



Supporting Online Material for

Mammalian Expression of Infrared Fluorescent Proteins Engineered from a Bacterial Phytochrome

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Supporting Online Material

Materials and Methods

Gene synthesis, mutagenesis and screening. A gene encoding IFP1.0 with codons optimized for *Escherichia coli* was synthesized by overlap extension PCR (S1). Genetic libraries were constructed by saturation and random mutagenesis as described (S2) and DNA shuffling (S3). IFP1.0 and mutants were cloned into a modified pBAD vector containing the heme oxygenase-1 gene from cyanobacteria. Libraries were expressed and screened as described (S2). A 676 nm laser was used for FACS screening of large libraries, with 710 – 900 nm emission filter.

Protein expression and characterization. IFPs in the modified pBAD vector were expressed in *E. coli* strain TOP10. Protein purification, fluorescence characterization and photobleaching experiments were done as described (S2). For quantum yield determinations, the integral of the emission spectrum (corrected for the wavelength-dependence of detection sensitivity) of a solution of IFP in PBS was compared with the analogous integral for an equally absorbing solution of Cy5 in PBS, whose quantum yield was assumed to be 0.27 (S4). For extinction coefficient determination, the holoprotein concentration was calculated based on the assumption that the extinction coefficient of holoprotein at 388 nm was equal to that of free BV, which was measured to be 39,900 $M^{-1} cm^{-1}$ in PBS. This is based on the result that the absorbance by the Q band (670 – 700 nm) decreased by ~10-fold after denaturation while the absorbance at 388 nm did not change.

Chimera construction and imaging. DNA encoding IFP1.0 with codons optimized for mammals was synthesized by overlap extension PCR (S1). Other IFPs were created by QuickChange Multi site-directed mutagenesis. AKT1's PH domain was fused to the C terminus of IFP1.4 to generate chimeras IFP1.4-PH^{AKT1}. All the IFPs and chimera were cloned into pcDNA3.1 vector. HEK293A cells were transfected with IFP cDNAs using Fugene, then imaged 24-48 hr later on a Zeiss Axiovert microscope with redshifted Cy5.5 filter set (Chroma) and a cooled CCD camera (Photometrics, Tucson, AZ), controlled by MetaFluor 2.75 software (Universal Imaging, West Chester, PA).

Adenovirus construction. To create adenoviruses expressing IFP1.1 or mKate and GFP, a transcription unit comprising the IFP1.1 or mKate coding sequence, the poliovirus IRES, and GFP was constructed by assembly PCR, cloned into pENTR1a (Invitrogen), and transferred into pAd-CMV-DEST (Invitrogen) by Gateway recombinase (Invitrogen). Viruses were produced in HEK293 cells by transfection followed by one round of amplification, purified by anion exchange chromatography (FastTrap purification kit, Millipore), resuspended in HBSS + 10% glycerol, and stored in aliquots at -80°C. Titers as assessed on HEK293 cells by GFP fluorescence were 5×10^{10} infectious units (IU) per mL for each virus.

Mouse imaging. The University of California San Diego Institutional Animal Care and Use Committee approved the protocol. Albino C57BL/6 mice (Jackson Labs) were injected with 2×10^9 infectious units of adenovirus via tail vein. After 5 days, belly fur was removed using a depilatory cream. Mice were imaged on a spectral imager (Maestro,

Cambridge Research Instruments). The IFP channel was excited with a 650/50 nm (center wavelength/full width at half maximum) bandpass filter with a 700 nm long pass filter in series with the imager's tunable emission filter at 710/40 nm. The mKate channel was 590/24 nm bandpass for excitation and 620/20 in series with 630/40 nm for emission. Images for GFP were acquired with 467/45nm excitation and a 515 nm long pass filter in series with the imager's 530/40 nm for emission. Images were taken with 3 seconds exposure. Images for Fig. 3A were acquired before BV and 1 hr after injection of 250 nanomoles of BV, then scaled so that the brightest pixels after BV administration would display at maximum intensity. mKate and GFP images were first scaled with the same parameters as the IFP images, then the mKate images were further brightened 5-fold to make them visible. For fluorescence time course measurement, background-subtracted images of averaged liver intensity of the same region over liver at different time points after 250 nmol biliverdin injection was divided by the fluorescence intensity after 1 hour (Image J, NIH).

For spectral deconvolution, an image cube was collected on the Maestro with excitation at 620/20 nm and emission at 650-800 nm taking an image every 10 nm. Fluorescence region and autofluorescence regions were identified and spectrally unmixed using the instrument's software, revealing true fluorescent protein signal (displayed in red) and autofluorescence (displayed in grey).

For fluorescence molecular tomographic imaging (FMT), an Ad5I infected mouse was anesthetized with ketamine and midazolam, then injected IV with 250 nmol biliverdin in 10% DMSO. One hour later, the mouse was placed in a FMT 2500 imaging system (VisEn Medical, Bedford MA) and imaged in channel 1 with Prosense 680 settings. Images were reconstructed and windowed (106 – 167 nM apparent concentrations depicted in blue to red pseudocolors) to show the 3D distribution of fluorescence viewed from two different angles (Fig. S9).

After mice were killed, they were dissected and imaged using both IFP and mKate filter sets at 3 levels during dissection: with the skin on, with the skin removed, and then with the peritoneum and rib cage removed. Using Image J software, regions of 80x300 pixels were selected from below the liver up to the mid thorax. These regions were analyzed by plotting the profile. The values were normalized by dividing each pixel by the average of the last 30 vertical pixels over the thorax. These data represent contrast of liver to adjacent thoracic background.

Liver histology. Livers were frozen for cryohistology to compare fluorescence protein signal strength and relative expression. Sections were cut at 10 μ m and then imaged on a fluorescence stereomicroscope (Lumar, Zeiss). Filter sets used were ex 470/40 nm and em 525/50 nm for GFP, ex 560/25 nm and em 607/36 for mKate, and ex 665/45 and em 725/50 for IFP. Images were acquired at 15s exposures for IFP and mKate channels and 3s exposure for the GFP channel, then displayed with intensity enhancements of 2, 1, and 1, respectively. Therefore the relative gains for the IFP, mKate, and GFP channels were 10, 5, and 1, respectively.

SOM Text

Evolution of IFP1.4. Random mutagenesis of IFP1.1 with fluorescence activated cell sorting using a 676 nm laser resulted in IFP1.2 with 32% increase in quantum yield (QY), due to an additional M54V mutation. However, the parent of IFP1.2, *DrCBD*, was previously shown to be a dimer. Multiple angle light scattering at 785 nm (Dawn 8+, Wyatt Technology, Santa Barbara CA) of IFP1.2 gave an apparent molecular weight 80 kDa, about twice the predicted monomeric size of 36.5 kDa, suggesting that IFP1.2 was also a dimer. To monomerize IFP1.2, Leu311 was rationally mutated to a lysine since it is in the hydrophobic dimer interface based on the crystal structure of *DrCBD* (Fig. S3). Size exclusion chromatography (SEC) showed that the resulted mutant (named as IFP1.3) was eluted later than IFP1.2 (Fig. S3B), suggesting that IFP1.3 is possibly a monomer. SEC of IFP1.2/IFP1.3 mixture confirmed the result (Fig. S3B). However, the QY of IFP1.3 was slightly decreased (8%). Another round of random mutagenesis and screening generated IFP1.4 with increased brightness. SEC (Fig. S2) and multiple angle light scattering at 12 μ M concentration (apparent molecular weight 41.5 kDa $\pm$ 10%) confirmed that IFP1.4 is monomeric. A sequence alignment of IFP1.4 with *DrCBD* is shown below with internal mutations shaded in blue. The S2A mutation (shaded in red) is to optimize the Kozak sequence when expressed in mammalian cells, and the C-terminal hexahistidine motif is to enable immobilized metal ion affinity chromatography.

<i>DrCBD</i>	MSRDPLPFFFPPLYLGGPEITTENCEREPIHIPGSIQPHGALLTADGHSGEVLQMSLNAAT	60
IFP1.4	M ^S RDPLPFFFPPLYLGGPEITTENCEREPIHIPGSIQPHGALLTADGHSGEVLQ ^V SLNAAT	60
<i>DrCBD</i>	FLGQEPTVLRGQTLAALLPEQWPALQAALPPGCPDALQYRATLDWPAAGHLSLTVHRVGE	120
IFP1.4	FLGQEPTVLRGQTLAALLPEQWPALQAALPPGCPDALQYRATLDWPAAGHLSLTVHRV ^{AE}	120
<i>DrCBD</i>	LLILEFEPTEAWDSTGPHALRNAMFALESAPNLRALAEVATQTVRELTFGDRVMLYKFAP	180
IFP1.4	LLILEFEPTEAWDS ^T GPHALRNAMFALESAPNLRALAEVATQTVRELTFGDRVMLYKFAP	180
<i>DrCBD</i>	DATGEVIAEARREG ^{LH} AF ^{LG} HRFPASDIPAQARALYTRHLLRLTADTRAAAVPLDPVLNP	240
IFP1.4	DATGE ^M IAEARREG ^{MC} AF ^{LG} HRFPAS ^{HT} IPAQARALYTRHLLRLTADTRAAAVPLDPVLNP	240
<i>DrCBD</i>	QTNAPTPLGGAVLRATSPMHMQYLRNMGVGSLSVSVVVGGLWGLIACHHQTTPYVLPDP	300
IFP1.4	QTNAPTPLGGAVLRATSPMHMQYLRNMGVGSLSVSVVVGGLWGLI ^V CHHQTTPYVLPDP	300
<i>DrCBD</i>	LRTTLEYLGRLLSLQVQVKEA	321
IFP1.4	LRTTLE ^E LGR ^K LS ^G QVQ ^R KEA ^{EFHHHHHH}	329

Increase of cellular IFP fluorescence by exogenous BV. HEK293A cells were transiently transfected with IFP1.4 in one 10-cm dish and incubated for 24 hours, trypsinized and replated into 5 wells of 6-well plate with $\sim$ 400,000 cells per well. After another 24 hours incubation, different amounts of BV (final concentrations 5, 10, 20, 40 μ M) were added, followed by 90 minutes incubation. Then cells were trypsinized and washed with PBS and resuspended for fluorescence measurement by a 96-well plate reader with monochromators (Safire, TECAN). Untransfected HEK293A cells with additions of exogenous BV were used as controls. Infrared fluorescence of transfected HEK293A cells increased upon the increase of added exogenous BV concentration and was saturated at 20 μ M, while the untransfected cells did not show infrared fluorescence either with or without BV (Fig. S6A). Addition of exogenous BV rapidly (within 10 minutes) led to infrared fluorescence of matured P2 cortical neurons, two weeks after

transfection of IFP1.1 (Fig. S6B), which were practically nonfluorescent before addition of BV.

Half-life of IFP1.4. Cycloheximide (30 µg/ml final concentration) was added to HEK293A cells 24 hours after transfection of IFP1.4. Infrared fluorescence was fitted with single exponential decay assuming first-order decay kinetics of protein degradation (S5) (Figs. S7 and S8). The half-life of IFP1.4 in HEK293A cells with or without exogenous BV was calculated to be 4.44 and 3.61 hours, whose average is 4.03 ± 0.41 hours.

Multiple sequence alignment. Below, 130 bacteriophytochrome-like sequences from NCBI database are aligned using DrCBD as the query (S6). It is suggested that readers zoom in to read the alignment.

Query 1 MSRDpfpffplyggpEITTENCEREPIHIGSIOPHGALLTADGHSGEVLQMSLNAAT 60
NP 285374 1 60
YP 488045 30V.IALAEPLRPVEV.A.IEA 66
YP 569534 29M.IALAEPLRPVEV.A.IQA 65
NP 513633 18K.....M.AVPELELRPVEV.V.EA 64
NP 442237 20TAHL.....LVVVLQEPDLTIS.I.A.CTG 51
ZP 01628825 12DISR.NK.....V.FALKEPDLTI..V.D.TFN 53
NP 486939 11 QVDLT..SK.....L.....V.VLQEPDLTI..V.N.TFN 53
YP 321522 12 VDLS..NK.....L.....V.ALQIDTLTI..V.N.TFN 53
NP 488044 7 DVLDT..D.....I.FNDLKAIVAV.S.I.. 48
ZP 01459223 7 LDLSQ.D.....L.G.S.V.AFR.FORLEVV.A.QA 49
NP 948356 28D.....A.....Y.FVSETDLRIASV.A.VED 64
DVLDT..D.....C.....I.FNDELK.VAV.S.IGN 48
NP 779569 17 ADLTD.D.....A.....I.FTDLQTVAA.A.LFD 51
ZP 01623500 7 DQ...Q.S.QV...I.VLEBP.LKI..V.K.TQS 52
YP 324352 10 NVTSLKEA...LS.Q.....V.VLEPELKI..V.N.TWS 51
NP 948355 15D.....A.....L.ALAADM-TVAG.D.LFE 50
NP 487197 10 NVTSLKEA...LS.Q.....V.VLEPELKI..V.N.TWG 51
YP 779570 28DK.....YVFLERLERLIVSV.A.VEK 64
ZP 01630443 10 NVNLSKEA...LPHK.....V.VLEPELKI..I.M.TWS 51
YP 001894923 15 D.A...Q...G...F.FSISA-T.TL..V.A.T.. 51
YP 001863766 19SSQ.....V.VLEPELKI..V.T.TLK 51
NP 001174646 14 DR.AD.....A.....V.VLSEPGQLVTHA.E.PA 54
ZP 01461267 12 VDLT..D.....A.....V.VLSEPGQLVTHA.E.PA 54
ZP 01730981 10 D.NLQRL.ELQ.LW.KT...V.FVL.ESNLKIV.T.S.TQ 52
NP 354963 9 DG.GA.....A.E.....VLSAREFS.V.A.D.L.N 47
YP 608942 13R.AQ...QV.....F.VL.NT.LRI..A.E.TER 51
YP 001668195 12R.AQ...L.S.....F.VL.ATDLR..A.E.VEH 50
NP 001769101 16D.....L.T.T..F.AVQ.PDLRI..I.D.VFD 52
YP 001748669 19R.AQ...QV.....F.VL.BQDLRI..A.D.VER 57
NP 744505 19R.AQ...QV.....F.VL.ATDLR..A.E.VEH 57
NP 945475 15 VDLS.S.D.LVQY.EA.....M.V.DQ.BK.HA.A.C.A 66
YP 001268649 12R.AQ...QV.....F.VL.ATDLR..A.E.VEH 50
NP 791725 10 VLLA..AD...QF..A.....L.F.LKEPELTI..V.A.VQS 52
YP 569510 12 VDLST.D.LVQY.EA.....M.V.DQ.HLMDA.A.C.E 66
NP 347894 15AD...RF..A.....L.....LSRPELII.I.A.V.E 52
YP 001864851 16 HD.K.....I..ALSTQL..IV.V.N.TOV 52
NP 460007 6 DV.LD...HK.....I..LPEPILLQIC.E.T.D 48
YP 236574 10 VLLA..AD...QF..A.....L.F.LAEPELTI..V.A.VQ 52
NP 792460 15 JALAE.A...R...A.....V.SVA.DPLCIE.V.A.C.K 57
ZP 01623038 12 VLLA..AD...QF..A.....L.F.LAEPELTI..V.A.VQ 52
NP 262277 15AD...RS..A.....V.....LSRPELRIQ.I.A.V.EA 52
ZP 01050418 25 K.DLT..DK.....LL.QV.S.AY.FVI.VLMQVLVRA.E.VFD 67
ZP 01623038 12 DLT..D.....Q.....V.FORLDTI..A.Q.ID 53
ZP 01094090 8 KVDLD..D.....Q.....V.AF.PQ.LVLRVM.Q...M 50
NP 235462 20 JALAE.A...RV..A.....V.SVA.DPLCIE.V.A.C.. 62
YP 780275 18E.LA.....V.VSEPEHRII.A.A.D 55
NP 001900695 26A..SR.V..CLSEPELRIH.V.E.LEA 52
YP 861991 10 KVDLS.S.K.....I.TS.A..VIVAY.RK.SKIT.IGE.CKE 52
ZP 02008304 26A..SR.V..CLSEPELRIQ.V.E.LE 52
YP 001242244 14LA.....Q..LA.....I..AVKEPDLN.I.A.T..A 54
NP 676783 12 VNMQ..DO.....Q.....F.IAITKEM.TRPC.E.VID 53
ZP 570910 21D..A..LA.....AVPELQIVMAGDET.A 57
YP 001203744 14LA.....Q..LA.....I..AVKEPDLN.I.A.T..A 54
NP 001196150 12 V.LT..DO.....Q.....F.IGLT.NMWDICTS.ISS 52
ZP 01061101 12 VSLT..DK.....L.K.H..F.VAC.ASMHTTIC.D.ISA 54
ZP 01884257 5 QVDTL..DK.....K..SQ.F.IAV.IQ.YQ.TTV.D.ISA 47
NP 531190 17E.LA.....V.VSEPEHRII.A.A.D 55
NP 487638 24A.....L.ALAADLTVAGAT.S 50
YP 001135775 22DLD..A.....V.R.V.VVREPEF..R.V.A.VEE 63
NP 001361736 20 DL.....A.....V.AVBERGDR.VVA.A..G 60
NP 001196265 12 V.LT...H.....F.IGL.SDL-KTCTA.ISL 52
YP 715500 20D.....V.....V.AVRADDT.V.V.A.I.E 58
ZP 01227360 24D.....M.V..RGTL.VA--G.GD 57
NP 252806 7 V.LA...D...V..A.....V.LEA-D.M..AA.E.IQA 47
YP 789005 7 V.LA...D...V..A.....IALRA-D.M..AA.E.IQA 47
ZP 001417343 20D.....R..A.....L.V..SRT--KVVA.GD 53
NP 00973293 7 V.LA...D...V..A.....IALRA-D.M..AA.E.IQA 47
ZP 02055103 182NAV.M.A..AA.L.I.AF.A..D 218
NP 001641522 182NAV.M.A..AA.L.I.AF.A..D 218
ZP 00974888 7 V.LA...D...V..A.....V.LEA-D.M..AA.E.IQA 47
NP 570862 18Q.Q..LA.....VISEPDRH.I.A.A..E 55
ZP 001239104 30D.....RT..A.....L..QL.PEELRITHAGDG.V 66
ZP 01439288 14D.....LL.AV.DP..F.V.VSFMQLQV.A.LES 49
NP 245299 13A.....L..Y.V..VI.PAD.RIV.A.TT..D 49
NP 639488 13A.....L..Y.V..VI.PAD.RIV.A.TT..D 49
NP 487589 19Q.Q..LA.....VISEPDRH.I.A.A..E 55
YP 001770032 151DPDL.A...V..A.....AV..AC.AATL.AAC.A..E 192
NP 001238408 4 QVNLTL..D.....V..F.F..ALVSDP-TICMA.E..PD 45
NP 485786 15D.....A.....A..A..RVDSV.THA.A.LEE 51
YP 001346361 7 V.LA...D...V..A.....IALRA-D.M..AA.E.IQA 47
NP 198931 13A.....L..Y.V..VIEPAD.RIV.A.ST..E 49
NP 366123 13A.....L..Y.V..VIEPAD.RIV.A.ST..D 49
NP 355125 12D.....Y.....C.IAC.NAMM..RH.E.CGE 49
NP 644587 13AQ.....L..Y.V..VIEPAD.RIV.A.ST..D 49
ZP 01302507 17 VDLS..D.....L.AV..F.F.....ML--ABA.A.E.Q 57
NP 001170431 10T..AT..A.....V..QAD..L.AHA.A.L.E 46
NP 001204102 5 VNLT..D.....V..V..F.F..ALVSDP-TICMA.E..PD 45
NP 971319 14LD.....D.....VAF..D.V.RVA.E.D 53
ZP 01017885 17D.....QL.H..SF.F.FCLSS-DMSI.KV.E.VNQ 53
ZP 01157401 11 V.L...A.....VL.R..F.C.IAVS..A-----A.TD 52
NP 742336 14A.A..V..AV..S.C.VSL.QLRLR..A.A.LHR 52
ZP 00988358 24D.....KL.AV.SY.C.VLSA-DWI..HA.A.VKS 60
NP 946341 4 KVDLTS.D.....C.C..AC.AQAVITRI.E..GA 46
NP 611325 16 LDS.DS...LS.L.NT..TVQ.PET.LIIGA.E.HL 56
YP 001749437 6 DVNLT..D.....Q.....C..AC.ASASL..RH.V.TSQ 48
NP 192038 26 S.D.....R.DA..Y.L..VV.ST.LKIIG--G.GD 60
NP 001668821 6 QVNLTL..D.....Q.....C..AC.ASATV..RH.V.PQ 48
ZP 01545930 3 QVDTL..D.....LL.KV.SF.F.I..TTPDWIISHV.R.VSQ 44
NP 001523831 11D.....C.IACTPDA.HI.CH.A..D 48
NP 745504 6 QVNLTL..D.....Q.....C..AC.ASATV..RH.V.PQ 48
YP 345327 13M...AT..A.....M..RAD..R.AHA.V.LGE 49
NP 001520152 5 LAIS..D...V...R..IF..IAT.K.LESITHV.S.ISD 47
NP 001267717 7 VNLT..D.....Q.....C..AC.ASATV..RH.V.PQ 48
YP 345384 62M...AT..A.....M..RAD..R.AHA.V.LGE 98
NP 001372987 7 VNLT..D.....M.V..QSQNL.PA.A.IPC 48
ZP 01011420 19D.....LL.RV.SY..V..LSFOWTQHA.E.LG 55
ZP 01864718 7 DVLDT..D.....RL.H..AF..IAVNA-DWFLA.R.A.LQS 48
NP 769292 12D.....QL..V..F.F..AVSS-DWI.MRA.A.L.E 48
ZP 00208733 13 QC..S.H.LS.E..F..RL.P-D.Q.SHA.A.CDA 50
ZP 01726549 7 QLNFRD..D.....EL..GY.Y.FALEEQ...KIV.D.IS 49
NP 946838 30L..A.....A..ALAPDLTVHAGAT.S 59
NP 745313 24D.....W..A.....F.I..VSLRW.TRV.A.I.D 59
ZP 01041311 13D.....QL.RV.SY...AVTT-DWI.QHA.E.LGD 54
ZP 01748188 18R..D.....E.V.SY.F.IAFSYDLMSIV.E.CE 49
ZP 01109672 11GSL.LQHINL..DY.Y..IL.IDELK.I.A.E.TDA 48
NP 01884592 12GSL..SPVNG..DY.F..VC.P-ALI...V.D..EA 46
ZP 01110306 12 Q...D.A..LIE.V.SF.VIVAINPTKQ.VF.E.HD 50
NP 01109728 15DK.....LL.K..NQ.FMVIV..KA.V.THL.E.LEH 50
ZP 427322 17DQ.....V..LV...F.VVL.SK..RIA.VTGPRA 53
NP 00514108 20M.....V..I..KFTFI..V.E.TK 48
ZP 01043109 10 .EAMK..AK...VQA.QCV.EN.M..AV.KRG.QIIAV.D.SDA 53
ZP 01051640 19NN...EWIE.V.S..V..AF.FD.LI.THA.K.LD.. 57
ZP 01736013 12KKLQET.F..Q.V..AL.SQFF-IGAC.E.VIE 46
ZP 01446334 18 D.D.....QL.RV.SY.....LSTDWI.QHA.E.LEA 54

[illegible]

[illegible]

NP 285374	181	GNVIAERAREGLHAGHGFPSADIPQARALVTLhrllatdraasVFLDPLVN	2400
YP 488045	191	WS.Q.Q.D.D.D.SISPDL.DPH.S.S.S.SMT.VIP.IGYRFS.L.DR	2501
YP 488046	191	WG.Q.Q.D.D.VDP.VFYSA.L.C.A.R.R.VNRI.VIP.VLAVR.L.DR	2502
YP 533633	191	WN.Q.V.D.D.SSDISF.DPH.S.S.S.SNT.IIP.IGYRFS.L.DR	2503
NP 442237	181	NNH.D.D.D.D.DMDEPY.LHY.E.QP.R.FHNNI.VIP.VYV.T.AV	2404
YP 0121627	181	GN.S.G.S.V.P.TS.VY.LHY.Q.Q.K.QQW.VIP.VYV.T.AV	2405
NP 486393	191	OWN.K.W.V.K.P.Y.TS.VY.LHY.Q.Q.K.QQW.VIP.VYV.T.AV	2406
YP 321522	181	OWN.K.W.V.P.Y.TS.VY.LHY.Q.Q.K.QQW.VIP.VYV.T.AV	2407
NP 01459223	166	WH.L.L.S.K.G.MDG.MH.T.V.N.N.P.I.A.RP.L.P.VHV	225
NP 948356	190	WS.Q.Q.D.D.D.SISPDL.DPH.S.S.S.SMT.VIP.IGYRFS.V.DI	2499
YP 779569	177	LS.Q.Q.D.D.S.VESY.LH.P.K.K.AINPV.IPMIDIVR.IAD	236
NP 01429350	182	BGH.V.D.KT.E.EPY.LHY.E.KP.K.L.ENMP.IIP.AQ.O.A.F.K.D	241
NP 948355	176	FS.D.D.CAEVSEY.LH.F.R.R.INPV.IIP.VYV.T.AV	235
NP 487197	181	GH.S.S.EKLS.EPY.LHY.E.KP.K.FISNIS.VIPMAQ.O.IOMI.A	240
NP 0130443	181	GH.S.S.EKLS.SMEPY.LHY.E.KP.K.FVANSI.VIP.SH.OIQI.AQ	240
NP 01894923	184	TGS.H.H.VADP.VASYADQN.L.C.A.Q.V.NRI.II.ADVYR.V.P.H	243
NP 01304983	180	EDN.L.L.VADP.VSYA.L.C.A.Q.R.RDN.I.VIQ.ANYKFS.L.DR	242
NP 01471664	180	EDN.L.L.VADP.VSYA.L.C.A.Q.R.RDN.I.VIQ.ANYKFS.L.DR	242
NP 01461267	182	ENH.S.S.DADADPY.LHY.R.E.Q.INLM.IIPIIDIPQARV.L.D	241
NP 01304983	180	EDN.L.L.VADP.VSYA.L.C.A.Q.R.RDN.I.VIQ.ANYKFS.L.DR	242
NP 354963	162	WS.H.V.V.GSGA.PSV.L.G.Q.Q.QR.MIP.VDYK.P.IR.EV.A	237
YP 608942	178	BGN.T.L.VADP.VPOY.LC.A.R.R.RINRI.VIP.ADY.S.V.AA	237
NP 01769101	183	WN.T.V.DGNGV.PSV.DL.R.R.E.NRI.VIP.ADY.S.V.AA	237
NP 01748669	188	BGN.Q.L.VADA.VFYR.LC.A.R.R.RVNI.VIP.ADY.S.V.AA	237
NP 01398195	183	GN.Q.Q.V.D.D.VDP.VFYSA.L.C.A.R.RVNI.VIP.VLAVR.L.DR	237
NP 945475	183	GS.Q.Q.V.D.D.EPY.LHY.T.P.P.R.FALSW.HLP.VYTV.LA	236
NP 01266649	183	BGN.Q.L.VADP.VFYSA.LC.A.R.R.RVNI.VIP.ADY.S.V.AA	236
NP 01304983	180	EDN.L.L.VADP.VSYA.L.C.A.Q.R.RDN.I.VIQ.ANYKFS.L.DR	242
YP 569510	183	GS.H.K.G.D.EPY.LHY.T.P.P.R.FSLSW.HLP.VYTV.IA	236
YP 347894	177	BGH.Q.Q.SDSMEVN.N.L.F.E.E.R.TNIN.IIPMAPOV.V.K.R	236
NP 01600001	183	GN.Q.Q.V.D.D.VDP.VFYSA.L.C.A.R.RVNI.VIP.VLAVR.L.DR	236
NP 660007	197	YN.V.V.KKE.NS.KQH.E.K.E.VNPI.L.VN.QKGS.V	236
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NP 01505418	185	WN.NI.T.S.E.V.E.SV.MH.V.T.Q.KSFLQGV.II.VASOT.FSK.E	244
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NP 01620308	182	GS.Q.Q.V.D.D.KKE.EPY.LHY.T.L.K.K.QCM.I.M.IPNV.DIV	241
NP 235462	167	BGH.Q.V.Q.LTGP.PSVS.LN.G.T.E.RLWV.IIP.VYTV.IA	236
NP 780275	174	BGH.F.S.R.KPE.EPY.N.Y.S.QM.R.E.TRV.VLV.VYTV.Q.R	235
NP 01398195	183	GN.Q.Q.V.D.D.VDP.VFYSA.L.C.A.R.RVNI.VIP.VLAVR.L.DR	236
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NP 01196265	175	LYN.V.F.S.C.D.EPY.LHY.LT.V.E.IK.N.VIG.VYTV.II.VTID	232
NP 01227360	177	GA.S.V.D.APH.VS.N.Q.T.K.V.NRI.VIP.VYTV.IA	236
NP 01728373	121	WH.K.V.S.KITIDISY.LH.E.PLS.H.FSLOP.FIPVNVYS.KFL.N	180
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NP 789005	168	DS.V.S.D.E.SV.QY.Q.Q.NQPI.II.VAIVEMVL.T	227
NP 001417343	171	EA.V.L.L.KAPEMS.N.H.O.G.V.R.V.NRV.VIP.VAIVR.A	228
NP 01645222	146	WN.V.D.D.E.SV.QY.Q.Q.NQPI.II.VAIVEMVL.T	227
NP 01645222	146	WN.V.D.D.E.SV.QY.Q.Q.NQPI.II.VAIVEMVL.T	227
NP 570862	174	BGH.F.S.RVVP.EPY.N.Y.S.QM.R.E.TRV.VLV.VYTV.Q.R	235
NP 01239104	189	GS.A.V.AAPDES.LHY.K.E.R.TNIN.IIP.A.V.PA.II.LSDA	248
NP 245299	173</		

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YP 533633	251	V.GG.DD.SVS.SV.I.VE.MV.A.YGAM.I.R.R.R..M.S.NTL.RFYAVE	310
NP 442237	241	S..RAVD.TS1.SIYAH.LT.K..A..TI.LIKDK..K..I.FE	300
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NP 486399	241	LNQD.LD.SRS.SV.L.IE.MQ..TA.M.I.I.MKNQ..S.KYI.YE	300
YP 321522	241	LN.Q.LD.SRS.SV.L.IE.MQ..TA.M.I.I.MKNQ..S.KYI.YE	300
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ZP 01459223	226	ALGR.LD.SNSA.SV.L.E.MQ..A.F.L.LLKE..L.LHSIHE	285
NP 948356	250	LQGD.D.SFS.SV.T.L.E.MV..MHAAM.I.I.RDNR..M.NL.RFYVE	300
YP 324792	241	LRG.DD.SVS.SV.I.VE.MV.A.YGAM.I.R.R.R..M.S.NTL.RFYAVE	310
YP 779569	241	LOG.DD.SFT.SV.T.L.E.MV..MGRGT..ILR.ER..V.SRE.RPVH	296
ZP 0162350	242	LIER.LD.THST.SA.SC.L..K..A..TI.LIDRK..S.KYI.YE	301
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NP 01769101	241	SSGR.LD.SQSI.SV.V.V.E.M..TIA.M.M..I.L.D.A..S.NKI.RFVQ	302
NP 01748669	248	R.GK.LDMS.A.SV.L.V.L.M..TIA.M.L.I..V.SD.QD.RPVDL	307
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NP 569510	243	LVTV.DVMSF.A.SV.V.V.TG..K..D.R.T.VMPI.K.E.K..M.A.ARHIS	303
YP 347894	241	Q.TD.LT.SFT.SV.I.C.M.K..L.M..I.LK.DK..S.GNRO.LHV	296
NP 01600001	241	NDR.VD.SVTV.SV.I.C..NA.M.I.I..S.K..YRSH.V.FE	211
YP 236574	237	P.QOQD.SFST.SV.I.C.M.K..L.M..I.LK..S.GNR.LYSHVE	296
NP 757602	237	P.QOQD.SFST.SV.I.C.M.K..L.M..I.LK..S.GNR.LYSHVE	296
NP 0516991	237	UNDTAL.THST.SA.C.I.E.H..A..TI.LIDRK..L.KYV.YE	134
ZP 01505418	245	..QQ.LD.THES.S.I.E.H..E.K..AT.TAAT.SN.I..YSSKFYV	310
ZP 01623038	241	LN.Q.LD.SNS.SV.I.VE.Q..AA.C.I.LIKD..M.V.YEAKYVYSE	301
YP 235462	242	A.QO.LD.FST.SV.V.C.E.K..R.M.I.LLD..V.G.FE.LYRVE	301
YP 780276	234	G.LGRLDMSFT.SM.I.L.Q..RAT.V.LD.M.K..V..YF.RFVH	293
NP 861991	232	VN.K.LD.TNSK.QV..A..TAALIN.S..L.N.SKFYN	291
ZP 02008304	241	S.E.GRA.D.TY.A..A.RAQRGT.G.ARA..TALAT.R.S..A.C.SF	298
NP 676783	239	NRQD.LD.SVS.SV.I.VE..H.I..AT.TI.LIHKK..S.YI.KYE	292
YP 570910	241	R.G.LD.LD.SQS.I.SV.A.RL.H.A.VA.M.L.IOHR..SSK.YR	300
NP 01196150	238	SKDNKL.SI.I.SV..VE.K..AT.TI.LIHRD..YSSKYS.A	292
ZP 01601160	238	QBNJ.DD.SISET..S.I.E..K..A..TAAIST.T..YF.KPNVY	292
YP 531190	233	G.LGRLDMSFT.SM.L.L.Q..RAT.V.LD.M.K..YF.RFVH	292
NP 487368	244	G.LGRLD.SVS.SV.I.VE..H.A.VA.M.L.IOHR..S.SA.YR	303
NP 01361735	248	G.G..LD.SHS.SV.I.VE.K..A.M.I.LI.E.R..V.YGHR.G	308
NP 01196265	235	KE.KNLD.SLSI.SV.I.VE.K..AT.TI.LIHRD..YSSKYS.E	294
ZP 01227360	235	AGLSEVD.SVSG.SV.I.R.Q..A.A.A.I.I.M.H.V..DEKASIST	296
YP 01728737	218	I.EAERL.SYCI.GVA.V.L.E.Q..IAA.M.TI.LKNN..NK.KLVISG	240
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NP 01417343	231	GAGSDLDMSFT.SV.I.V.K..A.A.A.I..KD..V.R.ARI.YE	293
ZP 02055103	406	AG..ID.TF.QN.TL..I.E.Q..LDG.M.I.M.NNR..MIG.R.HYVA	465
NP 01641522	406	AG..ID.TF.QN.TL..I.E.Q..LDG.M.I.M.NNR..MIG.R.HYVA	465
YP 570862	234	F.QOQDMSFT.SM.L.L.Q..RAT.V..L.I.LK..YF.RFVH	293
NP 01239104	249	PAA.ALD.SQST.SV.I.R.E..A.VA.M.M.IHR..RE.RY	308
YP 245299	233	LGT.VD.SDVS.SV.L.E.A..TAT.VA.I..NDA..S.YF.HPTNIA	292
NP 639488	233	LGT.VD.SDVS.SV.L.E.A..TAT.VA.I..NDA..S.YF.HPTNIA	292
NP 01770032	375	DAPG.DI.TF.QS.SL..I.E.Q..L.NG.M..ILLR..MIG.RK.HYVA	434
NP 01239408	228	VH.GLD.SMS..V.I.IE..M..AA.M..LLRD.K..F.YA.HSFE	288
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[illegible]

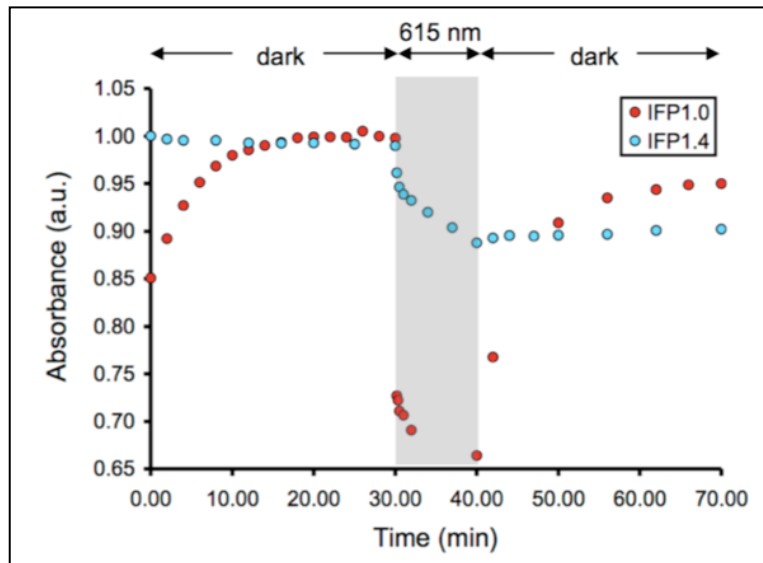


Fig. S1 Dark and light adapted behavior of IFP1.0 and IFP1.4. Absorbance spectra at different time points were taken in the dark (0 – 30 min.), upon 615 nm light illumination by solar simulator (30 – 40 min.), and then in the dark again (40 – 70 min.).

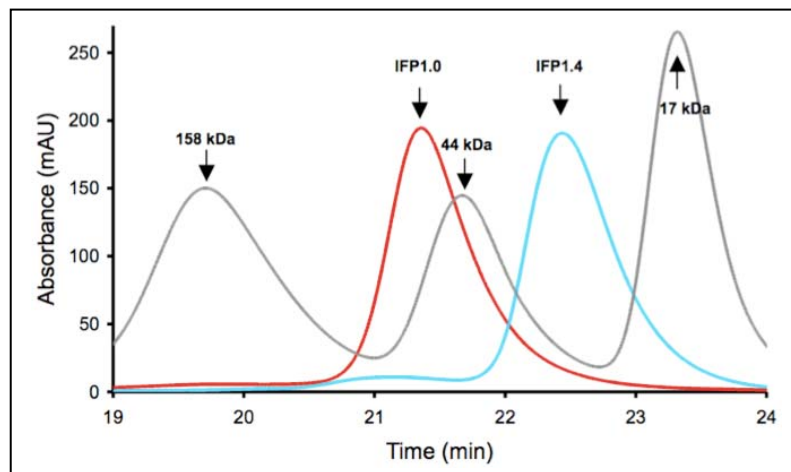


Fig. S2 Size exclusion chromatography of IFP1.0 and IFP1.4. Three standards are also shown: γ -globulin (158 kDa), ovalbumin (44 kDa) and myoglobin (17 kDa). Absorbance was measured at 280 nm.

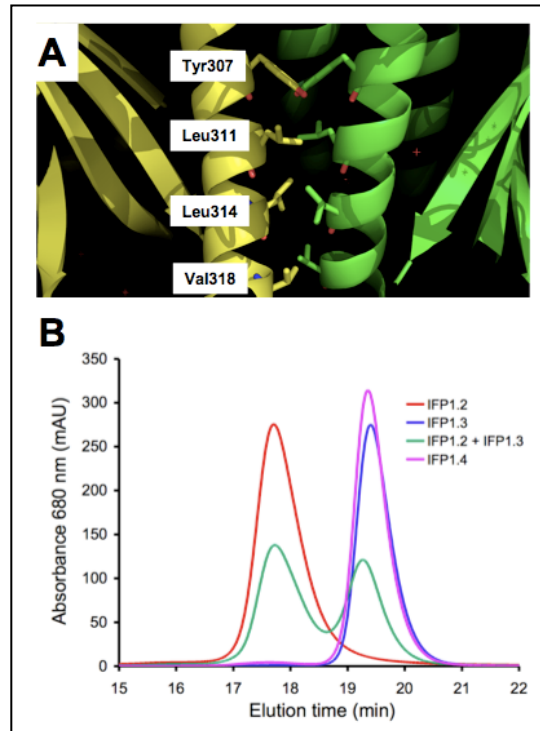


Fig. S3 IFP monomerization by L311K mutation. (A) The dimer interface in DrCBD is formed from 4 residues (Y307/L311/L314/V318). (B) Size exclusion chromatography of IFP1.2, 1.3, 1.4 and a mixture of IFP1.2 and 1.3.

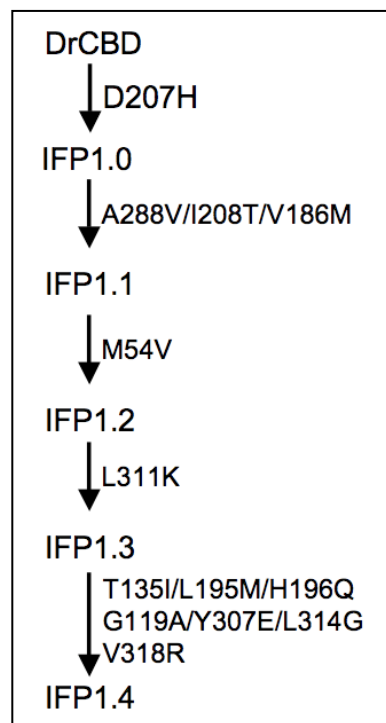


Fig. S4 Evolution of IFPs showing the mutations introduced at each stage.

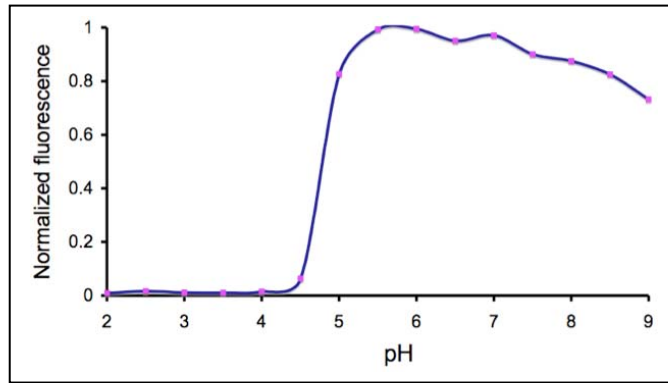


Fig. S5 pH dependence of IFP1.4 fluorescence. IFP1.4 was excited at 640 nm and its emission at 700 - 800 nm was integrated, normalized and plotted against pH.

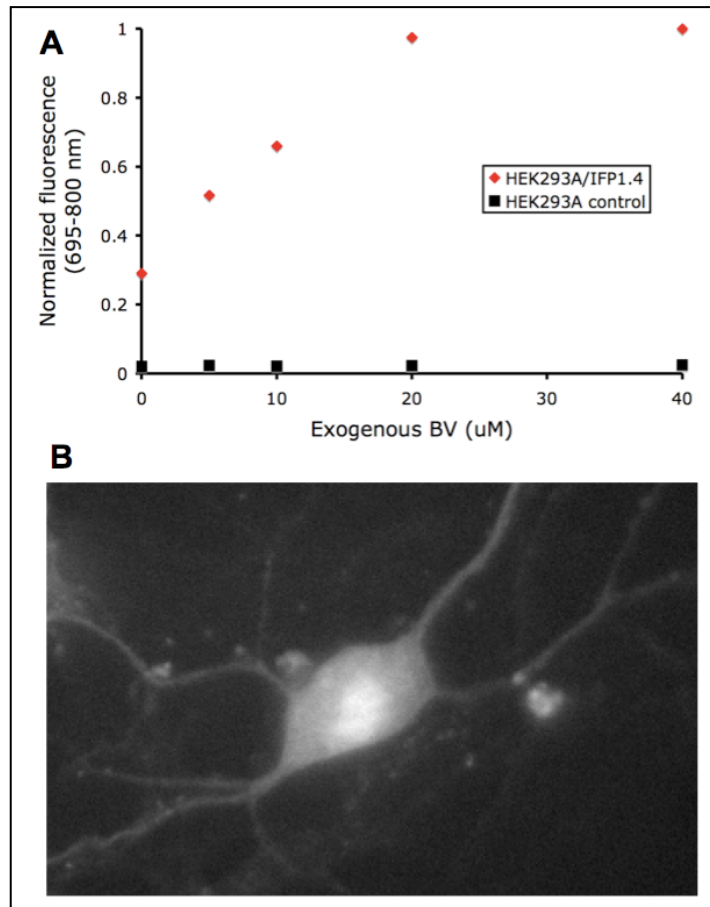


Fig. S6 Increase of IFP fluorescence by addition of exogenous BV. **(A)** IFP1.4-transfected HEK293A cells indicated increase of fluorescence upon addition of exogenous BV, with untransfected cells as the control. IFP fluorescence was integrated from 695 to 800 nm upon excitation at 660 nm. **(B)** Fluorescence image of a P2 cultured cortical neuron 2 weeks after transfection of IFP1.1, 10 minutes after addition of 25 uM BV.

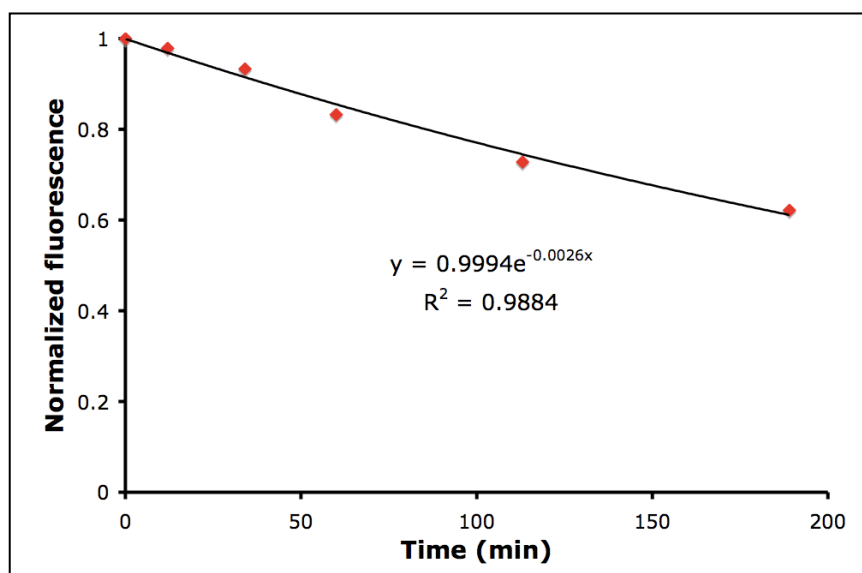


Fig. S7 IFP1.4 degradation in HEK293A cells with 20 μ M BV added.

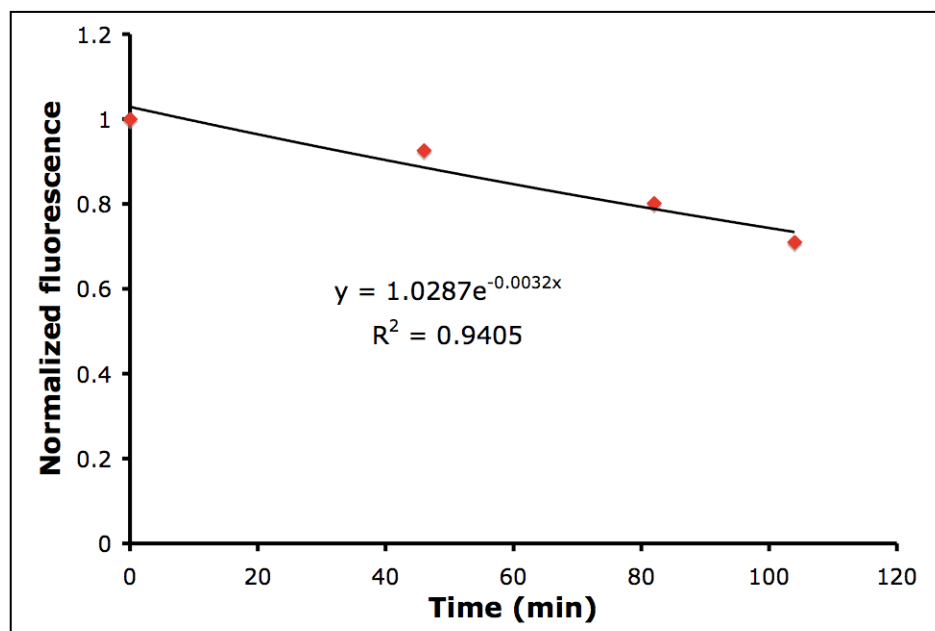


Fig. S8 IFP1.4 degradation in HEK293A cells without exogenous BV.

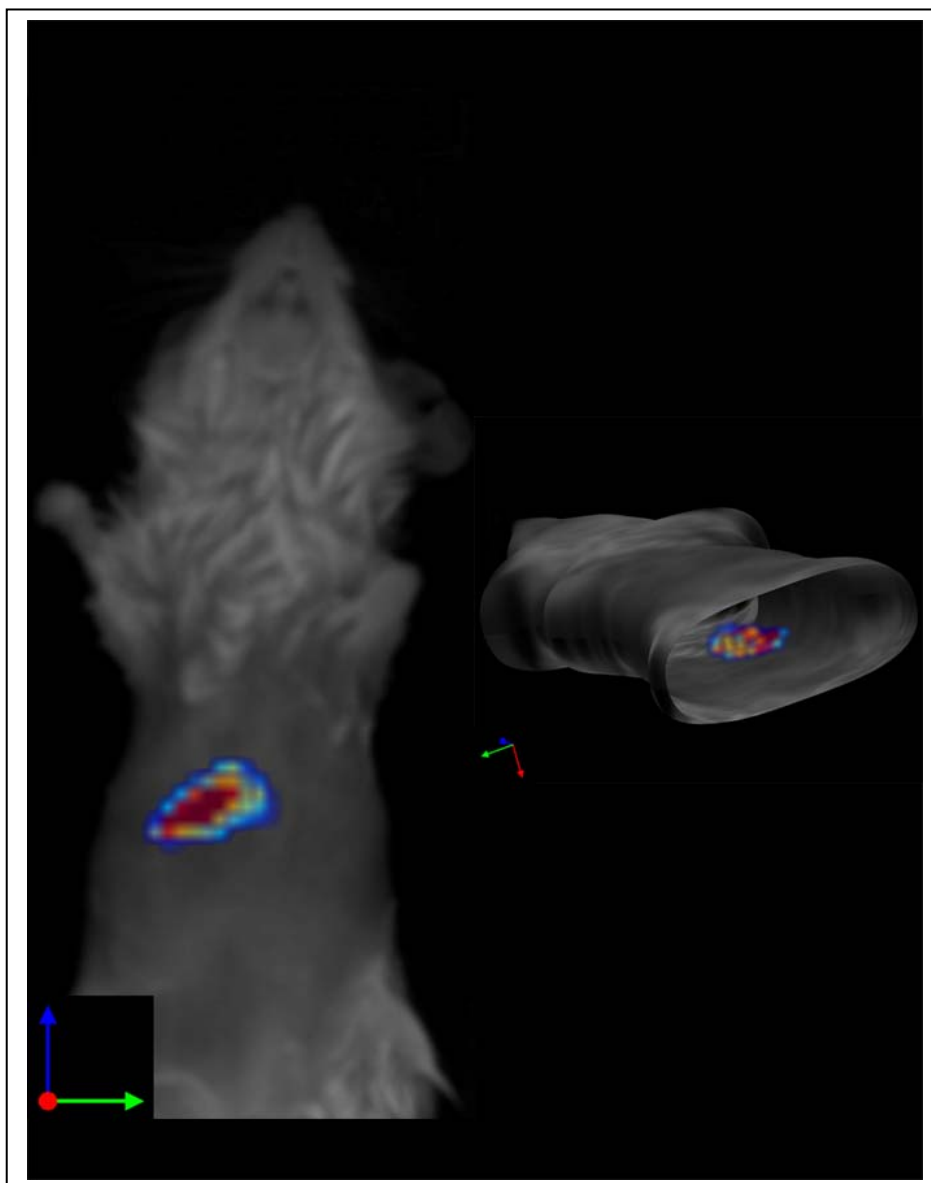


Fig. S9 Noninvasive fluorescence molecular tomographic (FMT) imaging of IFP-expressing mouse liver. Blue, green, and red arrows indicate rostral-caudal, left-right, and dorsoventral axes, respectively. Left: top view. Right: tilted view to show the 3D localization of fluorescence within the mouse.

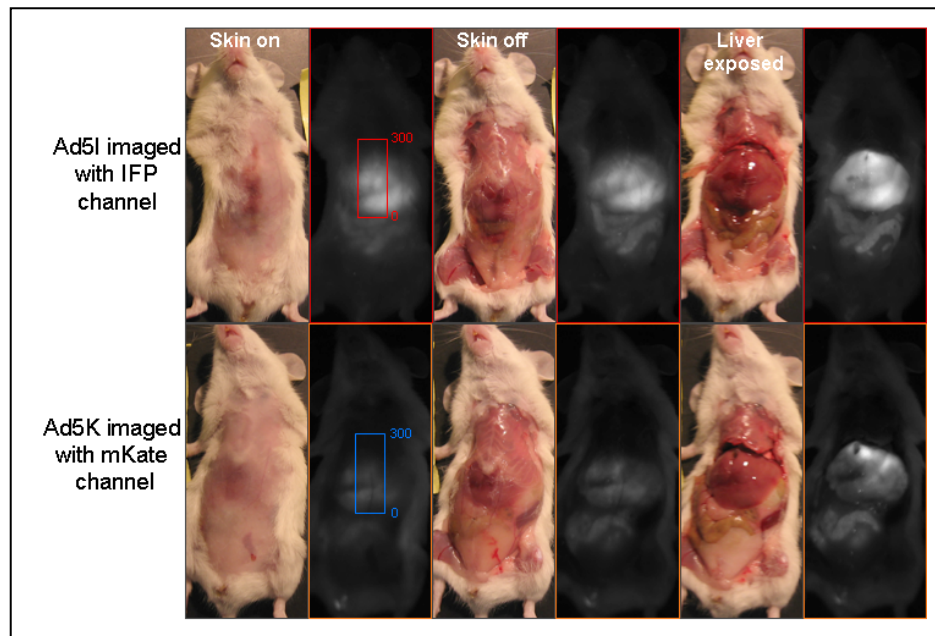


Fig. S10 IFP/mKate fluorescence images before dissection (skin on), after removal of skin (skin off), and after removal of overlying peritoneum and ribcage (liver exposed). mKate images were 2.5X brightened relative to IFP images. Note that Ad5I infected mouse was imaged after 250 nmol IV injection of BV.

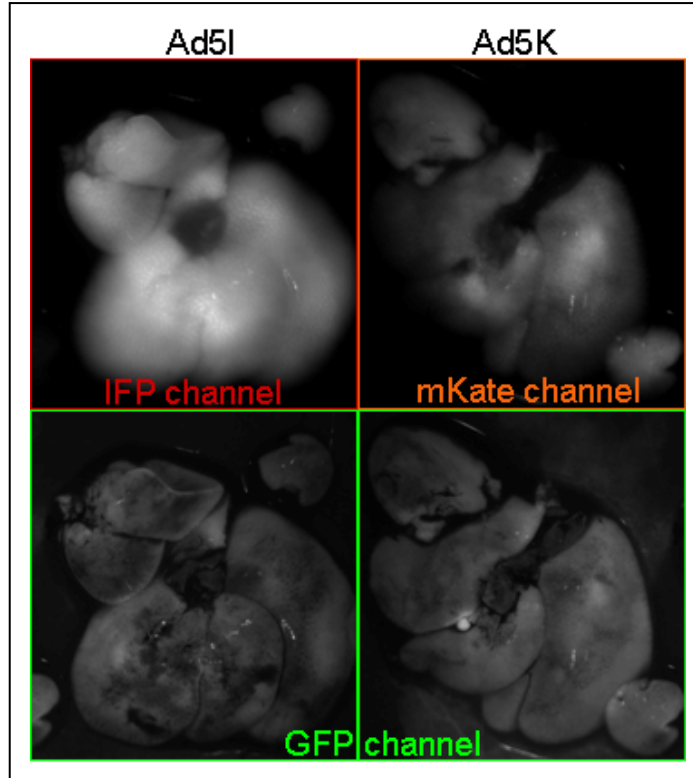


Fig. S11 Imaging of extracted livers infected with Ad5I and Ad5K.

Table S1 Properties of infrared fluorescent protein variants.

Fluorescent Protein	Absorption max. (nm)	Extinct. coeff. ($M^{-1} \text{ cm}^{-1}$)	Emission max. (nm)	Quantum yield	Relative brightness (%)	Stoichiometry
IFP1.0	699	60,000	722	0.028	100	Dimer
IFP1.1	686	86,000	713	0.050	256	Dimer
IFP1.2	684	86,000	707	0.066	338	Dimer
IFP1.3	684	84,000	707	0.061	305	Monomer
IFP1.4	684	92,000	708	0.070	383	Monomer

Supporting References

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