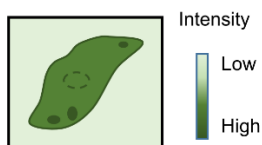


Supplementary Material

1 Supplementary Figures and Tables

1.1 Supplementary Figures



1. ROI selection in green intensity image

1.1. Intensity thresholding

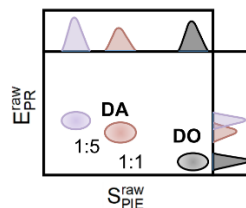
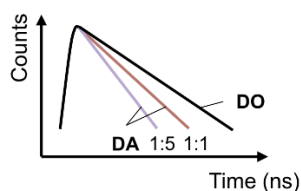
1.2. Manual “vesicles” clearing



2. Data analysis (Time-resolved)

2.1. Ensemble analysis

2.2. Pixel-by-pixel analysis



3. Parameter – readout

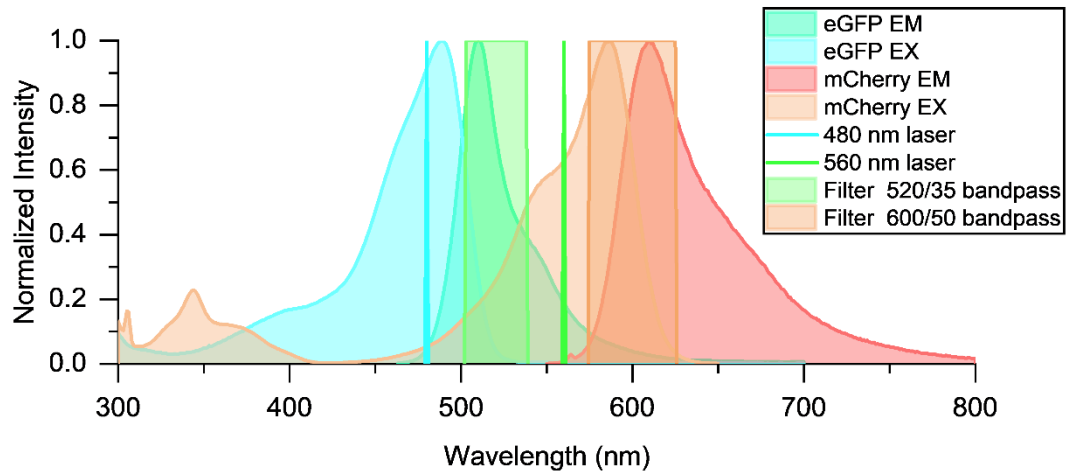
3.1. Ensemble analysis

3.2. Pixel-by-pixel analysis

	Parameter		Parameter
$\tau_1, x_1, \tau_2, x_2, \dots$	Lifetime and fraction of species 1,2,...	τ_{FF}	Average photon arrival time
τ_{avg}	Species-weighted average lifetime	$I^{D D}, I^{A D}, I^{A A}$	Signal intensities
E_τ	Lifetime-based FRET efficiency	S_{PIE}^{raw}	Stoichiometry
$I_\Sigma^{D D}, I_\Sigma^{A D}, I_\Sigma^{A A}$	Signal intensities sum of all pixels	E_{PR}^{raw}	Proximity ratio
$S_{\Sigma PIE}^{raw}$	Stoichiometry of summed intensity	I_Σ	Sum of signal intensities

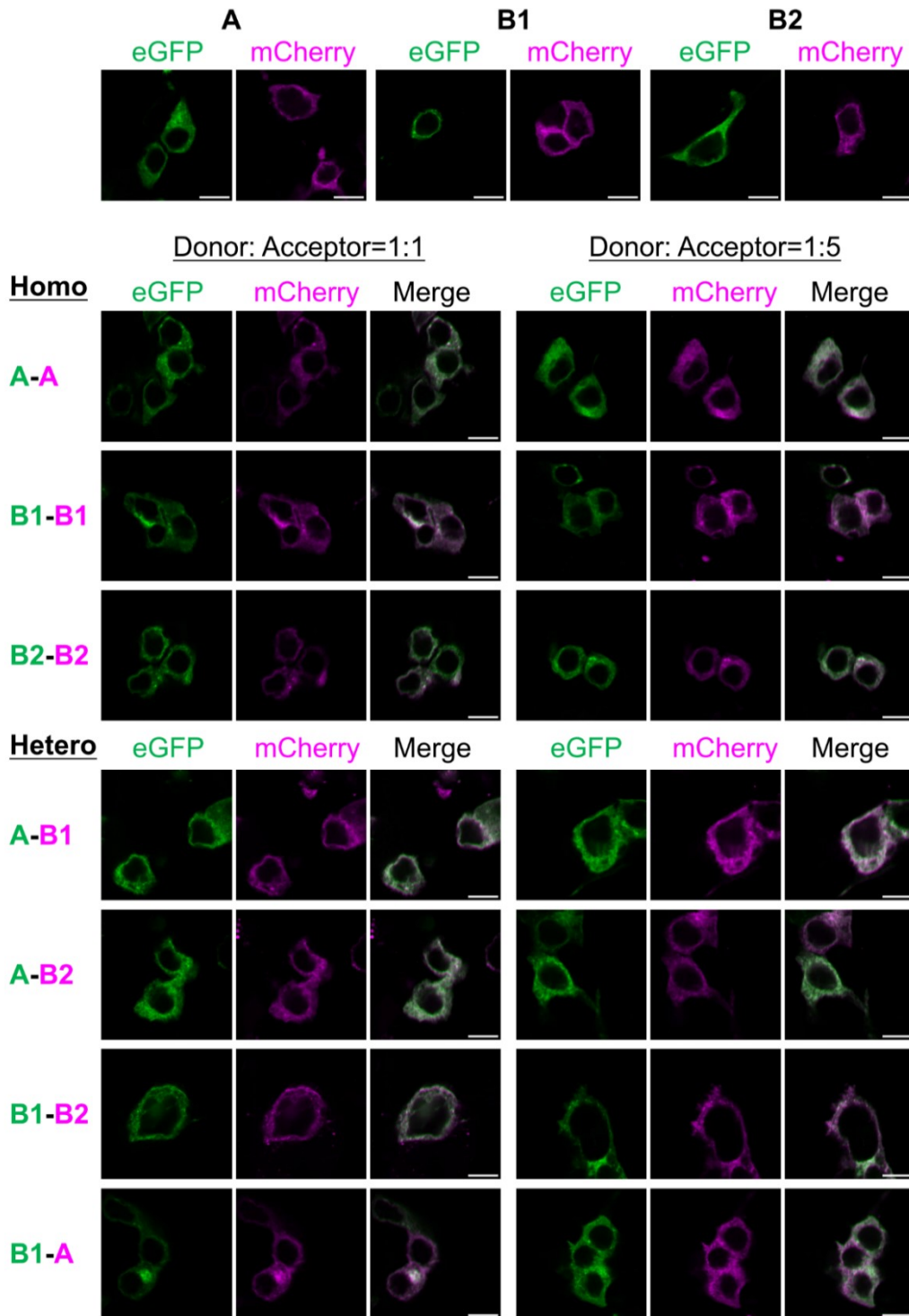
Supplementary Figure 1. Schemes of PIE-based FRET-FLIM experimental design.

After ROI selection, two analysis pipelines were performed: ensemble analysis and pixelwise analysis. PIE mode allows for retrieving stoichiometry information. The ensemble approach concludes the dimerization based on overall changes of donor lifetime and stoichiometry. In the pixelwise approach, the parameters such as donor lifetime and stoichiometry are calculated per pixel and displayed as one- or two-dimensional distributions. Here, the ensemble approach robustly provides information of multiple lifetime species while the pixelwise approach was only used to reveal heterogeneity in live-cell agonist activation experiment.



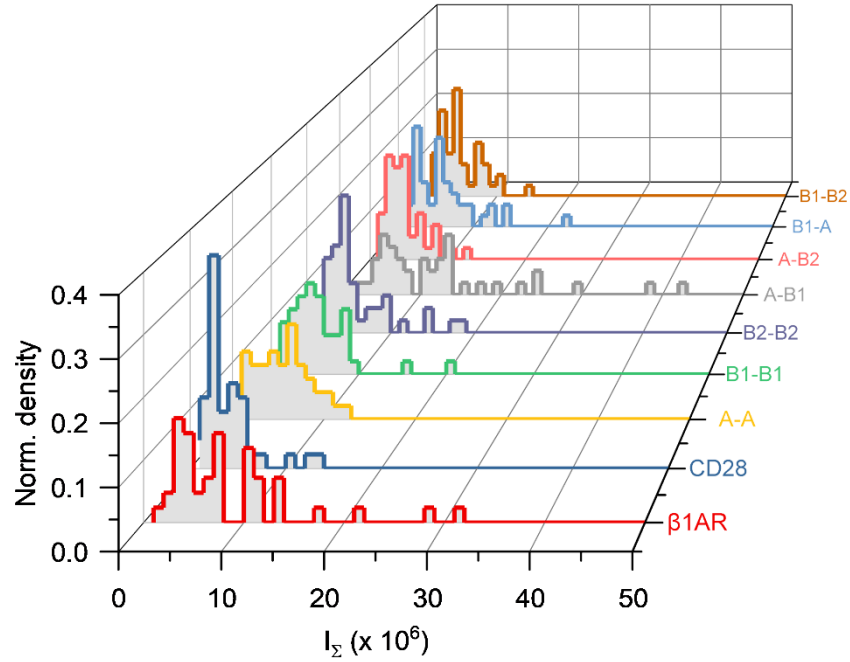
Supplementary Figure 2. Spectral information of fluorescent proteins used in FRET.

Excitation and emission spectra of eGFP and mCherry. Laser lines (480 nm, 560 nm) and filters (520/35 and 600/50) used in the FLIM measurement were indicated.



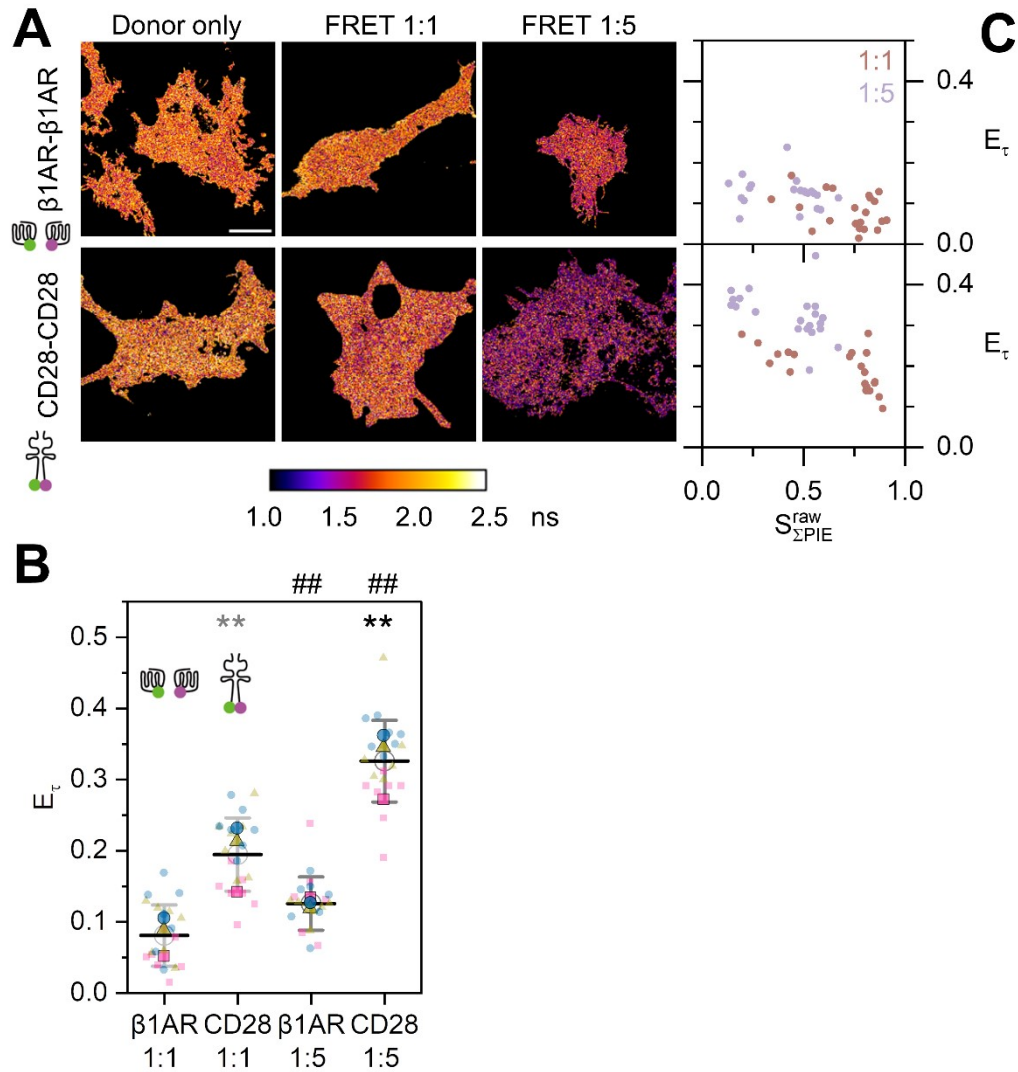
Supplementary Figure 3. Membrane expression of single and double transfected Mc4r.

Mc4r A, B1, B2 expression, by confocal microscopy. Single transfection of eGFP or mCherry fused Mc4r isoforms all show clear cell membrane expression. Double transfection of two Mc4r isoforms with one eGFP tag (donor) and one mCherry tag (acceptor) also exhibit correct cell membrane expression. Scale bar: 10 μm.



Supplementary Figure 4. Total fluorescent signal intensity of all protomers combinations.

Total fluorescent signal intensity I_{Σ} were calculated by **equation (7)**, from all measured cells. Histogram were plotted with 50 bins for each protomer combination. All I_{Σ} were distributed within one order of magnitude.

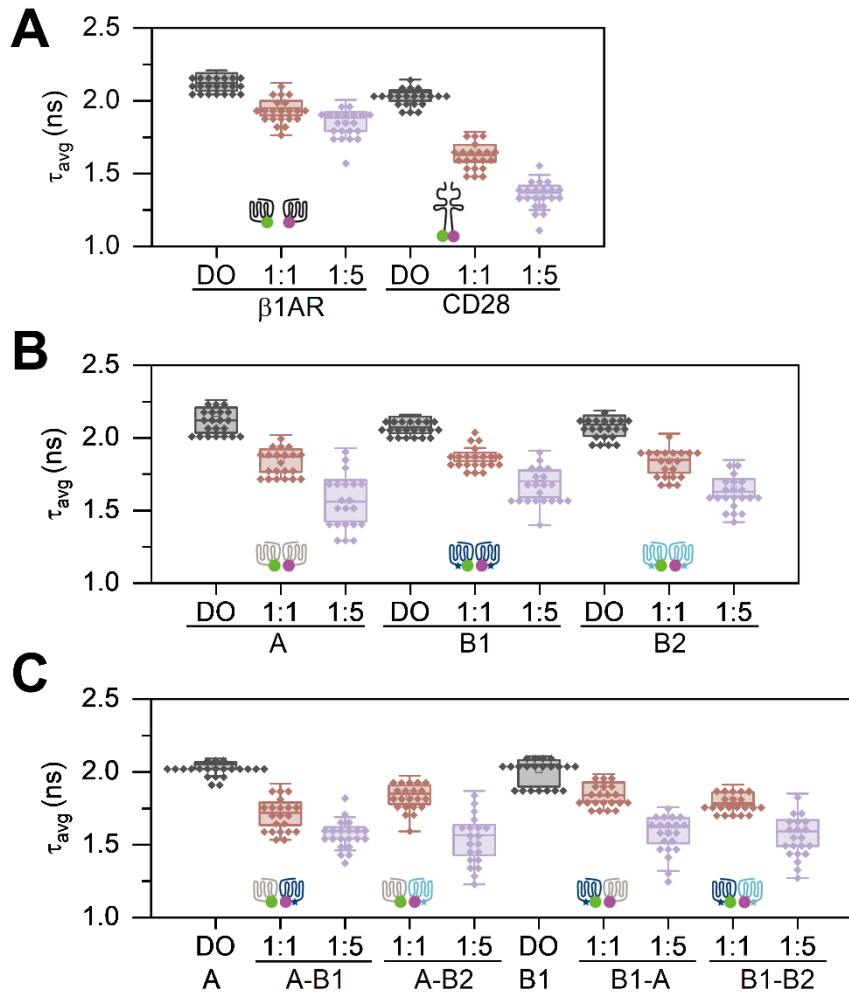


Supplementary Figure 5. Monomer $\beta 1AR$ and dimer CD28 controls.

(A) Lifetime images show species-weighted average lifetime of eGFP. Scale bar: 10 μm .

(B) Superplot of lifetime-based FRET efficiency E_{τ} . In the superplot, data from three replicates are plotted in red squares, yellow triangles, and blue circles, respectively. The mean of each replicate is indicated with the larger sign of same color pattern. The overall mean (horizontal bar), and standard deviation of the mean (error bars) are indicated. Two tails unpaired t -test are used in statistical analysis. Grey *: compared to $\beta 1AR$ 1:1. Black *: compared to $\beta 1AR$ 1:5. Black #: 1:1 vs. 1:5 for each pair. Statistics (summary in **Supplementary Table 2**): ns: not significant, */#: $p < 0.05$, **/##: $p < 0.01$.

(C) 2D scatter plots of lifetime-based FRET efficiency to effective stoichiometry for controls.



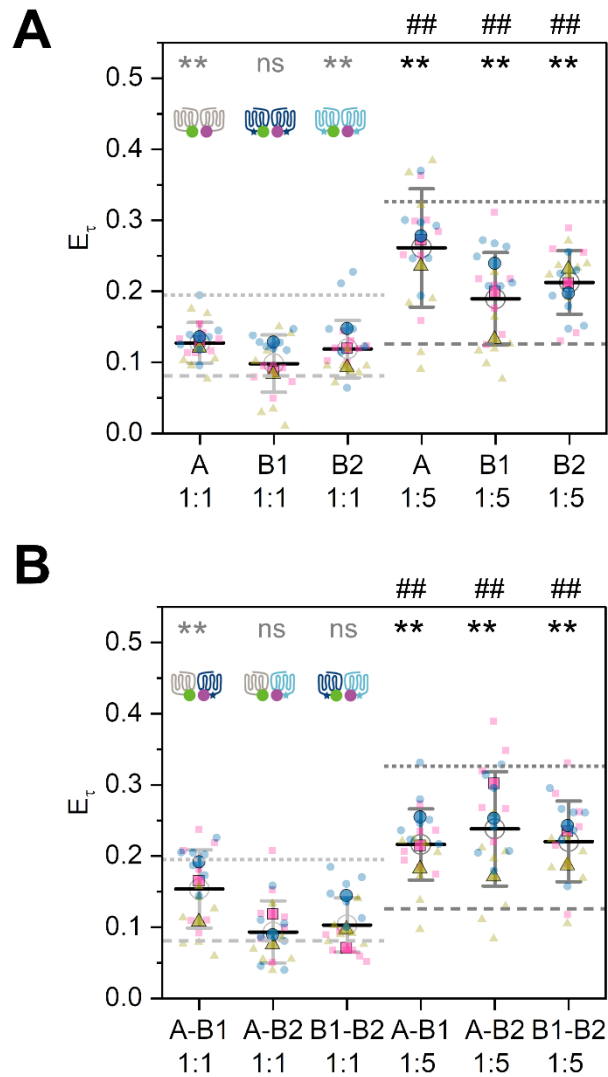
Supplementary Figure 6. Average fluorescence lifetime for two controls and all fish samples.

(A) Controls.

(B) Mc4r homodimers.

(C) Mc4r heterodimers.

In each pair, donor-only samples (black), and samples of donor/acceptor ratio 1:1 (dark orange) and 1:5 (light purple) were displayed. All data are presented as individual points, and the boxes represented median with 25th and 75th percentiles, with whisker at 1.5 interquartile range.

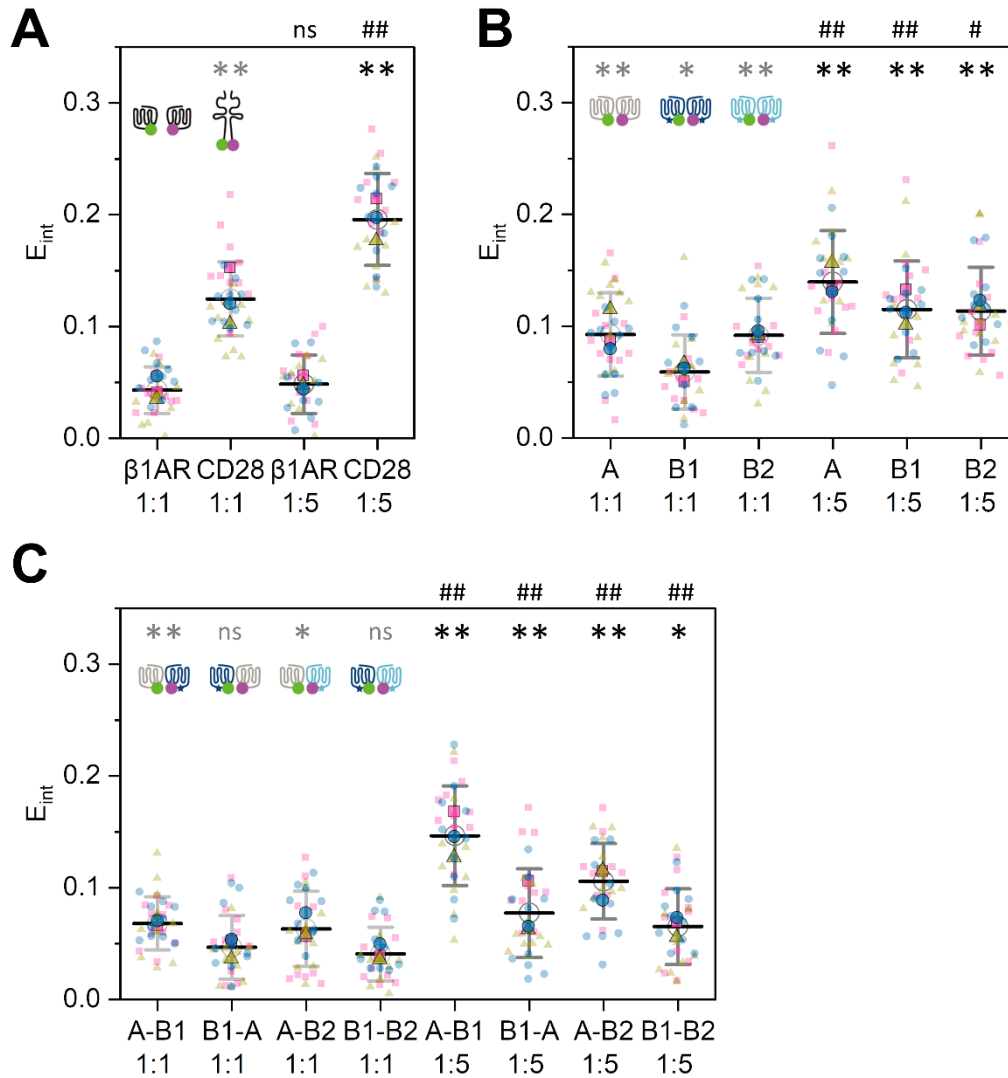


Supplementary Figure 7. Superplots of Mc4r homodimer and heterodimer pairs.

(A) Mc4r homodimers.

(B) Mc4r heterodimers.

Superplot of lifetime-based FRET efficiency E_{τ} . The superplots were designed same as in **Supplementary Figure 5B**. Dotted and dashed lines represent reference lines from CD28 and β 1AR, respectively, at 1:1 (grey) and 1:5 (black). Two tails unpaired t -test are used in statistical analysis. Grey *: compared to β 1AR 1:1. Black *: compared to β 1AR 1:5. Black #: 1:1 vs. 1:5 for each pair. Statistics (summary in **Supplementary Table 2**): ns: not significant, */#: $p < 0.05$, **/###: $p < 0.01$.



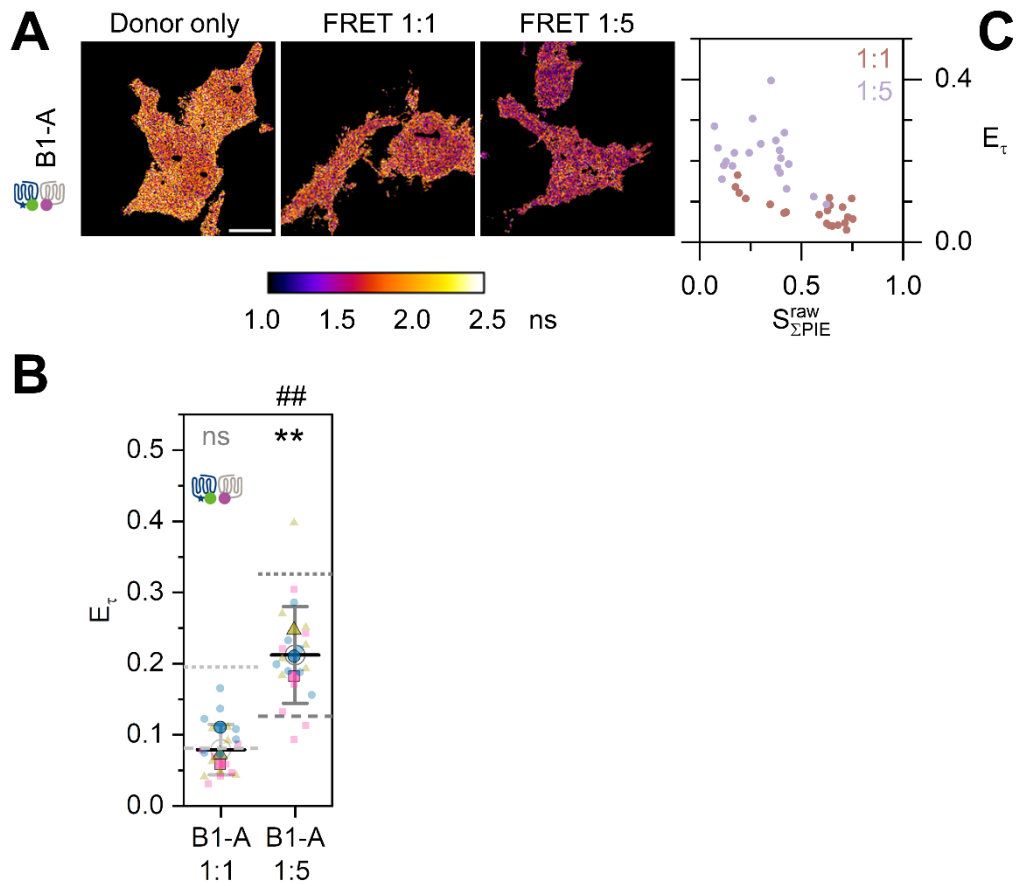
Supplementary Figure 8. FRET-AB measurement of various receptors combinations.

(A) Controls.

(B) Mc4r homodimers.

(C) Mc4r heterodimers.

In each pair, samples with donor/acceptor ratio 1:1 and 1:5 were measured. Superplots were designed same as **Supplementary Figure 5B**. Two tails unpaired *t*-test are used in statistical analysis. Grey *: compared to $\beta 1AR$ 1:1. Black *: compared to $\beta 1AR$ 1:5. Black #: 1:1 vs. 1:5 for each pair. Statistics (summary in **Supplementary Table 3**): ns: not significant, */#: $p < 0.05$, **/###: $p < 0.01$.

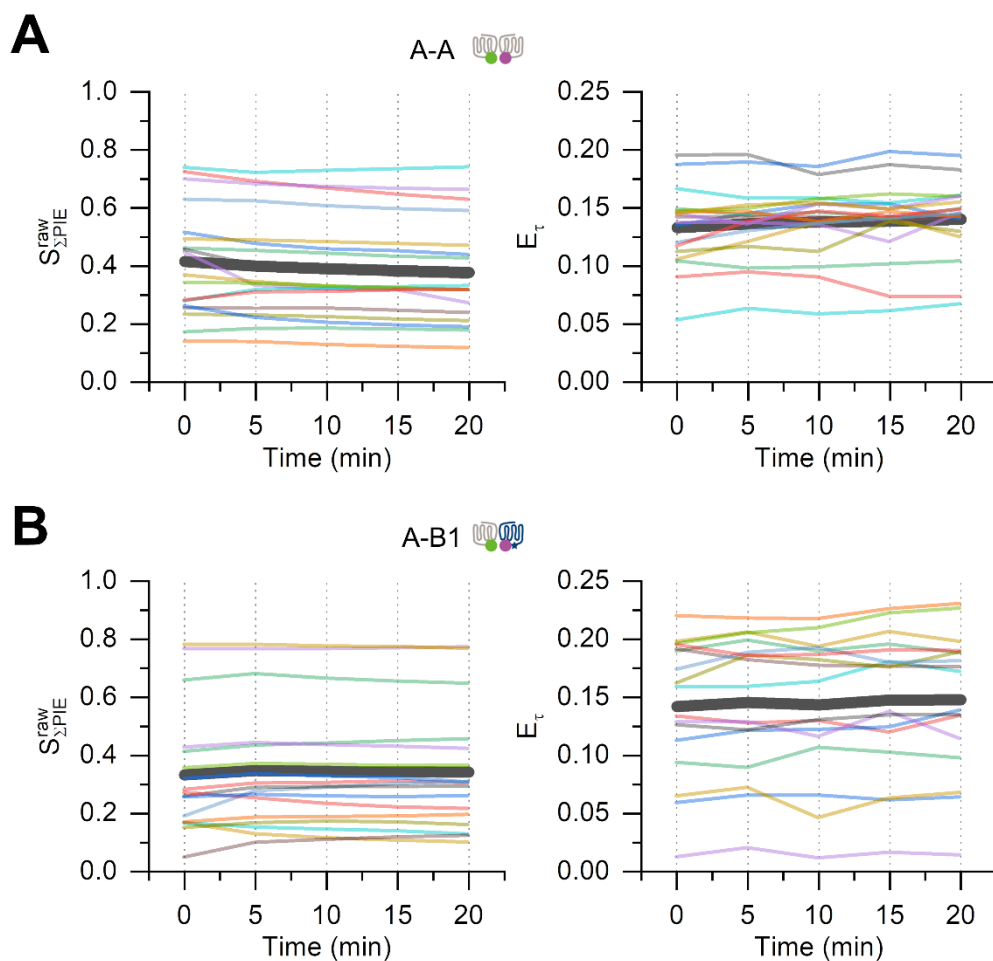


Supplementary Figure 9. Fish Mc4r B1-A heterodimerization.

(A) Lifetime images show species-weighted average lifetime of eGFP. Scale bar: 10 μm .

(B) Superplot of lifetime-based FRET efficiency E_{τ} . The superplots were designed same as in **Supplementary Figure 5B**, **Supplementary Figure 7A**. Dotted and dashed lines represent reference lines from CD28 and $\beta 1\text{AR}$, respectively, at 1:1 (grey) and 1:5 (black). Two tails unpaired t -test are used in statistical analysis. Grey *: compared to $\beta 1\text{AR}$ 1:1. Black *: compared to $\beta 1\text{AR}$ 1:5. Black #: 1:1 vs. 1:5 for each pair. Statistics (summary in **Supplementary Table 2**): ns: not significant, */#: $p < 0.05$, **/##: $p < 0.01$.

(C) 2D scatter plot of lifetime-based FRET efficiency to effective stoichiometry for B1-A.

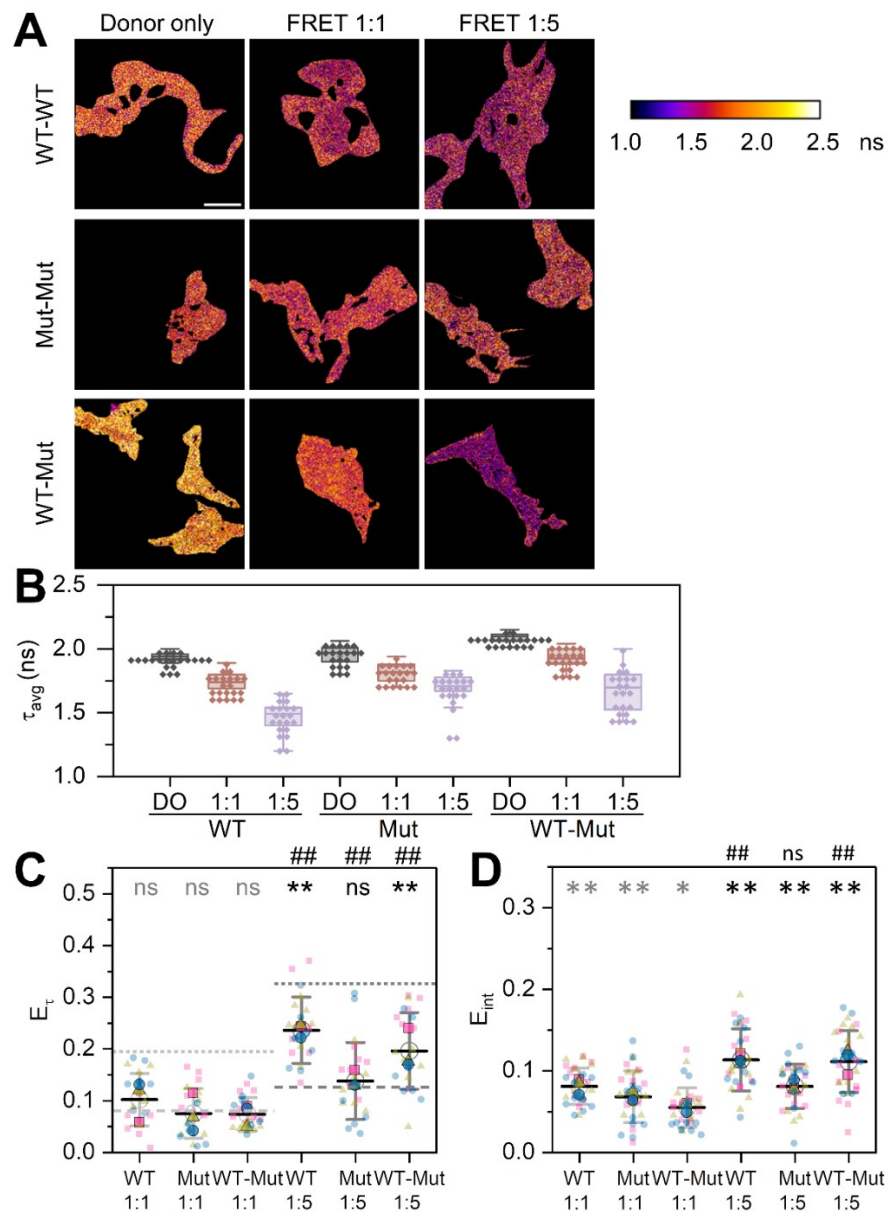


Supplementary Figure 10. Analysis of activated homo- and heterodimers.

(A) Samples A-A with donor/acceptor ratio 1:1.

(B) Samples A-B1 with donor/acceptor ratio 1:1.

Time-course changes were plotted for each cell in transparent thin color lines and the average of all cells are plotted as the thick black lines. Left panels are change of stoichiometry through time and right panels are change of lifetime-based FRET efficiency through time.



Supplementary Figure 11. Dimerization of human MC4R as a comparison.

(A) Lifetime images show species-weighted average lifetime of eGFP. Scale bar: 10 μ m.

(B) Lifetime plots.

(C) FRET-FLIM measurement.

(D) FRET-AB measurement.

Superplots were designed as **Supplementary Figure 7** and **Supplementary Figure 8**. Two tails unpaired *t*-test are used in statistical analysis. Grey *: compared to β 1AR 1:1. Black *: compared to β 1AR 1:5. Black #: 1:1 vs. 1:5 for each pair. Statistics (summary in **Supplementary Table 2** and **Supplementary Table 3**): ns: not significant, */#: $p < 0.05$, **/###: $p < 0.01$.

1.2 Supplementary Tables

Supplementary Table 1. Primers.

Primers	Sequences	Comments
hMC4R_HindIII_kATG_F	CACC AAGCTT GCCGCCATG GTGAACTCCACCCACCGTG	Cloning for human MC4R wild-type/mutant
hMC4R_XbaI_R	TATA TCTAGA ATATCTGCT AGACAAGTCAC	Cloning for human MC4R wild-type/mutant
Mc4rA_HindIII_kATG_F	CACC AAGCTT GCCGCCATG AACTCCACGGCTCAGCAAG	Cloning for <i>Xiphophorus</i> Mc4r A/B1/B2
Mc4rA_XbaI_R	TATA TCTAGA CAGAAAGCT AATACACGAGA	Cloning for <i>Xiphophorus</i> Mc4r A
Mc4rB1_XbaI_R	TATA TCTAGA CAGAAAGCT AATACACCAGA	Cloning for <i>Xiphophorus</i> Mc4r B1
Mc4rB2_XbaI_R	TATA TCTAGA CAGATGAAC TACCTGGAGCG	Cloning for <i>Xiphophorus</i> Mc4r B2
CD28_HindIII_kATG_F	CACC AAGCTT GCCGCCATG CTCAGGCTGCTCTTGGCTC	Cloning for CD28
CD28_XbaI_R	TATA TCTAGA CCTGCTCCT CTTACTCCTCA	Cloning for CD28
b1AR_HindIII_kATG_F	CACC AAGCTT GCCGCCATG GGCGCGGGGGTGCTCGTCC	Cloning for β 1AR
b1AR_XbaI_R	TATA TCTAGA CACCTTGGA TTCCGAGGCCA	Cloning for β 1AR

Note: Red letters indicate restriction sites and blue letter are the Kozak sequence.

Supplementary Table 2. FRET-FLIM statistics summary.

		E_{τ}	\pm	N	t -test 1:1 vs. 1:5		t -test each vs. β 1AR	
β 1AR	1:1	0.081	0.043	21	8.60E-04	***		
	1:5	0.126	0.038	21				
CD28	1:1	0.195	0.051	21	1.43E-09	****	1.61E-09	****
	1:5	0.326	0.057	21			2.26E-16	****
A	1:1	0.127	0.029	21	2.01E-08	****	1.88E-04	***
	1:5	0.261	0.083	21			3.49E-08	****
B1	1:1	0.098	0.040	21	2.73E-06	****	1.88E-01	ns
	1:5	0.189	0.065	21			3.79E-04	***
B2	1:1	0.119	0.040	21	1.26E-08	****	5.46E-03	**
	1:5	0.212	0.045	21			3.55E-08	****
A-B1	1:1	0.154	0.055	21	4.08E-04	***	2.31E-05	****
	1:5	0.216	0.050	21			6.00E-08	****
B1-A	1:1	0.079	0.036	21	9.80E-10	****	8.92E-01	ns
	1:5	0.212	0.068	21			8.80E-06	****
A-B2	1:1	0.093	0.044	21	1.08E-08	****	3.61E-01	ns
	1:5	0.238	0.080	20			1.06E-06	****
B1-B2	1:1	0.103	0.038	21	1.21E-09	****	8.44E-02	ns
	1:5	0.221	0.057	21			1.37E-07	****
WT	1:1	0.102	0.051	21	3.74E-09	****	1.52E-01	ns
	1:5	0.236	0.064	21			3.70E-08	****
Mut	1:1	0.075	0.048	20	2.80E-03	**	6.96E-01	ns
	1:5	0.138	0.074	21			5.03E-01	ns
WT-Mut	1:1	0.074	0.033	22	1.02E-08	****	5.41E-01	ns
	1:5	0.196	0.074	22			3.38E-04	***

Note: E_{τ} represents average lifetime-based FRET efficiency, and \pm indicates SD.

ns $p \geq 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Supplementary Table 3. FRET-AB statistics summary.

		E_{int}	\pm	N	t -test 1:1 vs. 1:5		t -test each vs. β 1AR	
β 1AR	1:1	0.043	0.021	30	3.89E-01	ns		
	1:5	0.048	0.026	30				
CD28	1:1	0.125	0.033	30	6.82E-10	****	1.88E-16	****
	1:5	0.196	0.041	30			1.16E-23	****
A	1:1	0.093	0.037	33	3.48E-05	****	2.24E-08	****
	1:5	0.140	0.046	30			2.67E-13	****
B1	1:1	0.059	0.033	30	6.10E-07	****	2.95E-02	*
	1:5	0.115	0.043	30			1.37E-09	****
B2	1:1	0.092	0.033	30	2.52E-02	*	5.46E-09	****
	1:5	0.113	0.039	30			3.41E-10	****
A-B1	1:1	0.068	0.024	30	8.16E-12	****	6.06E-05	****
	1:5	0.147	0.045	30			6.71E-15	****
B1-A	1:1	0.047	0.028	30	1.09E-03	**	5.69E-01	ns
	1:5	0.077	0.040	30			1.47E-03	**
A-B2	1:1	0.063	0.034	30	8.05E-06	****	7.10E-03	*
	1:5	0.106	0.034	30			7.17E-10	****
B1-B2	1:1	0.041	0.024	30	1.93E-03	**	6.79E-01	ns
	1:5	0.065	0.034	30			3.49E-02	*
WT	1:1	0.081	0.022	30	1.69E-04	***	5.49E-09	****
	1:5	0.113	0.038	30			1.87E-10	****
Mut	1:1	0.068	0.032	30	1.01E-01	ns	5.70E-04	***
	1:5	0.081	0.027	30			1.40E-05	****
WT-Mut	1:1	0.055	0.024	33	1.32E-09	****	4.00E-02	*
	1:5	0.111	0.038	31			3.37E-10	****

Note: E_{int} represents intensity-based FRET efficiency, and \pm indicates SD.

ns $p \geq 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

2 Supplementary Data

Supplementary Script 1. Export Symphotime-based ROIs for grouped/ensemble analysis.

```
#####
# Katherina Hemmen ~ Core Unit Fluorescence Imaging ~ RVZ
# katherina.hemmen@uni-wuerzburg.de
#####

# Converting Symphotime ROIs export for grouped / ensemble analysis

# Input:
# (1) ASCII export of green channel data, where the ROI has been marked in Symphotime
# Data is in txt format, where the top-half of the file corresponds to the green
# photon count and the bottom-half corresponds to the tau(FastFLIM) calculated by Symphotime
# (2) Tif-images exported from Symphotime for the following channels and time windows:
# (i) green prompt, (ii) red prompt and (iii) red delay

# Output:
# (1) Intensity images of the three channels & time windows
# (i) green prompt, (ii) red prompt and (iii) red delay
# where intensity values outside the marked ROI have been set to 0
# (2) Table of summarizing values, with first column being the filename:
# (i) sum of number of green photons in prompt time range (gp)
# (ii) sum of number of red photons in prompt time range (rp)
# (iii) sum of number of red photons in delay time range (rd)
# (iv) green to red intensity ratio in prompt time window
# (v) effective stoichiometry
# (vi) apparent FRET efficiency (proximity ratio)

# 1. Importing of all required modules
from skimage import io
import glob
import os
import numpy as np

# 2. Data to be processed
path = 'C:/Users/Downloads/DATA/*.ptu'
# All files within this folder in PTU format will be used to generate a list of filenames.
save_file_as = 'C:/Users/Downloads/Results.txt'
# The relevant parameter such as sum of photons are saved into a txt document.

# Initialize list of parameters to be saved at the end
list_filenames = list() # filenames
list_nr_green_ph = list() # number of green photons in prompt time range (gp)
list_nr_red_p_ph = list() # number of red photons in prompt time range (rp)
list_nr_red_d_ph = list() # number of red photons in delay time range (rd)
list_SgSr = list() # green to red intensity ratio in prompt time window
list_effSpie = list() # effective stoichiometry
list_appE = list() # apparent FRET efficiency (proximity ratio)

# Loop over all files in folder
for file in glob.glob(path):
    filename = os.path.abspath(file).split(".")[0] # Get the filenames

    print('Processing...' + filename)

# 3. Read and convert Symphotime ASCII file into binary ROI
ROI = filename + '.txt'
ROI_int = np.genfromtxt(ROI, skip_header=3, skip_footer=259) # First half of txt file
ROI_mean_tau = np.genfromtxt(ROI, skip_header=262) # Second half of txt file
io.imsave(filename + '_green_int.tif', ROI_int, check_contrast=False)
io.imsave(filename + '_tauFF.tif', ROI_mean_tau, check_contrast=False)

# ROI_int and ROI_mean_tau have values identical to number of green photons
# / mean lifetime inside the ROI and values = 0 outside the ROI
# To generate the binary ROI (an image filled with 0 outside ROI and 1 inside),
# ROI_int is used and all values > 0 are set to 1
binary_ROI = np.where(ROI_int > 0, 1, 0)
io.imsave(filename + '_ROI.tif', binary_ROI, check_contrast=False)

# 4. Use ROI to generate masks on the intensity images
```

```

# Read the exported tif-files for channels/time windows
# All images have two channels: channel 0 = intensity, channel 1 = tau(FastFLIM)
int_gp = io.imread(filename + '_gp.tif')[0]
int_rp = io.imread(filename + '_rp.tif')[0]
int_rd = io.imread(filename + '_rd.tif')[0]
# All values where the binary ROI = 1 are kept, other pixel values are set 0
masked_intensity_gp = np.where(binary_ROI ==1, int_gp, 0)
masked_intensity_rp = np.where(binary_ROI ==1, int_rp, 0)
masked_intensity_rd = np.where(binary_ROI ==1, int_rd, 0)

# 5. Calculate and save sum of photons
sum_green_ph = np.sum(masked_intensity_gp)
sum_red_prompt_ph = np.sum(masked_intensity_rp)
sum_red_delay_ph = np.sum(masked_intensity_rd)

# 6. Calculate FRET-related parameters
# Note: apparent FRET efficiency and stoichiometry are calculated as no corrections are applied
SgSr = sum_green_ph / sum_red_prompt_ph
effSpie = (sum_green_ph + sum_red_prompt_ph) / (sum_green_ph + sum_red_prompt_ph
+ sum_red_delay_ph)
appE = sum_red_prompt_ph / (sum_red_prompt_ph + sum_green_ph)

# Append all values to a growing list
list_filenames.append(str(file)) # filenames
list_nr_green_ph.append(sum_green_ph) # number of green photons in prompt time range
list_nr_red_p_ph.append(sum_red_prompt_ph) # number of red photons in prompt time range
list_nr_red_d_ph.append(sum_red_delay_ph) # number of red photons in delay time range
list_SgSr.append(SgSr) # green to red intensity ratio in prompt time window
list_effSpie.append(effSpie) # effective stoichiometry
list_appE.append(appE) # apparent FRET efficiency (proximity ratio)

# Column header of saved txt file
header = 'Filename\tNr Green ph\tNr Red ph (prompt)\tNr Red ph (delay)\tSgSr\teffSpie\tappE'

# Save results as txt file
np.savetxt(
save_file_as,
np.vstack(
[
list_filenames,
list_nr_green_ph,
list_nr_red_p_ph,
list_nr_red_d_ph,
list_SgSr,
list_effSpie,
list_appE
]
).T,
delimiter='\t', fmt="%s", header=header
)

```


3 Supplementary Information

Xiphophorus multilineatus Mc4r A

>Xmulti_Mc4r_A_nt

ATGAACTCCACGGCTCAGCAAGGCTTGATCCCATGCTACCTGAACGGGAGCCTGTGCCCGGGAAC TC
TGCCAGAGAAAGACGTTTCCGGAGAGGAGAAGGATTCCCTCTGCTGGCTGCTCTGAGCAGCTGCTGAT
TTCCACCGAGGTCTTCCTCACTTTGGGCATCATCAGCCTGCTGGAGAACATCCTAGTGGTCGCCGCC
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>Xmulti_Mc4r_A_aa

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ASICSLLAIAIDRYITIFYALRYHNI VTI RRALLVIAGIWTCC TVSGILFIIYSESTMVLICLITMF
FTMLVLMASLYVHMFLARQHMKRIAALPGNAPIQQRANMKGAITLTLILLGVFVVCWAPFFLHLILM
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Xiphophorus multilineatus Mc4r B1

>Xmulti_Mc4r_B1_nt

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TGCCAGAGAAAGACGTTTCCGGAGAGGAGAAGGATTCCCTCTGCTGGCTGCTCTGAGCAGCTGCTGAT
TTCCACCGAGGTCTTCCTCACTTTGGGCATCATCAGCCTGCTGGAGAACATCCTAGTGGTCGCCGCC
ATCATCAAGAACAAGAACCTCCATTCCCCGAAGTACTTCTTCATCTGCAGCCTGGCGGTCGCTGACA
TGCTGGTCAGCGTCTCCAACGCCTCGGAGACAATCGTCATAGCGCTGTTCAACGGCGGCAGCCTCAC
CATCCCTGTCACGTTTCATCAAAAGCATGGACAATGTGTTCAACTCCATGATCTGCAGCTCTCTGCTC
GCCTCCATCTGCAGCTTGCTCGCCATCGCCATCGACCGCTACATCACCATCTTCTACGCCCTGCGGT
ACCACAACATCGTCACCATCCGGCGGGCGTTGCTGGTCATCGGCAGCATCTGGACGTGCTGCACCGT
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>Xmulti_Mc4r_B1_aa

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Xiphophorus multilineatus Mc4r B2

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