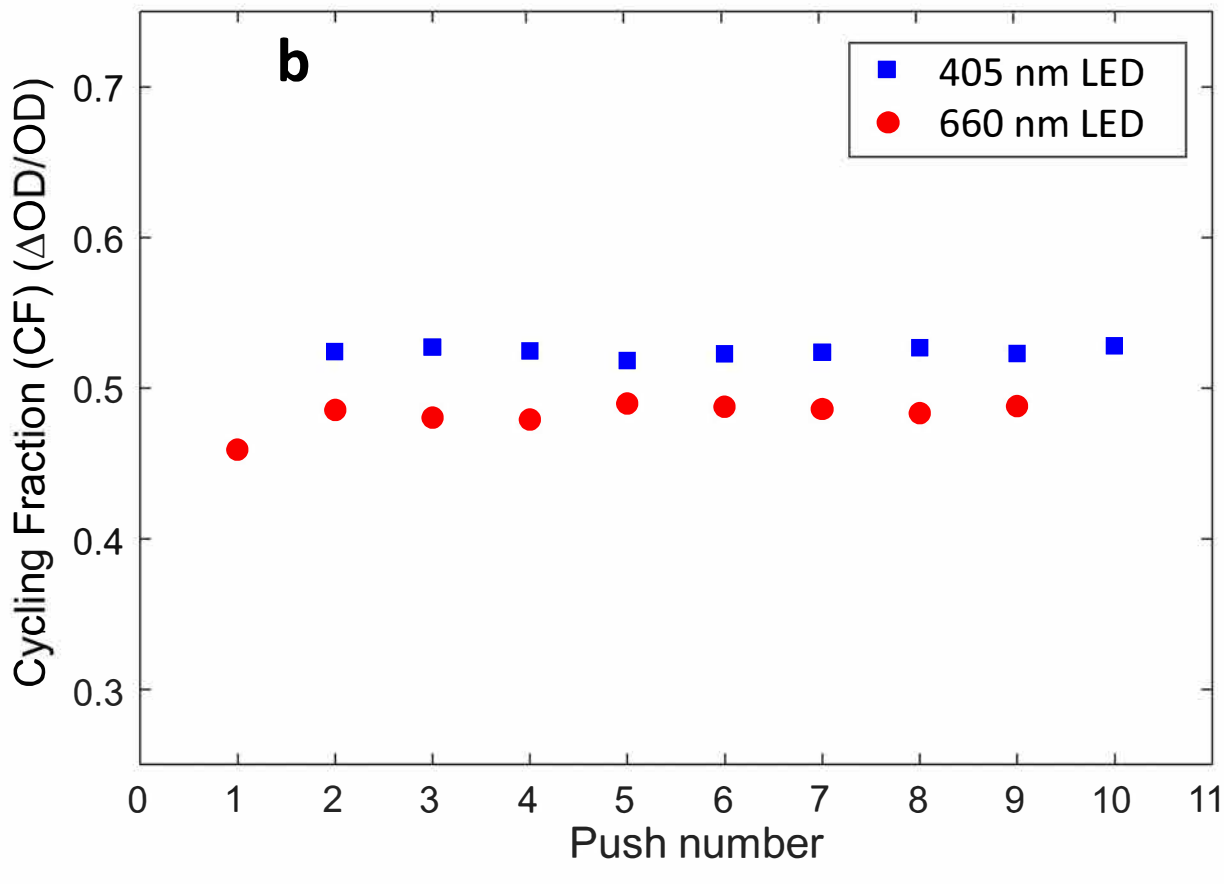
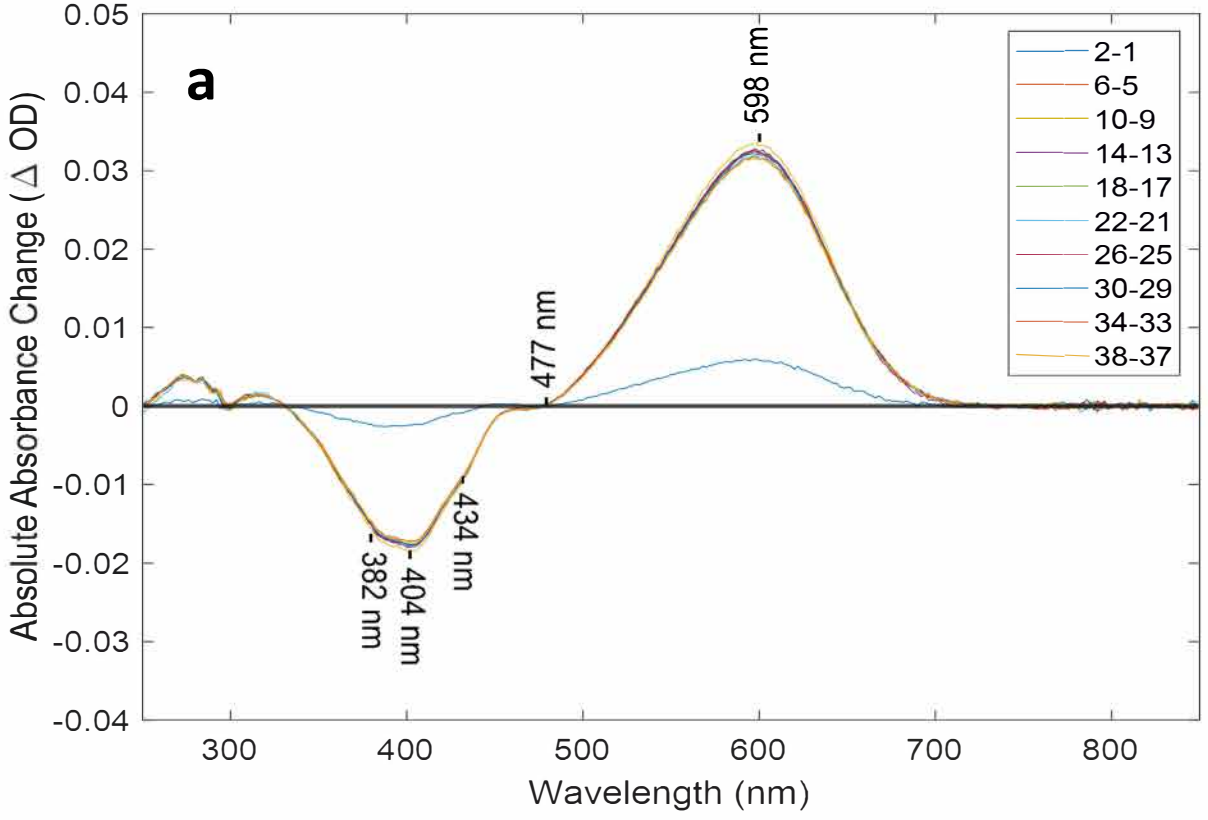
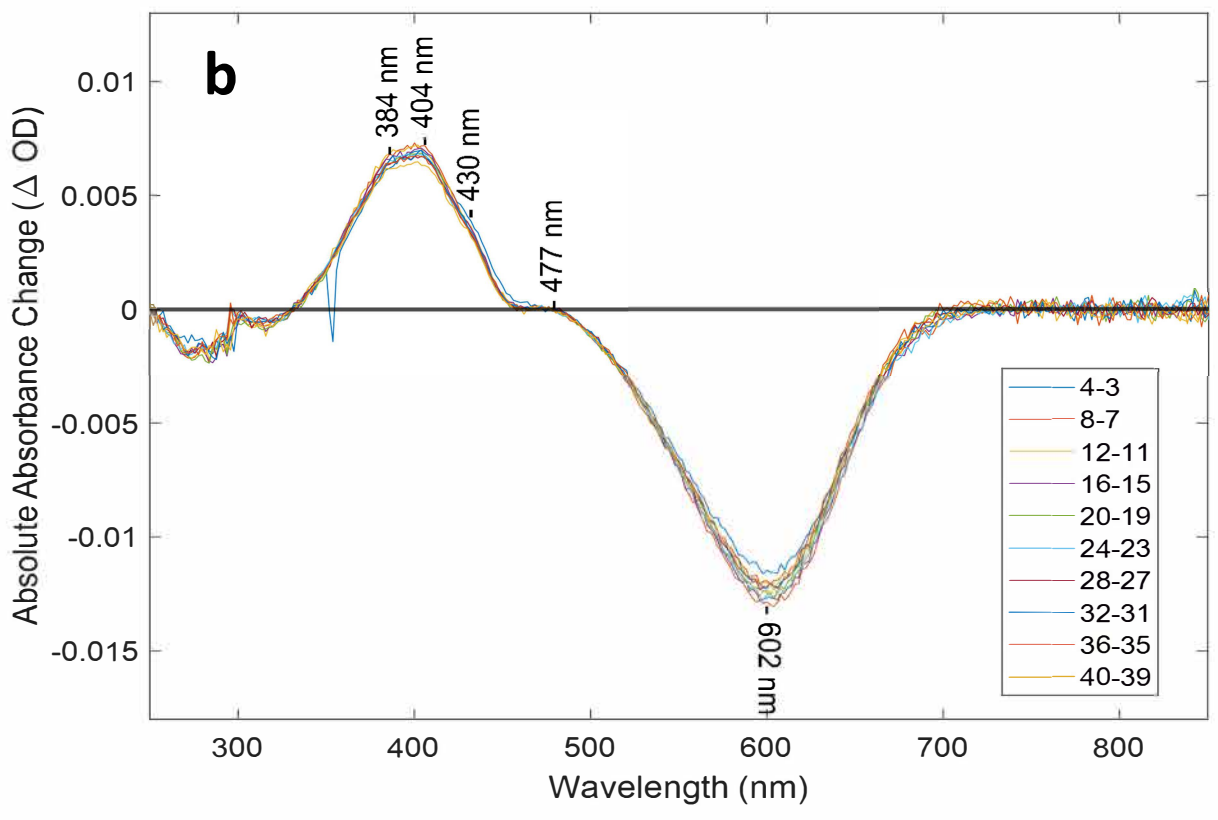



**Figure S5. Difference spectra of QuasAr2 cycled 10 times between O-like and M-like states measured during illumination.** In contrast to Figures S3A and B, differences reflect measurements between dark and illuminated samples. Timing of individual steps in the cycle was similar to those shown in Figure S2 but extended to 10 cycles instead of 3. A) Base-line corrected differences obtained using 405 nm light for each of 10 cycles. Differences spectra shown are differences between spectra recorded in dark before and during 405 nm illumination (e.g. #2-#1, #6-#5 ….. #38-#37). The blue trace (#2-#1) reflects the lower level of M-like intermediate present after sample is place in spectrometer compared to subsequent cycles. B) Same as A showing differences recorded in dark before and during 660 nm illumination (e.g. #4-#3, #8-#7 …….#40-#39).

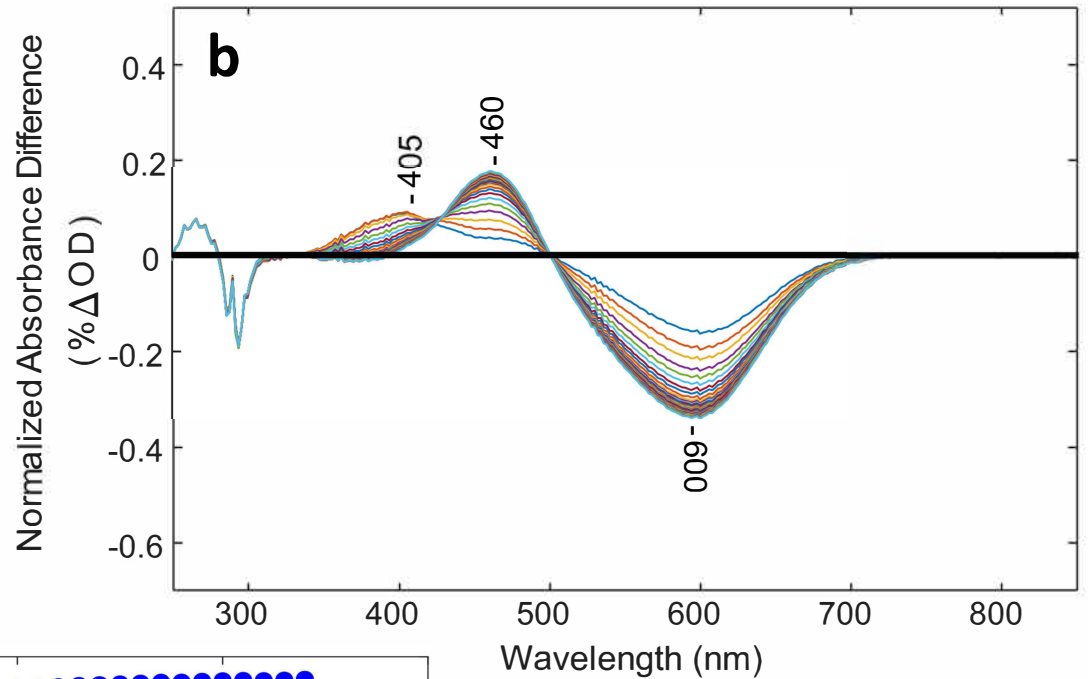


**a** QuasAr2 Extended Red-light Sequence

| 5min | 5min | 5min | 5min |
|---|---|---|---|
| dark | 405LED | dark | 660 LED |
| 1 | 2 | 3 | 4 |
| 5 | 6 | 7 | 8 |
| | ... | | |
| 37 | 38 | 39 | 40 |

**Red-light illumination for 3 hours (from scans #41 to #76)**



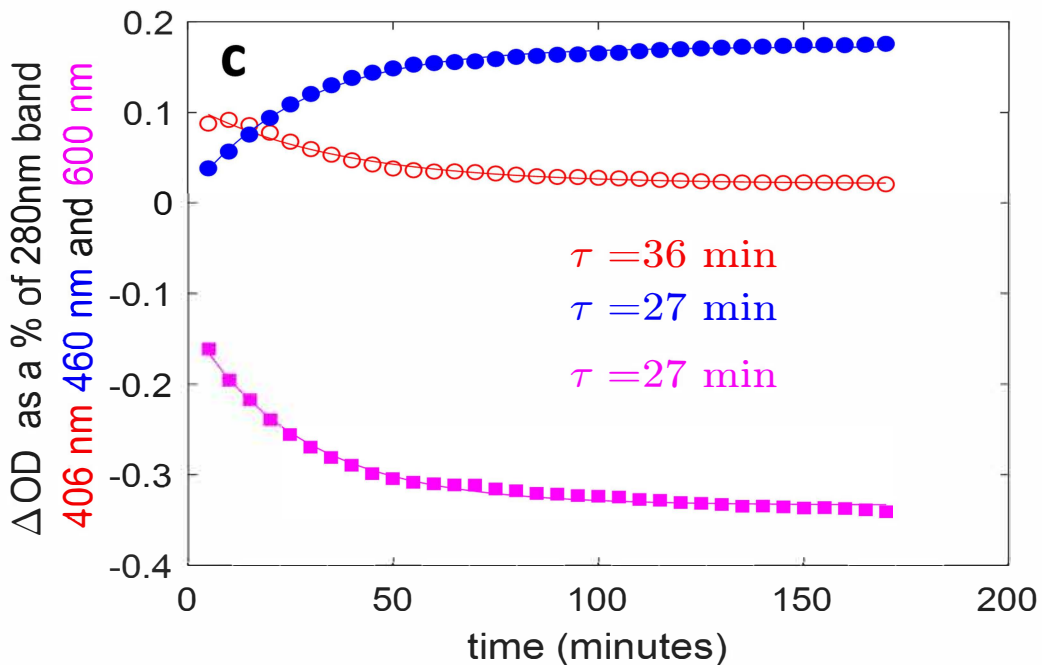




**Figure S6.** (A) Schematic showing the sequence of LED illumination used for switching between M-like and O-like states of QuasAr2 followed by 3 hours of measurements during red-light illumination. Note in contrast to 3 cycles of illumination used for data shown in Figure 2B, 10 cycles were used. Each measured spectrum is sequentially numbered. In addition, 4 times (400%) intensity was used for 660 nm illumination compared to the experiment described in Figure S2. The duration of the time for each measurement is listed in row 2 and illumination condition in row 3. The sequence shown was continued up to spectrum #40. After spectrum #40 was recorded the sample remained under the 660 LED illumination for 3 hours (from scans #41 to #76). The columns show the number of the measured spectrum in the sequence. (B) Difference absorption spectra of QuasAr2 during prolonged-light (660 nm) illumination. Base-line corrected difference spectra correspond to protocol shown in Figure S6A. Light blue trace with lowest amplitude negative band at 600 nm corresponds to difference (#41-#39) where red light was on during #40 and #41. Subsequent differences while red light is on [(N-#42 to #76) minus #39] show increasing loss of the 600 nm band as the O-like state is depleted and increase of the 460 nm band as the pink membrane species is formed up to difference (#76-#39). All spectra are scaled to 280 nm band in the absolute absorption spectrum. (C) Kinetics of pink membrane formation: Time course of the difference peak amplitudes in Figure S6B measured at 406 nm (red circles), 460 nm (blue solid dots) and 600 nm, (pink squares) as a percentage of the 280 nm band reflecting decay of the O-like and M-like species and formation of a species absorbing near 460 nm (pink membrane). Time constants were calculated using a single-exponential function (see Materials and Methods).

**Figure S7: Base-line Corrected Difference absorption spectra of NovArch during extended red-light (660 nm) illumination using protocol shown in Figure S6A.** Light green trace with lowest amplitude negative band at 581 nm corresponds to difference (#41-39). Subsequent differences show increasing loss of the 581 nm band up to difference (#76-39). All spectra are scaled to the 280 nm band in absolute absorption spectrum. Linear 2-point correction was used (334 nm and 800 nm) for base-line correction.

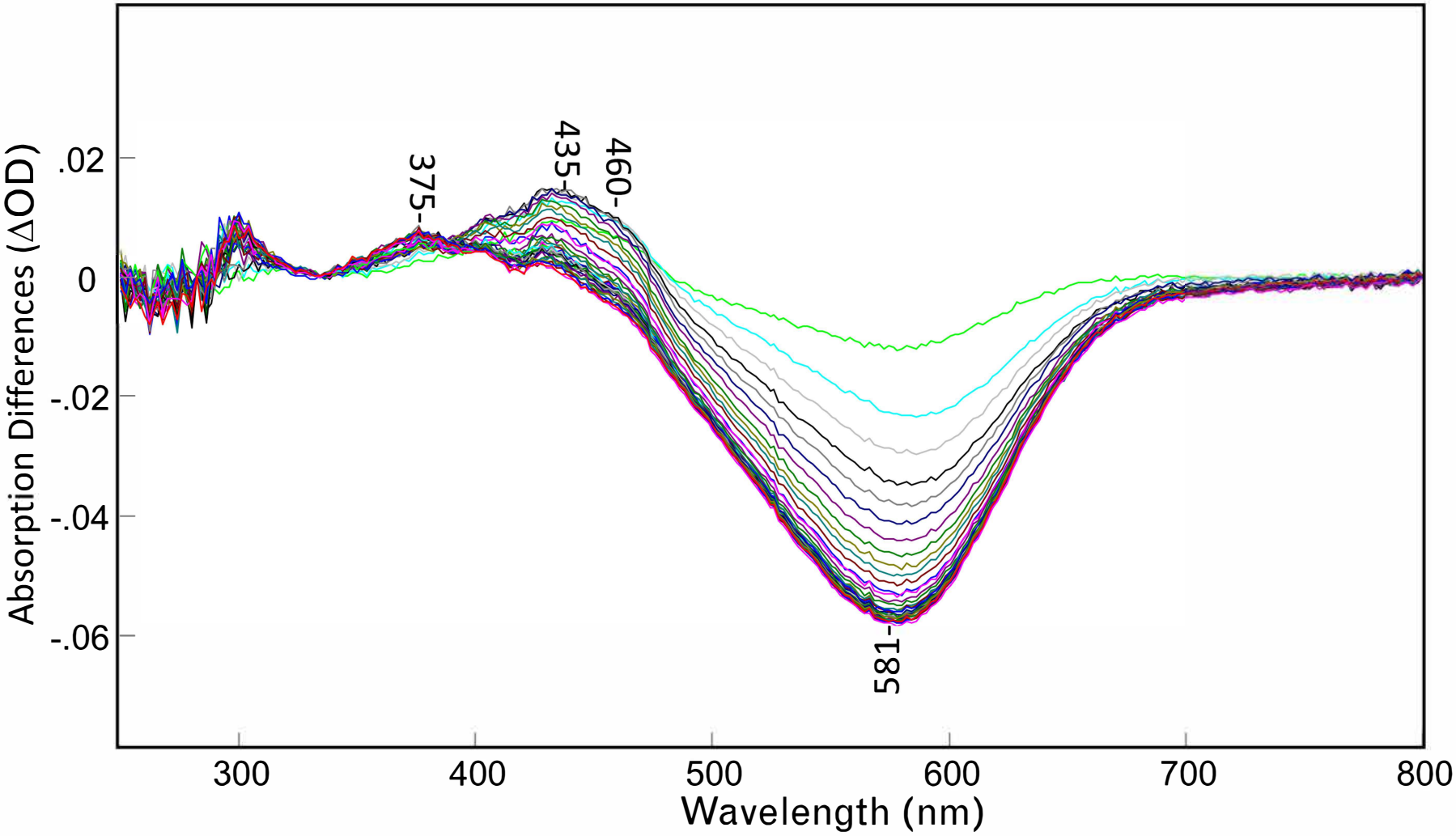
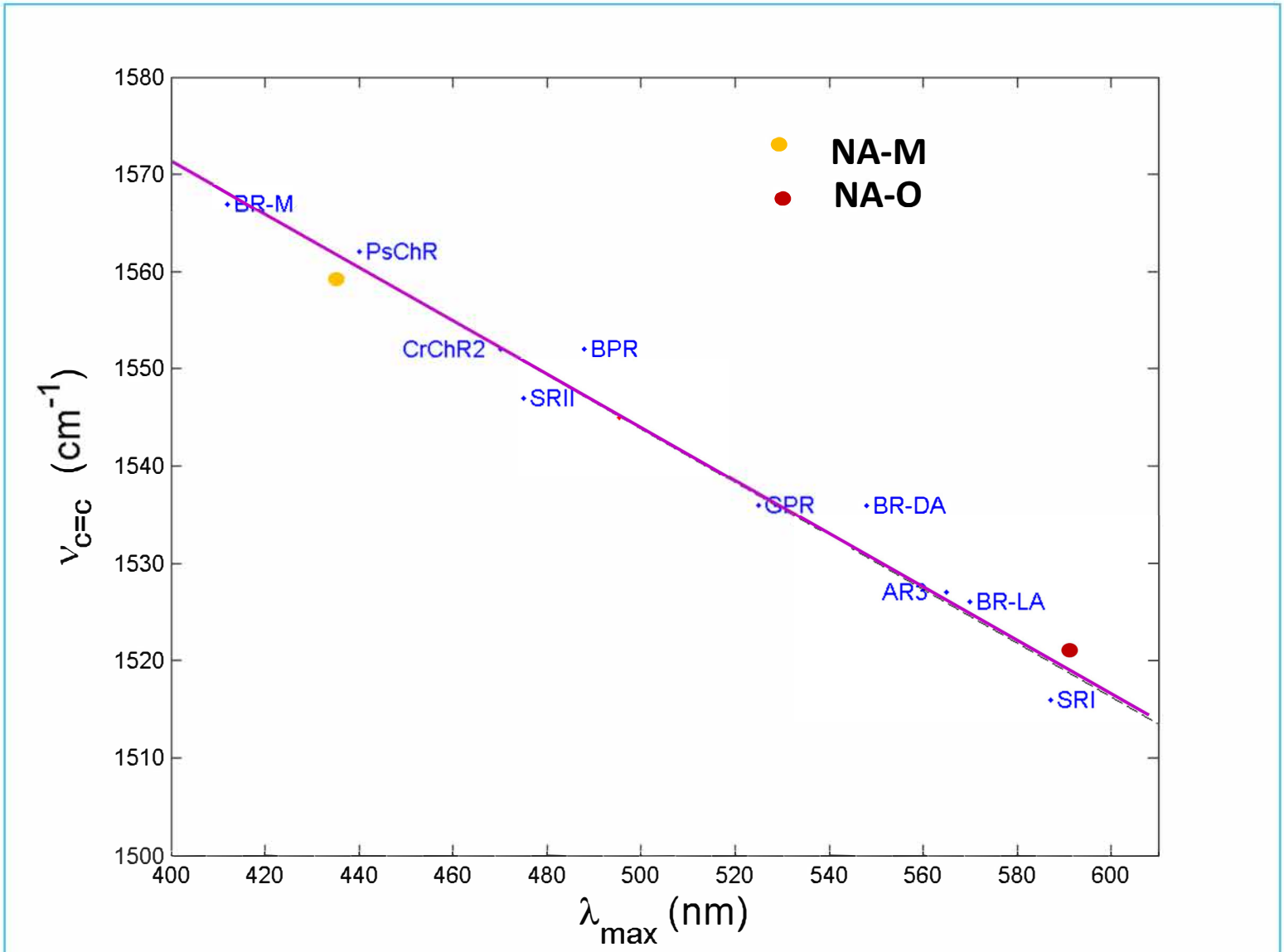
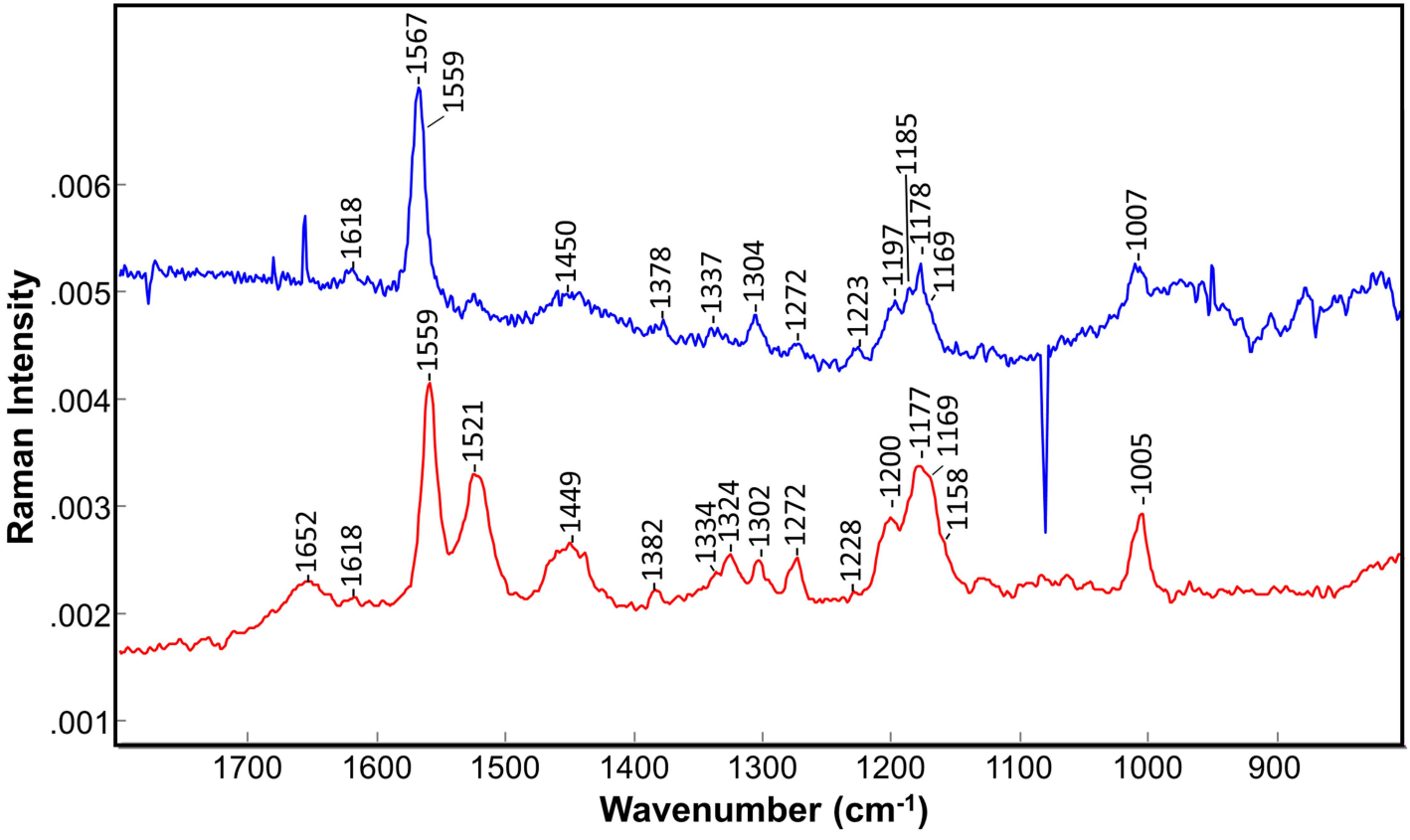


**Figure S8.** Inverse linear correlation plot (purple line) between ethylenic frequency and visible absorption wavelength maximum for various microbial rhodopsins (small blue dots and accompanying blue colored labels along with the FT-Raman measurement (Figure 5) for assigned ethylenic frequency for NovArch M-like (amber dot) and O-like (red dot) species). Adapted from reference (24), supplementary Figure S6 (see accompanying caption for further information).



# Figure S9


Raman Intensity
.006
.005
.004
.003
.002
.001
1700
1600
1500
1400
1300
1200
1100
1000
900
Wavenumber (cm-1)
1567
1559
1618
1450
1378
1337
1304
1272
1223
1197
1185
1178
1169
1007
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1521
1652
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1200
1177
1169
1158
1005

**Figure S9: Comparison of the light-dark resonance Raman difference spectrum recorded using 785-nm excitation recorded for the mutant BR-D96N in intact purple membrane at pH 7.8 (blue trace) and the NovArch FT-Raman spectrum shown in Figure 6 (red trace).** BR D96N exhibits a slowed M-decay at a pH above 7. The slowed M-decay causes an accumulation of the M-intermediate under steady-state illumination. The difference shown consists of a visual interactive subtraction of the BR-D96N recorded during white light illumination and under dark conditions such that contributions from the resonance Raman spectrum of the BR-D96N in the dark (but still light adapted) are neutralized. Y-scale is for NovArch spectrum.