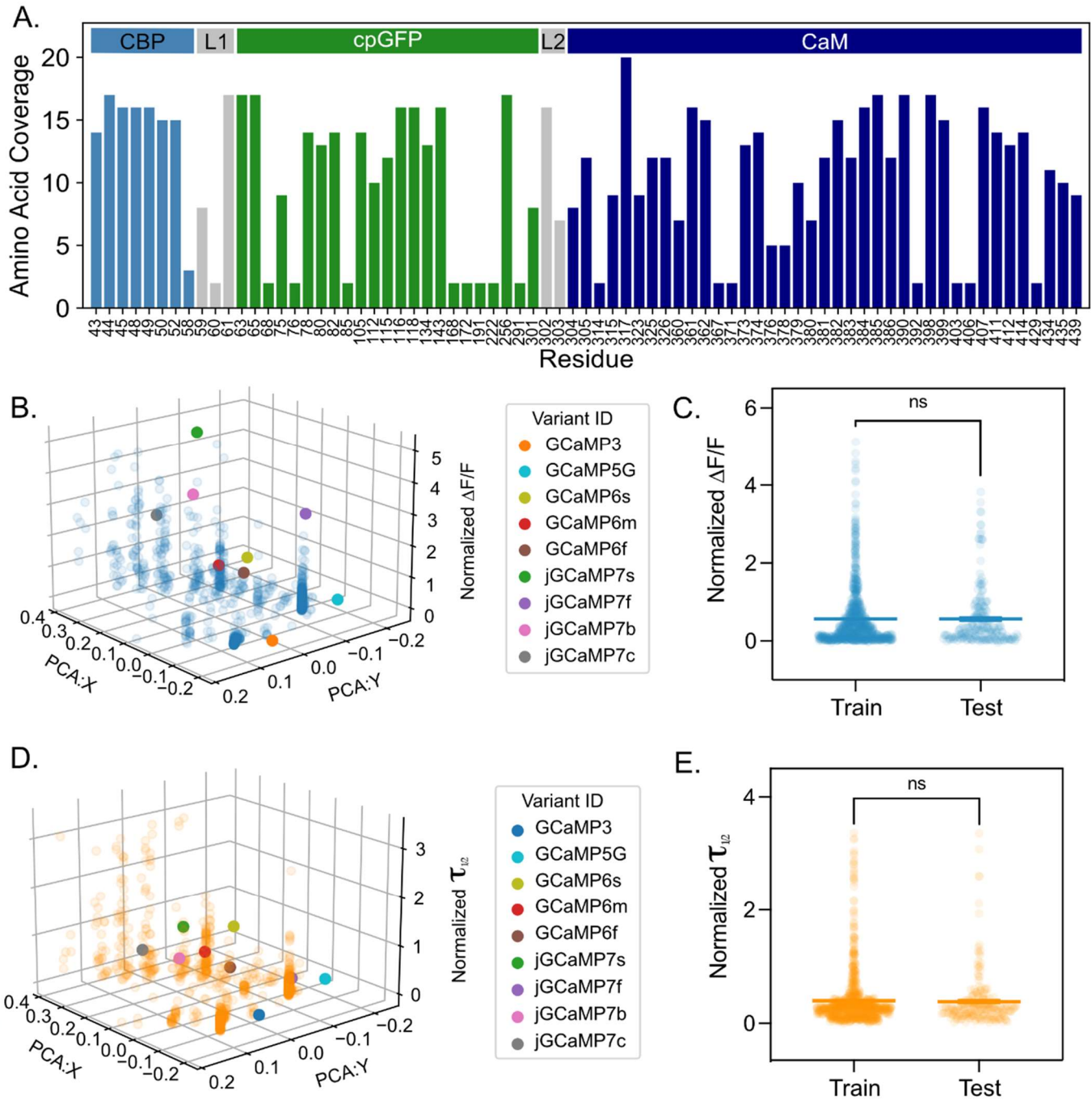


Machine learning-guided engineering of genetically encoded fluorescent calcium indicators

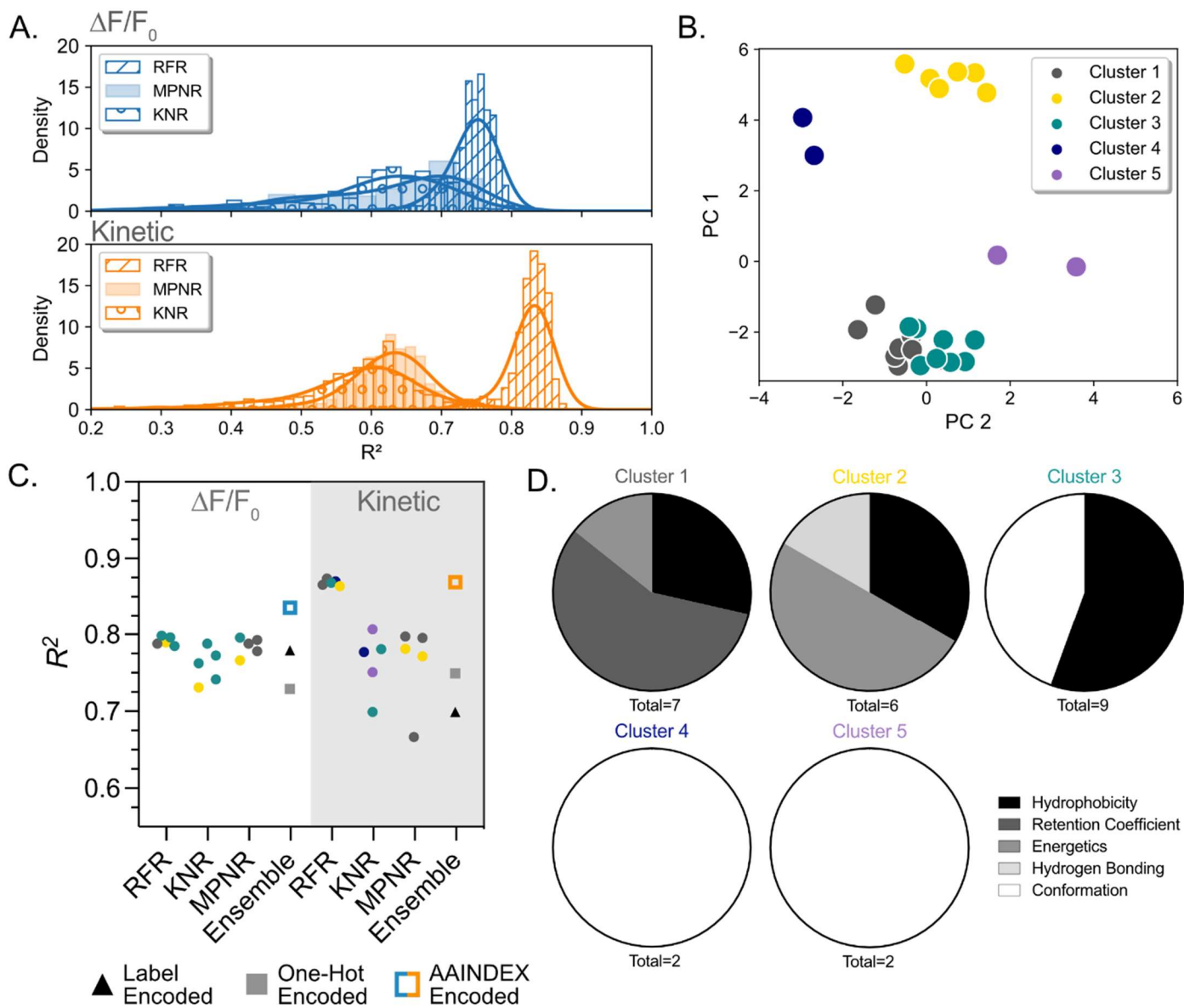
In the format provided by the authors and unedited



Supplementary Figure 1: *Mutation Scope of Chen & Dana dataset & Train/Test Breakdown*

A. Bar plot depicts the number of tested amino acids for each residue in the full variant library. Color coding indicates the location within the GCaMP protein, light blue indicates the residue is in the CBP, gray indicates the residue is in one of the two linkers, green indicates the residue is in the cpGFP, and dark blue indicates the residue is in the CaM (x-axis denotes residue number, bar height indicates # of amino acids).

- B. 2D principal component analysis (PCA) of sequences contained in the full variant library with the third dimension displaying the normalized $\Delta F/F_0$ of each variant. Published variants are included as differentially colored dots, indicated in the legend.
- C. Span of Normalized $\Delta F/F_0$ values in the train set (n=862) and the test set (n=216). Each dot indicates one variant, where the line designates the mean and error bars SEM. Average values and distribution did not differ between the two sets. (ns = P-value>0.05, unpaired t-test, two-tailed)
- D. 2D PCA of sequences contained in the full variant library with the third dimension displaying the normalized $\tau_{1/2}$ of each variant. Published variants are included as differentially colored dots, indicated in the legend.
- E. Span of Normalized $\tau_{1/2}$ values in the train set (n=862) and the test set (n=216). Each dot indicates one variant, where the line designates the mean and error bars SEM. Average values and distribution did not differ between the two sets. (ns = P-value>0.05, unpaired t-test, two-tailed)

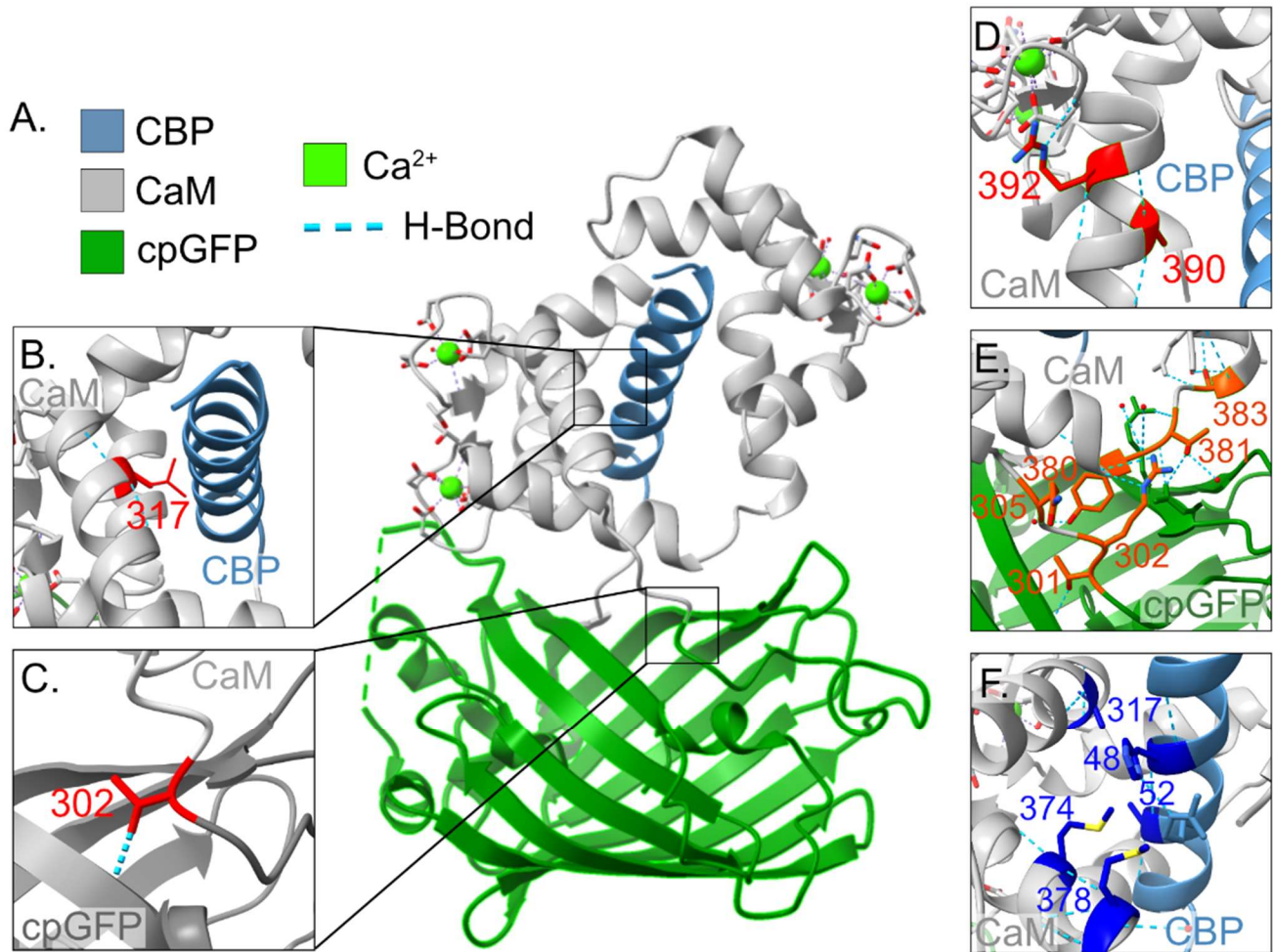


Supplementary Figure 2: $\Delta F/F_0$ and Kinetics Ensembles Display Amino Acid Property Preference

- Kernel density estimates depict the range of the max R^2 values saved for each of the 554 amino acid property datasets optimized for both ensembles. Bar patterning designates regressor type. The top five performing property datasets were advanced for downstream analysis.
- Principal component analysis of the values in the top datasets from each ensemble (2x15 datasets). (number of components = 4, number of clusters = 5).
- Scatter plot of R^2 values from the top five performing amino acid matrices for each regressor type within each ensemble. Color mapping is indicative of PCA cluster identity (B.). Ensemble R^2 indicates the final R^2 value of predictions from each contributor model after averaging for the indicated encoding method.

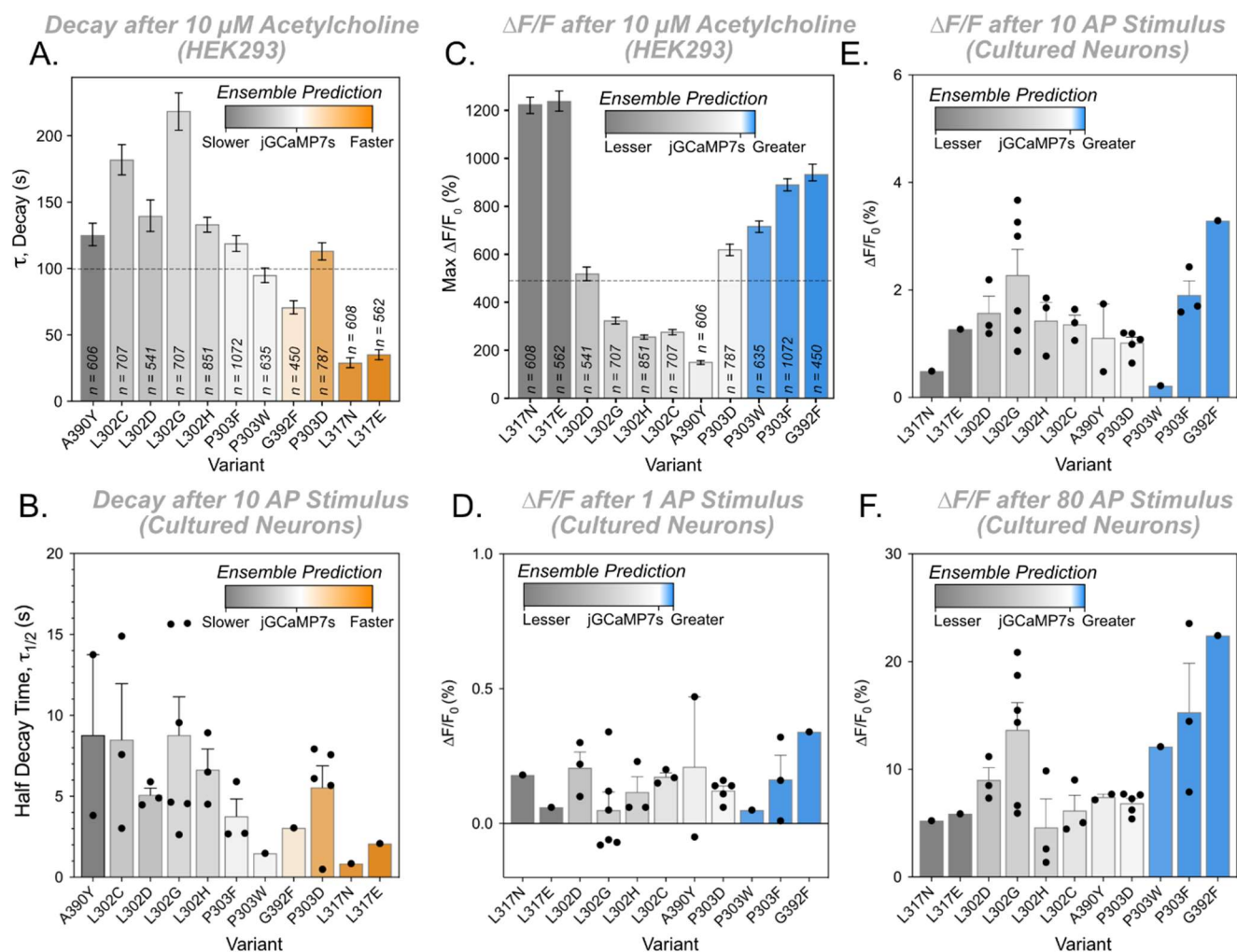
Models belonging to the $\Delta F/F_0$ library are plotted on a transparent background, and models belonging to the kinetic library are plotted on an opaque background.

- D. Pie-charts depict the amino acid properties that were found in each PCA cluster. Total indicates the number of property matrices within the cluster. Name and name color coordinate with B./C.



Supplementary Figure 3: *In Silico* Predictions Indicate Key Residues and Interactions Within GCaMP Protein

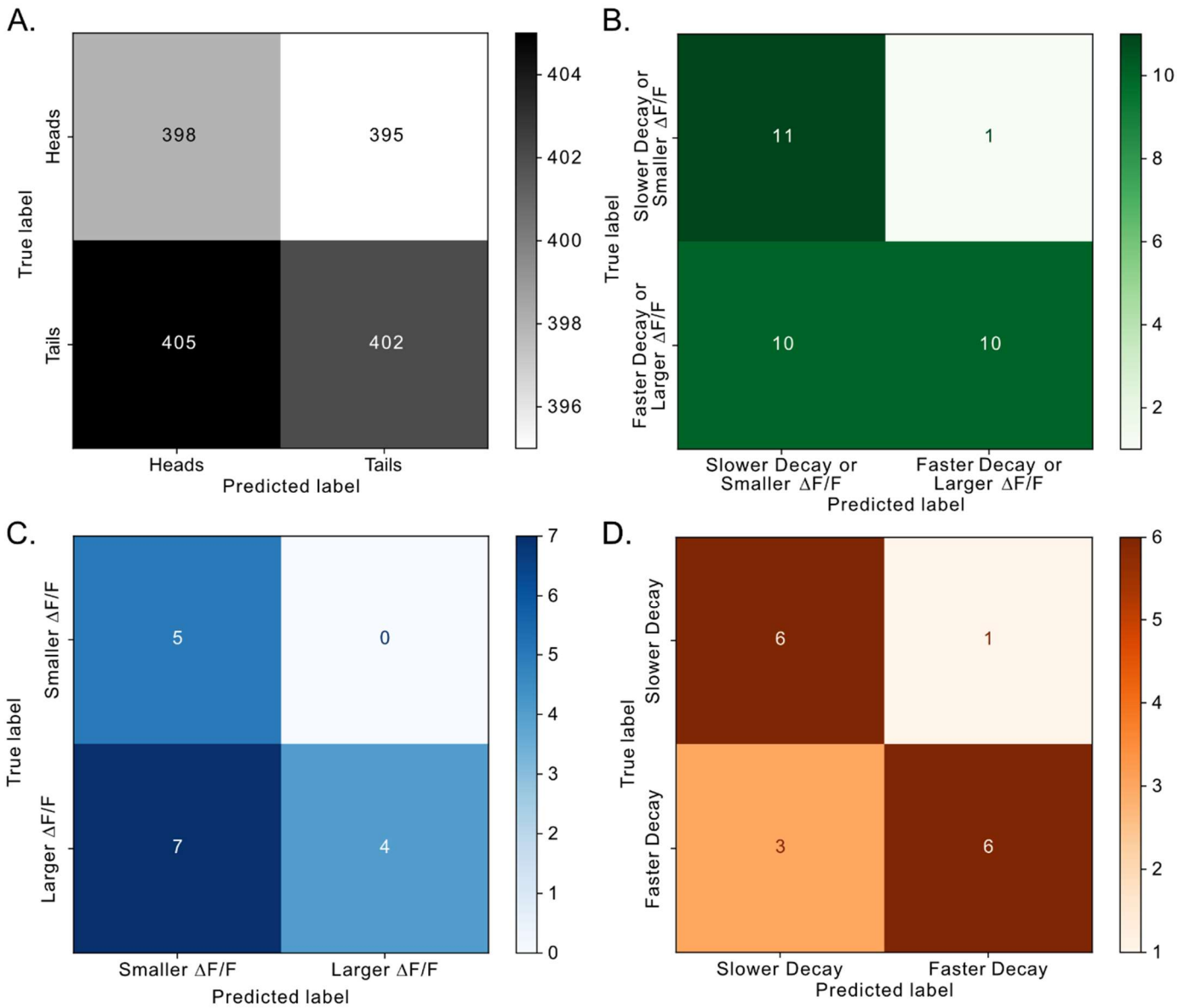
- A. Crystal structure of GCaMP3 D380Y (RCSB: 3SG3), with color mapped CaM (gray), CBP (light blue), cpGFP (dark green), Ca²⁺ (lime green), and hydrogen bonds (light blue dashed lines).
- B. Residue 317 (red) on the interface of CaM (gray) and CBP (light blue).
- C. Residue 302 (red), on linker between CaM (light-gray) and cpGFP (dark-gray), with hydrogen bonds (light blue dashed lines).
- D. Residue 390 (red, left), interfacing with the EF-hand motif, and residue 392 (red, right) interfacing with CBP (light blue), with color-mapped CaM (gray).
- E. Representative image of residues 301, 302, 305, 380, 381, and 383 (dark orange) proximity on the crystal structure with color-mapped CaM (gray) and cpGFP (dark green).
- F. Representative image of residues 48, 52, 317, 374, and 378 (dark blue) proximity on the crystal structure with color-mapped CaM (gray) and CBP (light blue).



Supplementary Figure 4: Comparison of Single Point Mutation Results in HEK293 and Cultured Neuron Screens

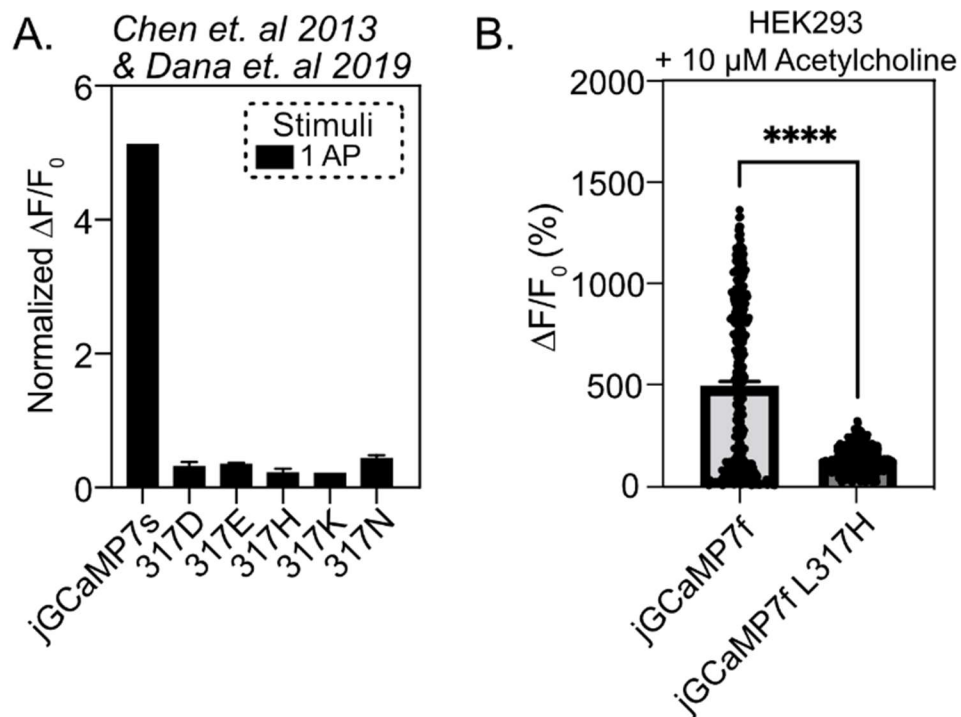
- Decay values (τ , tau, Eq.4) obtained from each mutant of jGCaMP7s expressed in HEK293 cells and stimulated with ten μM acetylcholine. Heat mapping indicates the ensemble's prediction. Mutations are sorted in order of the ensemble's predicted performance. Dotted line depicts mean performance of the base construct, jGCaMP7s. (n = number of cells quantified; bars depict mean + bootstrapped 95% CI).
- Half decay times of each variant of jGCaMP7s expressed in primary cortical neurons and stimulated with 10 APs. Heat mapping indicates the ensemble's prediction. Mutations are sorted in order of the ensemble's predicted performance. (Dots depict cells quantified; bars depict mean + bootstrapped 95% CI).
- Max fluorescent responses (Eq. 1) were obtained from each mutant of jGCaMP7s expressed in HEK293 cells and stimulated with ten μM acetylcholine. Heat mapping indicates the ensemble's prediction. Mutations are sorted in order of the ensemble's predicted performance. Dotted line depicts mean performance of the base construct, jGCaMP7s. (n = number of cells quantified; bars depict mean + bootstrapped 95% CI).
- Max fluorescent responses (Eq.1) of each variant of jGCaMP7s expressed in primary cortical neurons and stimulated with 1 AP. Heat mapping indicates the ensemble's prediction. Mutations are sorted in order of the ensemble's predicted performance. (Dots depict cells quantified; bars depict mean + bootstrapped 95% CI).

- E. Max fluorescent responses (*Eq. 1*) of each variant of jGCaMP7s expressed in primary cortical neurons and stimulated with 10 AP. Heat mapping indicates the ensemble's prediction. Mutations are sorted in order of the ensemble's predicted performance. (Dots depict cells quantified; bars depict mean + bootstrapped 95% CI).
- F. Max fluorescent responses (*Eq. 1*) of each variant of jGCaMP7s expressed in primary cortical neurons and stimulated with 80 AP. Heat mapping indicates the ensemble's prediction. Mutations are sorted in order of the ensemble's predicted performance. (Dots depict cells quantified; bars depict mean + bootstrapped 95% CI).



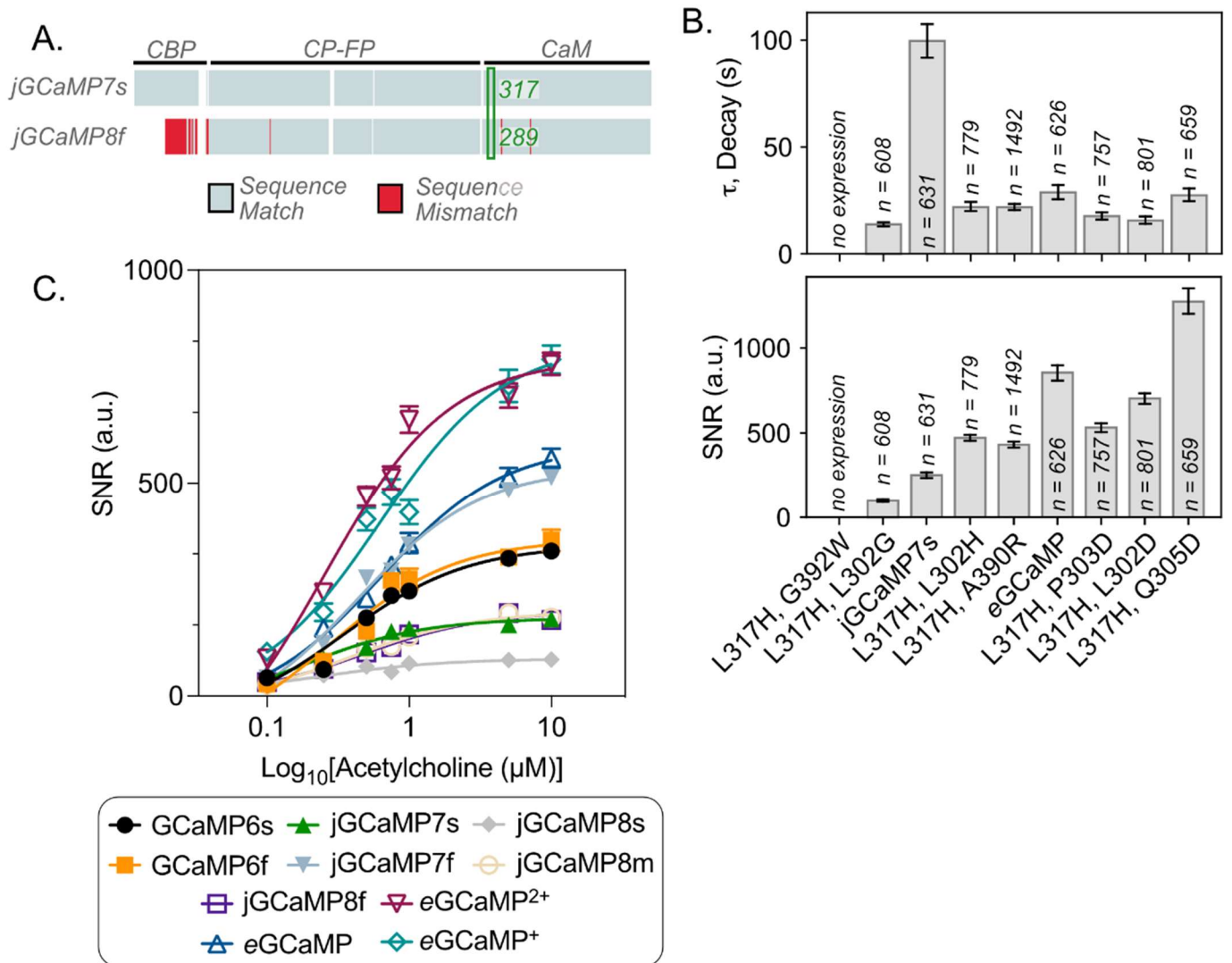
Supplementary Figure 5: *Estimation of Model Accuracy with Acetylcholine Results.*

- A. Confusion Matrix of 100 simulated coin flip experiments (each containing 16 samples).
- B. Confusion matrix of both models' predictions and acetylcholine performance.
- C. Confusion matrix of all fluorescence model's predictions and acetylcholine performance.
- D. Confusion matrix of all kinetics model's predictions and acetylcholine performance.



Supplementary Figure 6: *Retroactive Assessment of Ensemble Predictions Compared to In Vitro Behaviors*

- A. Normalized fluorescent responses to indicated stimuli in cultured neurons, obtained from the *Chen et al. 2013* and *Dana et al. 2019* variant library (bars depict mean + SEM (if applicable)).
- B. Max fluorescent responses obtained from listed variants expressed in HEK293 cells and stimulated with acetylcholine. (dots indicate analyzed cells; bars depict mean + SEM, **** = <0.0001 (unpaired t-test, two-tailed)). [jGCaMP7f = 497.6 (SEM: 22.15; n=353); jGCaMP7f L317H = 130.7 (SEM: 3.57; n=330)].



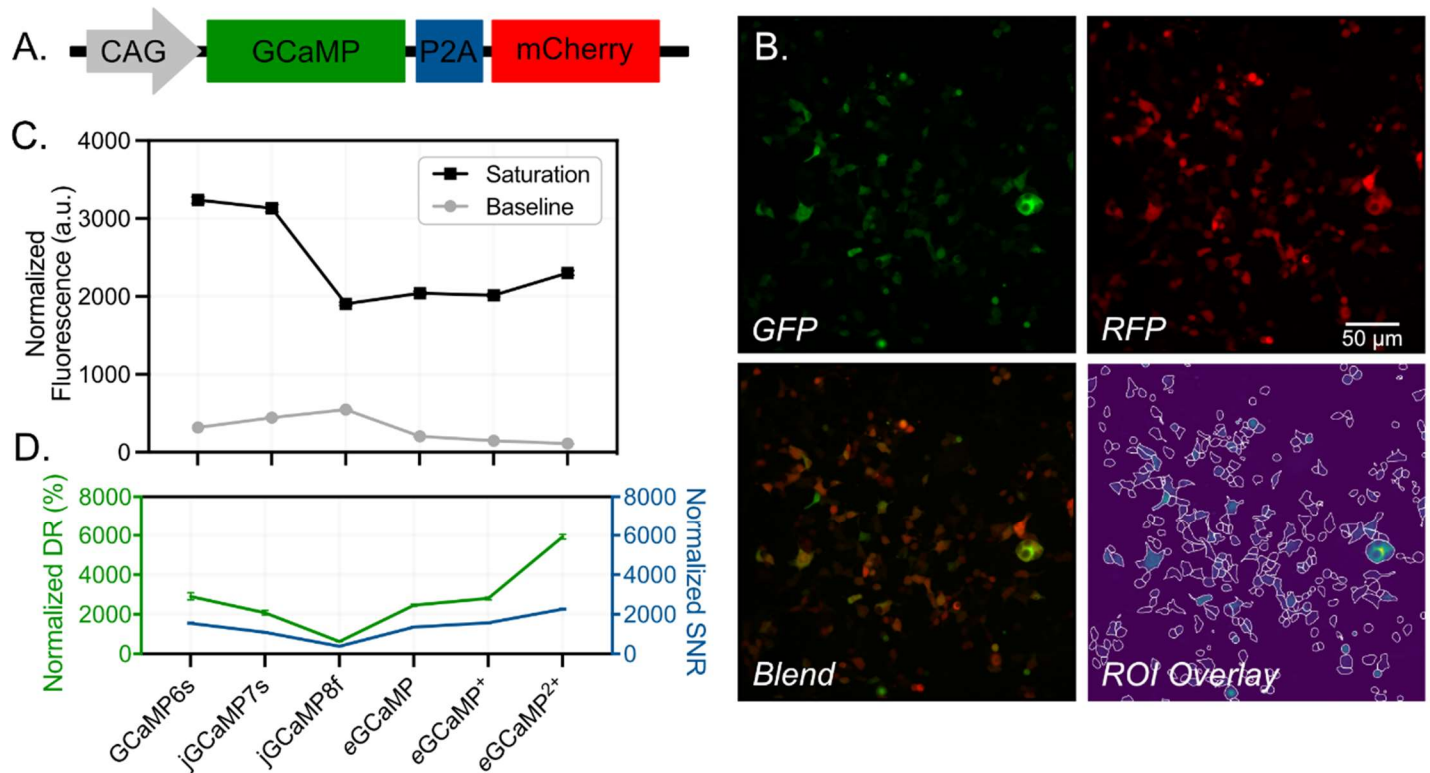
Supplementary Figure 7: *Combinatorial Mutation Biophysical Characteristics and Basis for Mutation Transfer*

A. Sequence alignment of jGCaMP7s and jGCaMP8f. Light gray indicates identical sequence alignment, and red indicates sequence dissimilarities. Breaks indicate a missing sequence portion caused by additional sequence portions in other constructs. The physical location of the residue L317 in jGCaMP7s is designated with the green box and the residue number of the matching location is included as green text over the sequence. Text along the top of the sequence depicts the physical location in the GCaMP protein: CBP, CaM, or circularly permuted fluorescent protein (cpFP).

B. Decay values (τ , tau, Eq. 4) obtained from each combinatorial mutant of jGCaMP7s expressed in HEK293 cells and stimulated with 10 μ M acetylcholine. Mutations are sorted according to $\Delta F/F_0$ performance (n = the number of cells quantified; bars depict mean + bootstrapped 95% ci). Signal-to-noise ratio (SNR, Eq. 4) obtained from each combinatorial mutant of jGCaMP7s expressed in HEK293 cells and stimulated with

10 μM acetylcholine. Mutations are sorted according to $\Delta F/F_0$ performance (n = the number of cells quantified; bars depict mean + bootstrapped 95% ci).

- C. Signal-to-noise ratio (SNR, *Eq.4*) of indicated variant, expressed in HEK293 cells and stimulated with different acetylcholine concentrations (x-axis). Plotted points indicate the mean SNR response for each variant to indicated stimuli, and error bars display the SEM. The solid line depicts the non-linear fit of scatter data.



Supplementary Figure 8: *Ratiometric analysis of baseline fluorescence for eGCaMP, eGCaMP⁺, and eGCaMP²⁺*

- GCaMP variants GCaMP6s, jGCaMP7s, jGCaMP8f, eGCaMP, eGCaMP⁺, eGCaMP²⁺ were cloned into a CAG driven vector, with a mCherry control fluorophore added after a self-cleaving motif (P2A) within the reading frame.
- Representative images taken with 488 nm wavelength excitation (GFP), 585nm wavelength excitation (RFP), an overlap of the two channels depicting the ratio of GFP intensity to RFP intensity (Blend), and the Cellpose derived ROIs used for analysis (ROI overlay). The scale bar depicts 50 μ m.
- Normalized fluorescence intensity of each ratiometric variant (x-axis shared with D.) at baseline (gray) and saturation conditions (black). Scatter points depict mean value and error bars depict SEM.
- Normalized dynamic range (DR, green, left y-axis) and signal-to-noise ratio (SNR, blue, right y-axis) for each ratiometric variant (x-axis). Scatter points depict mean value and error bars depict SEM. 4 independent biological replicates per construct in **B.**, **C.**, and **D.**

Fluorescence				
Model	AAINDEX	R ²	Descriptor	Cluster #
RFR	ROSM880102	0.88	Side chain hydropathy, corrected for solvation (Roseman, 1988)	2
	MANP780101	0.88	Average surrounding hydrophobicity (Manavalan-Ponnuswamy, 1978)	3
	KANM800104	0.88	Average relative probability of inner beta-sheet (Kanehisa-Tsong, 1980)	3
	JURD980101	0.87	Modified Kyte-Doolittle hydrophobicity scale (Juretic et al., 1998)	3
	MEEJ810102	0.87	Retention coefficient in NaH ₂ PO ₄ (Meek-Rossetti, 1981)	1
MPNR	BROC820101	0.79	Retention coefficient in TFA (Browne et al., 1982)	1
	ZIMJ680105	0.79	RF rank (Zimmerman et al., 1968)	1
	FAUJ830101	0.78	Hydrophobic parameter pi (Fauchere-Pliska, 1983)	1
	CIDH920104	0.78	Normalized hydrophobicity scales for alpha/beta-proteins (Cid et al., 1992)	3
	BULH740101	0.77	Transfer free energy to surface (Bull-Breese, 1974)	2
KNR	KANM800104	0.77	Average relative probability of inner beta-sheet (Kanehisa-Tsong, 1980)	3
	LIFS790102	0.77	Conformational preference for parallel beta-strands (Lifson-Sander, 1979)	3
	MANP780101	0.77	Average surrounding hydrophobicity (Manavalan-Ponnuswamy, 1978)	3
	BASU050101	0.77	Interactivity scale obtained from the contact matrix (Bastolla et al., 2005)	3
	MIYS990101	0.76	Relative partition energies derived by the Bethe approximation. (Miyazawa-Jernigan, 1999)	2

Supplementary Table 1: $\Delta F/F_0$ library encoding dataset information

The table contains the top five performing AAINDEX datasets for each model (RFR, MPNR, KNR). The 'AAINDEX' column pertains to the property dataset's unique identifier within the AAINDEX repository. 'R²' is the performance score of the model when trained on the 80% train set and validated with the 20% test set. 'Descriptor' is the description of the property obtained from AAINDEX. 'Cluster #' is derived from the principal component analyses on all property datasets.

Kinetics				
Model	AAINDEX	R ²	Descriptor	Cluster #
RFR	ZASB82010	0.88	Dependence of partition coefficient on ionic strength (Zaslavsky et al., 1982)	1
	QIAN880130	0.88	Weights for coil at the window position of -3 (Qian-Sejnowski, 1988)	4
	RADA880101	0.88	Transfer free energy from chx to wat (Radzicka-Wolfenden, 1988)	1
	NADH010103	0.87	Hydropathy scale based on self-information values in the two-state model (16% accessibility) (Naderi-Manesh et al., 2001)	3
	GUYH850104	0.87	Apparent partition energies calculated from Janin index (Guy, 1985)	2
MPNR	BULH740101	0.79	Transfer free energy to surface (Bull-Breese, 1974)	2
	FAUJ880110	0.79	Number of full nonbonding orbitals (Fauchere et al., 1988)	2
	ZIMJ680105	0.78	RF rank (Zimmerman et al., 1968)	1
	MEEJ800101	0.78	Retention coefficient in HPLC, pH7.4 (Meek, 1980)	1
	ROSM880101	0.77	Side chain hydropathy, uncorrected for solvation (Roseman, 1988)	2
KNR	PALJ810103	0.77	Normalized frequency of beta-sheet from LG (Palau et al., 1981)	3
	PALJ810107	0.77	Normalized frequency of alpha-helix in all-alpha class (Palau et al., 1981)	5
	PALJ810112	0.77	Normalized frequency of beta-sheet in alpha/beta class (Palau et al., 1981)	3
	LEVJ780103	0.77	Normalized frequency of reverse turn, with weights (Levitt, 1978)	4
	QIAN880105	0.76	Weights for alpha-helix at the window position of -2 (Qian-Sejnowski, 1988)	5

Supplementary Table 2: *Kinetics library encoding dataset information*

The table contains the top five performing AAINDEX datasets for each model (RFR, MPNR, KNR). The 'AAINDEX' column pertains to the property dataset's unique identifier within the AAINDEX repository. 'R²' is the performance score of the model when trained on the 80% train set and validated with the 20% test set. 'Descriptor' is the description of the property obtained from AAINDEX. 'Cluster #' is derived from the principal component analyses on all property datasets.

$\Delta F/F$ (%)			
Variant	Mean	95% CI	ROIs Measured (n)
L317N	1224.53	[1188.62, 1257.82]	608
L317E	1239.16	[1195.84, 1282.91]	562
L317K	436.49	[397.03, 477.46]	145
L317H	932.7	[897.68, 967.47]	626
L302D	519.48	[492.15, 549.41]	541
L302R	258.63	[248.38, 271.61]	769
L302G	322.53	[309.5, 335.76]	707
L302H	255.27	[246.09, 264.53]	851
L302C	276.18	[265.04, 287.55]	707
A390Y	149.82	[142.29, 157.56]	606
A390R	906.15	[884.21, 927.9]	882
P303D	620.06	[598.72, 641.78]	787
jGCaMP7s	489.93	[469.53, 510.23]	631
P303W	716.15	[691.97, 740.69]	635
P303F	889.13	[863.36, 914.67]	1072
G392F	932.41	[896.26, 969.28]	450
G392W	1119.84	[1085.01, 1154.64]	573

Supplementary Table 3: $\Delta F/F_0$ Descriptive Statistics of Ensemble Prediction Screen Results

The table contains the information displayed in Figure 3B. The table contains the construct (Variant), the mean $\Delta F/F_0$ (%), the 95% confidence interval (95% CI), and the number of samples (ROIs Measured (n)).

Model	Accuracy	Precision
Random Chance (50/50)	0.5	0.5
Fluorescence + Kinetics	0.66	0.91
Fluorescence	0.56	1
Kinetics	0.75	0.86

Supplementary Table 4: *Precision and Accuracy Quantifications of Model Predictions*
Quantification of each model's accuracy (Eq.6) and precision (Eq.7).

Tau (s)			
Variant	Mean	95% CI	ROIs Measured (n)
A390Y	124.72	[116.65, 133.5]	606
L302C	181.63	[170.57, 193.2]	707
L302D	139.24	[127.55, 151.71]	541
L302R	100.29	[93.8, 107.2]	769
A390R	66.75	[64.23, 69.38]	882
L302G	218.24	[204.23, 232.75]	707
L302H	132.81	[127.34, 138.61]	851
P303F	118.52	[112.54, 125.0]	1072
P303W	94.61	[89.19, 100.68]	635
jGCaMP7s	99.6	[92.02, 107.83]	631
G392F	70.51	[65.82, 75.64]	450
G392W	65.17	[60.42, 70.23]	573
L317H	28.75	[25.54, 32.31]	626
P303D	113.24	[107.3, 119.51]	787
L317K	4.65	[4.26, 5.08]	145
L317N	28.94	[25.49, 33.0]	608
L317E	35.16	[31.7, 39.14]	562

Supplementary Table 5: *Decay Descriptive Statistics of Ensemble Prediction Screen Results*

The table contains the information displayed in Figure 3C. The table contains the construct (Variant), the mean Tau (s), the 95% confidence interval (95% CI), and the number of samples (ROIs Measured (n)).

Variant ID	52	317	374	1 AP $\Delta F/F_0$
10.1473	V	L	M	5.120
10.915	A	D	M	0.380
10.1129	A	D	M	0.240
67	A	E	M	0.085
361	A	E	M	0.214
362	A	E	M	0.140
500	A	E	M	0.285
608	A	E	M	0.129
609	A	E	M	0.153
610	A	E	M	0.262
611	A	E	M	0.155
612	A	E	M	0.288
613	A	E	M	0.737
630	A	E	M	0.329
631	A	E	M	0.241
632	A	E	M	0.209
633	A	E	M	0.110
642	A	E	M	0.419
644	A	E	M	0.279
645	A	E	M	0.220
646	A	E	M	0.319
647	A	E	M	0.311
648	A	E	M	0.209
649	A	E	M	0.427
663	A	E	M	0.123
664	A	E	M	0.244
684	A	E	M	0.155
685	A	E	M	0.197
686	A	E	M	0.173
687	A	E	M	0.150
688	A	E	M	0.131
689	A	E	M	0.098
690	A	E	M	0.164
691	A	E	M	0.147
692	A	E	M	0.068
10.1083	A	E	M	0.610
10.1087	A	E	M	0.740

Variant ID	52	317	374	1 AP $\Delta F/F_0$
10.1088	A	E	M	0.650
10.1097	A	E	M	0.270
10.1098	A	E	M	0.640
10.1112	A	E	M	0.280
10.1113	A	E	M	0.760
10.1117	A	E	M	0.430
10.1118	A	E	M	0.430
10.1119	A	E	M	0.420
10.1124	A	E	M	0.490
10.1125	A	E	M	0.290
10.1126	A	E	M	0.340
10.1127	A	E	M	0.900
10.1128	A	E	M	0.320
10.1136	A	E	M	0.600
10.1206	A	E	M	0.260
10.1207	A	E	M	0.280
10.1212	A	E	M	0.160
10.1217	A	E	M	0.590
10.1227	A	E	M	0.740
10.1228	A	E	M	0.210
10.1229	A	E	M	0.510
10.124	A	E	M	0.140
10.1251	A	E	M	0.500
10.1253	A	E	M	0.320
10.1257	A	E	M	0.010
['GCaMP6f', 'GCaMP6f']	A	E	M	0.618
['68', '10.916']	A	E	M	0.205
['10.1175', '10.1182']	A	E	M	0.565
['10.1239', '10.1243']	A	E	M	0.375
['10.108', '10.123']	A	E	M	0.625
['10.1234', '10.1236']	A	E	M	0.550
['10.1241', '10.1258']	A	E	M	0.500
['10.1177', '10.1186']	A	E	M	0.355
10.1035	A	H	M	0.150
10.1259	A	H	M	0.280
10.92	A	K	M	0.210
10.923	A	N	M	0.380
10.1166	A	N	M	0.480

Supplementary Table 6: *Previously Described 317 Variants*

Values are derived from the Chen *et al.* 2013 and Dana *et al.* 2019 variant library, rows contain the identification number of the cataloged variant (Index), the variants' amino acid identities at residues 52, 317, and 374 (52, 317, 374), and the normalized $\Delta F/F_0$ at 1 AP ($\Delta F/F_0$: 1AP). jGCaMP7s is included in the first row as it's Dana *et al.* 2019 derived identity (10.1473).

SNR (a.u.)			
Variant	Mean	95% CI	ROIs Measured (n)
jGCaMP7s	251.74	[234.83, 269.59]	631
L302C	138.67	[131.24, 146.11]	707
L302D	319.8	[297.64, 342.55]	541
L302G	428.67	[405.3, 452.19]	707
L302H	146.09	[136.89, 155.66]	851
L302R	98.1	[92.87, 104.14]	769
P303D	455.22	[429.31, 482.85]	787
P303F	357.71	[341.91, 373.36]	1072
P303W	343.9	[325.42, 363.0]	635
L317E	810.13	[753.73, 866.7]	562
L317K	133.79	[117.73, 151.03]	145
L317H	854.35	[808.94, 899.55]	626
L317N	586.23	[541.01, 636.35]	608
A390R	559.12	[533.85, 585.68]	882
A390Y	41.99	[39.03, 45.06]	606
G392F	471.71	[441.75, 502.39]	450
G392W	590.15	[557.76, 623.86]	573

Supplementary Table 7: *SNR Descriptive Statistics of Ensemble Prediction Screen Results*

The table contains the information displayed in Figure 3D. The table contains the construct (Variant), the mean signal-to-noise ratio (SNR) (Mean), the 95% confidence interval (95% CI), and the number of samples (ROIs Measured (n)).

Performance Score (a.u.)			
Variant	Mean	95% CI	ROIs Measured (n)
jGCaMP7s	3.83	[3.51, 4.15]	631
L302C	1.22	[1.11, 1.34]	707
L302D	4.02	[3.6, 4.47]	541
L302G	3.65	[3.09, 4.45]	707
L302H	1.46	[1.34, 1.59]	851
L302R	1.68	[1.43, 2.06]	769
P303D	5.43	[5.03, 5.85]	787
P303F	4.42	[4.17, 4.69]	1072
P303W	5.02	[4.67, 5.38]	635
L317E	35.48	[32.71, 38.38]	562
L317K	32.71	[28.56, 37.26]	145
L317H	54.49	[51.17, 57.92]	626
L317N	34.89	[32.0, 37.85]	608
A390R	10.4	[9.84, 10.98]	882
A390Y	0.51	[0.46, 0.56]	606
G392F	9.31	[8.5, 10.13]	450
G392W	12.84	[11.83, 13.94]	573

Supplementary Table 8: *Performance Score Descriptive Statistics of Ensemble Prediction Screen Results*

The table contains the information displayed in Figure 3E. The table contains the construct (Variant), the mean Performance Score (Mean), the 95% confidence interval (95% CI), and the number of samples (ROIs Measured (n)).

Tau (s)				
Variant	Acetylcholine Stimuli (μM)	Mean	95% CI	ROIs Measured (n)
L317H, L302G	10	13.82	[12.91, 14.78]	608
jGCaMP7s	10	99.6	[92.15, 107.49]	631
L317H, L302H	10	21.86	[19.91, 24.12]	779
L317H, A390R	10	21.83	[20.57, 23.24]	1492
eGCaMP (L317H)	10	28.75	[25.57, 32.35]	626
L317H, P303D	10	17.61	[16.02, 19.49]	757
L317H, L302D	10	15.59	[14.03, 17.49]	801
L317H, Q305D	10	27.39	[24.62, 30.51]	659

Supplementary Table 9: *Decay Descriptive Statistics of Combinatorial Variants*

The table contains the information displayed in Supplementary Figure 7B. The table contains the construct (Variant), the concentration of acetylcholine (Acetylcholine Stimuli (μM)), the mean Tau (s) (Mean), the 95% confidence interval (95% CI), and the number of samples (ROIs Measured (n)).

$\Delta F/F_0$ (%)				
Variant	Acetylcholine Stimuli (μM)	Mean	95% CI	ROIs Measured (n)
L317H, L302G	10	401.8	[387.48, 416.08]	608
jGCaMP7s	10	489.93	[469.11, 510.71]	631
L317H, L302H	10	758.06	[738.7, 777.22]	779
L317H, A390R	10	892.11	[875.99, 908.27]	1492
eGCaMP (L317H)	10	932.7	[897.84, 967.47]	626
L317H, P303D	10	942.58	[916.89, 968.52]	757
L317H, L302D	10	1098.63	[1070.71, 1126.54]	801
L317H, Q305D	10	2407	[2335.38, 2478.47]	659

Supplementary Table 10: $\Delta F/F_0$ Descriptive Statistics of Combinatorial Variants

The table contains the information displayed in Figure 4E. The table contains the construct (Variant), the concentration of acetylcholine (Acetylcholine Stimuli (μM)), the mean $\Delta F/F_0$ (Mean), the 95% confidence interval (95% CI), and the number of samples (ROIs Measured (n)).

SNR (a.u.)				
Variant	Acetylcholine Stimuli (μM)	Mean	95% CI	ROIs Measured (n)
L317H, L302G	10	97.7	[91.52, 104.22]	608
jGCaMP7s	10	251.74	[234.96, 269.24]	631
L317H, L302H	10	471.92	[454.95, 489.27]	779
L317H, A390R	10	430.55	[413.14, 448.84]	1492
eGCaMP (L317H)	10	854.35	[809.76, 901.75]	626
L317H, P303D	10	531.58	[504.94, 558.54]	757
L317H, L302D	10	702.85	[672.35, 733.8]	801
L317H, Q305D	10	1271.48	[1200.07, 1343.99]	659

Supplementary Table 11: *SNR Descriptive Statistics of Combinatorial Variants*

The table contains the information displayed in Supplementary Figure 7B. The table contains the construct (Variant), the concentration of acetylcholine (Acetylcholine Stimuli (μM)), the mean SNR (Mean), the 95% confidence interval (95% CI), and the number of samples (ROIs Measured (n)).

SNR/Tau (a.u.)				
Variant	Acetylcholine Stimuli (μM)	Mean	95% CI	ROIs Measured (n)

L317H, L302G	10	10.35	[9.56, 11.17]	608
jGCaMP7s	10	3.83	[3.51, 4.15]	631
L317H, L302H	10	35.66	[33.73, 37.61]	779
L317H, A390R	10	27.58	[26.26, 29.05]	1492
eGCaMP (L317H)	10	54.49	[51.21, 57.96]	626
L317H, P303D	10	44.43	[41.78, 47.16]	757
L317H, L302D	10	62.97	[59.92, 66.04]	801
L317H, Q305D	10	74.62	[69.97, 79.53]	659

Supplementary Table 12: *SNR/Tau Descriptive Statistics of Combinatorial Variants*

The table contains the information displayed in Figure 4F. The table contains the construct (Variant), the concentration of acetylcholine (Acetylcholine Stimuli (μM)), the mean SNR/Tau (Mean), the 95% confidence interval (95% CI), and the number of samples (ROIs Measured (n)).

Concentration (μM)	Variant	Mean $\Delta F/F_0$ (%)	SEM	Samples (n)
10.0	GCaMP6s	542.0576957490350	16.3	1218
10.0	GCaMP6f	580.0599817630100	19.75	776
10.0	jGCaMP7s	309.66659364822100	8.69	1401
10.0	jGCaMP7f	776.2219775641050	8.83	1883
10.0	jGCaMP8s	189.8974402543510	4.34	1129
10.0	jGCaMP8m	257.06451324858200	4.63	1514
10.0	jGCaMP8f	268.4993953371440	5.21	1288
10.0	eGCaMP	724.4879489890530	14.36	1618
10.0	eGCaMP2+	1567.5173223331000	24.54	1924
10.0	eGCaMP+	1162.094675384620	21.91	1467

Supplementary Table 13: $\Delta F/F_0$ Descriptive Statistics of $10\mu\text{M}$ Acetylcholine Stimulus

The table contains the concentration of the acetylcholine stimulus (Concentration (μM)), the construct (Variant), the mean response (Mean $\Delta F/F_0$ (%)), the standard error of the mean (SEM), and the number of samples (Samples (n)).

Concentration (μM)	Variant	Mean $\Delta F/F_0$ (%)	SEM	Samples (n)
5.0	GCaMP6s	529.343955352201	14.26	1275
5.0	GCaMP6f	582.7062640702780	14.46	1355
5.0	jGCaMP7s	332.8529265633950	10.67	910
5.0	jGCaMP7f	743.6582692870790	7.57	1867
5.0	jGCaMP8s	194.30823094856800	4.17	1143
5.0	jGCaMP8m	250.46020535142500	5.08	1203
5.0	jGCaMP8f	256.31216255142000	4.69	1358
5.0	eGCaMP	715.4126616948510	14.6	1527
5.0	eGCaMP2+	1526.9948613182700	26.47	1448
5.0	eGCaMP+	1171.9621473829300	23.31	1161

Supplementary Table 14: $\Delta F/F_0$ Descriptive Statistics of $5\mu\text{M}$ Acetylcholine Stimulus

The table contains the concentration of the acetylcholine stimulus (Concentration (μM)), the construct (Variant), the mean response (Mean $\Delta F/F_0$ (%)), the standard error of the mean (SEM), and the number of samples (Samples (n)).

Concentration (μM)	Variant	Mean $\Delta F/F_0$ (%)	SEM	Samples (n)
1.0	GCaMP6s	389.1751755671520	18.02	785
1.0	GCaMP6f	447.78850813463900	19.66	584
1.0	jGCaMP7s	271.939682741784	11.82	686
1.0	jGCaMP7f	542.3482366328280	9.69	1524
1.0	jGCaMP8s	188.80805475510900	5.06	749
1.0	jGCaMP8m	203.66560060670000	6.75	760
1.0	jGCaMP8f	200.35393958757700	5.39	1065
1.0	eGCaMP	497.4989626023940	15.62	1000
1.0	eGCaMP2+	1389.2302765905300	29.65	1125
1.0	eGCaMP+	698.0678991857000	21.61	996

Supplementary Table 15: $\Delta F/F_0$ Descriptive Statistics of $1\mu\text{M}$ Acetylcholine Stimulus

The table contains the concentration of the acetylcholine stimulus (Concentration (μM)), the construct (Variant), the mean response (Mean $\Delta F/F_0$ (%)), the standard error of the mean (SEM), and the number of samples (Samples (n)).

Concentration (μM)	Variant	Mean $\Delta F/F_0$ (%)	SEM	Samples (n)
0.75	GCaMP6s	329.4149698176440	15.49	804
0.75	GCaMP6f	429.4997942849410	14.13	1016
0.75	jGCaMP7s	268.2510926313680	9.28	1006
0.75	jGCaMP7f	477.93217506889600	9.81	1361
0.75	jGCaMP8s	135.61875283648100	4.28	805
0.75	jGCaMP8m	173.8268266918370	5.27	983
0.75	jGCaMP8f	164.20390709877900	3.92	1671
0.75	eGCaMP	474.7734111076490	11.93	1666
0.75	eGCaMP2+	1079.6686913867400	26.0	1278
0.75	eGCaMP+	648.4762047315260	19.95	1034

Supplementary Table 16: $\Delta F/F_0$ Descriptive Statistics of $0.75\mu\text{M}$ Acetylcholine Stimulus

The table contains the concentration of the acetylcholine stimulus (Concentration (μM)), the construct (Variant), the mean response (Mean $\Delta F/F_0$ (%)), the standard error of the mean (SEM), and the number of samples (Samples (n)).

Concentration (μM)	Variant	Mean $\Delta F/F_0$ (%)	SEM	Samples (n)
0.5	GCaMP6s	290.844910478741	14.57	826
0.5	GCaMP6f	293.7001358695750	18.37	657
0.5	jGCaMP7s	223.09331472361800	11.05	846
0.5	jGCaMP7f	442.48651191810300	9.13	1574
0.5	jGCaMP8s	151.1790419548550	6.43	462
0.5	jGCaMP8m	157.38969324099700	5.01	1035
0.5	jGCaMP8f	128.5803460675330	4.69	1100
0.5	eGCaMP	350.11532966689300	12.21	1175
0.5	eGCaMP2+	1033.0644958159400	25.58	1353
0.5	eGCaMP+	644.6372960966310	19.25	1121

Supplementary Table 17: $\Delta F/F_0$ Descriptive Statistics of 0.5 μM Acetylcholine Stimulus

The table contains the concentration of the acetylcholine stimulus (Concentration (μM)), the construct (Variant), the mean response (Mean $\Delta F/F_0$ (%)), the standard error of the mean (SEM), and the number of samples (Samples (n)).

Concentration (μM)	Variant	Mean $\Delta F/F$ (%)	SEM	Samples (n)
0.25	GCaMP6s	105.54097548728200	15.68	302
0.25	GCaMP6f	128.21780442992800	12.6	491
0.25	jGCaMP7s	156.54839686698600	11.77	453
0.25	jGCaMP7f	214.02339937468600	7.26	1577
0.25	jGCaMP8s	102.7639405766460	5.92	477
0.25	jGCaMP8m	91.12941574591900	4.29	935
0.25	jGCaMP8f	79.27533088088380	4.02	934
0.25	eGCaMP	249.34280073848800	13.68	784
0.25	eGCaMP2+	546.5026204926650	18.21	1471
0.25	eGCaMP+	313.18723778004700	15.23	976

Supplementary Table 18: $\Delta F/F_0$ Descriptive Statistics of 0.25 μM Acetylcholine Stimulus

The table contains the concentration of the acetylcholine stimulus (Concentration (μM)), the construct (Variant), the mean response (Mean $\Delta F/F_0$ (%)), the standard error of the mean (SEM), and the number of samples (Samples (n)).

Concentration (μM)	Variant	Mean $\Delta F/F_0$ (%)	SEM	Samples (n)
0.1	GCaMP6s	78.83819697878870	14.76	474
0.1	GCaMP6f	50.79367750011170	13.63	356
0.1	jGCaMP7s	77.28006581708250	9.16	381
0.1	jGCaMP7f	81.90602739824190	4.79	1385
0.1	jGCaMP8s	58.181684834710300	5.5	300
0.1	jGCaMP8m	35.943127649074500	3.16	626
0.1	jGCaMP8f	40.4984093431018	3.1	937
0.1	eGCaMP	71.75163612188040	6.5	896
0.1	eGCaMP2+	205.68795282465300	14.34	807
0.1	eGCaMP+	145.3234198499200	10.61	808

Supplementary Table 19: $\Delta F/F_0$ Descriptive Statistics of 0.1 μM Acetylcholine Stimulus

The table contains the concentration of the acetylcholine stimulus (Concentration (μM)), the construct (Variant), the mean response (Mean $\Delta F/F_0$ (%)), the standard error of the mean (SEM), and the number of samples (Samples (n)).

Concentration (μM)	Variant	Tau Off (s)	SEM	Samples (n)
5	GCaMP6s	72.31	3.06	940
5	GCaMP6f	24.58	1.03	1357
5	jGCaMP7s	100.9	4.27	676
5	jGCaMP7f	45.97	1.42	2088
5	jGCaMP8s	30.44	0.69	1379
5	jGCaMP8m	32.28	1.19	1325
5	jGCaMP8f	33.52	1.13	1457
5	eGCaMP	34.02	1.49	1564
5	eGCaMP2+	39.02	1.74	1509
5	eGCaMP+	18.13	0.79	1297

Supplementary Table 20: *Decay Descriptive Statistics of 5 μM Acetylcholine Stimulus*

The table contains the concentration of the acetylcholine stimulus (Concentration (μM)), the construct (Variant), the speed of off-decay (Tau Off (s)), the standard error of the mean (SEM), and the number of samples (Samples (n)).

Sensor	Kd (nM)	Hill Coefficient	ϵ (Saturated) (x1000) (M ⁻¹ cm ⁻¹)	ϕ (Saturated)
GCaMP6s	120.8 [110.6, 132.2]	2.014 [1.716, 2.387]	N/A	N/A
GCaMP6f	291.3 [256.4, 333.1]	1.857 [1.544, 2.261]	65.276	0.6
jGCaMP7s	46.2 [39.3, 53.7]	2.138 [1.596, 1.918]	N/A	N/A
eGCaMP	354.8 [262.8, 516.4]	1.761 [1.087, 3.339]	62.726	0.68
eGCaMP2+	358.7 [310.4, 418.8]	1.925 [1.540, 2.461]	60.070	0.72
eGCaMP+	1885 [1.082, 34.02]	0.9976 [0.4875, 1.871]	58.988	0.63

Supplementary Table 21: *Photophysical Properties of Purified eGCaMP Proteins*

The table contains the construct (Sensor), dissociation constant [95% CI] (Kd (nM)), hill coefficient [95% CI], extinction coefficient (ϵ (Saturated)(x1000)(M⁻¹ cm⁻¹)), and quantum yield (ϕ (Saturated)). N/A indicates not acquired. All properties were measured in purified protein solutions (*Methods*).

1 AP							
Construct	GCaMP6s	GCaMP6f	jGCaMP7s	jGCaMP8f	eGCaMP	eGCaMP ⁺	eGCaMP ²⁺
Number of values	33	48	102	72	56	49	47
Mean	3.911	2.626	6.252	8.438	2.783	3.026	10.1
Std. Deviation	3.197	5.021	11.51	18.88	2.845	3.702	16.25
Std. Error of Mean	0.5564	0.7247	1.139	2.225	0.3802	0.5289	2.371

Supplementary Table 22: *Descriptive Statistics of Primary Neuron Responses to 1AP*

The table contains the construct (Construct), the number of samples (Number of Values), the mean $\Delta F/F_0$ (%) response (Mean), the standard deviation (Std. Deviation), and the standard error of the mean (Std. Error of Mean) to 1 AP stimulus.

10 AP							
Construct	GCaMP6s	GCaMP6f	jGCaMP7s	jGCaMP8f	eGCaMP	eGCaMP ⁺	eGCaMP ²⁺
Number of values	49	55	134	50	82	77	53
Mean	43.77	20.99	48.53	31.94	37.81	30.54	113.8
Std. Deviation	53.41	34.86	57.84	35.49	50.59	64.68	207.4
Std. Error of Mean	7.63	4.701	4.996	5.019	5.587	7.371	28.49

Supplementary Table 23: *Descriptive Statistics of Primary Neuron Responses to 10AP*

The table contains the construct (Construct), the number of samples (Number of Values), the mean $\Delta F/F_0$ (%) response (Mean), the standard deviation (Std. Deviation), and the standard error of the mean (Std. Error of Mean) to 10AP stimulus.

80 AP							
Construct	GCaMP6s	GCaMP6f	jGCaMP7s	jGCaMP8f	eGCaMP	eGCaMP ⁺	eGCaMP ²⁺
Number of values	49	63	111	72	88	86	54
Mean	276.1	114.6	142.4	84.28	275.3	190.4	502.5
Std. Deviation	252.1	173.6	126.7	64.44	243.7	193.1	370.1
Std. Error of Mean	36.01	21.87	12.03	7.594	25.97	20.82	50.37

Supplementary Table 24: *Descriptive Statistics of Primary Neuron Responses to 80AP*

The table contains the construct (Construct), the number of samples (Number of Values), the mean $\Delta F/F_0$ (%) response (Mean), the standard deviation (Std. Deviation), and the standard error of the mean (Std. Error of Mean) to 80AP stimulus.

40 mM KCl							
Construct	GCaMP6s	GCaMP6f	jGCaMP7s	jGCaMP8f	eGCaMP	eGCaMP ⁺	eGCaMP ²⁺
Number of values	82	44	121	15	96	29	92
Mean	940.5	333.3	546.4	233.2	928.8	833.5	2110
Std. Deviation	465.3	253.7	239.1	89.34	445.1	572.1	1009
Std. Error of Mean	51.39	38.24	21.74	23.07	45.43	106.2	105.2

Supplementary Table 25: *Descriptive Statistics of Primary Neuron Responses to 40mM KCl*

The table contains the construct (Construct), the number of samples (Number of Values), the mean $\Delta F/F_0$ (%) response (Mean), the standard deviation (Std. Deviation), and the standard error of the mean (Std. Error of Mean) to 40mM KCl.

10 AP Decay							
Construct	GCaMP6s	GCaMP6f	jGCaMP7s	jGCaMP8f	eGCaMP	eGCaMP ⁺	eGCaMP ²⁺
Number of values	16	44	70	47	75	62	43
Mean	4.37	0.9547	9.479	1.49	1.168	0.7355	2.099
Std. Deviation	2.583	0.4496	5.487	0.9786	0.5577	0.6476	1.595
Std. Error of Mean	0.6457	0.06778	0.6558	0.1427	0.0644	0.08225	0.2433

Supplementary Table 26: *Descriptive Statistics of Primary Neuron Responses Half Decay to 10AP*

The table contains the construct (Construct), number of samples (Number of Values), the mean half decay time (s) (Mean), the standard deviation (Std. Deviation), and the standard error of the mean (Std. Error of Mean) after 10 AP stimuli.