Live-cell imaging and measurement of intracellular pH in filamentous fungi using a genetically encoded ratiometric probe

(pH imaging in fungi using a genetically encoded probe)

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Supplementary material

Materials and Methods

RaVC gene construction

Genes encoding for Venus, Citrine and pHluorin were used as templates to construct RaVC. Replacing some fragments of pHluorin with Citrine or Venus enabled substantial codon optimization. The primers were constructed with overhangs to enable consecutive PCR ligation of the obtained PCR fragments derived from Venus, Citrine and pHluorin (Supplementary Table 1). Six fragments: 1-195 bp of Citrine, 196-244 bp of pHluorin, 245-396 bp of Citrine, 397-507 pHluorin, 508-606 bp of Venus, 607-720 pHluorin were PCR amplified. After PCR ligations we obtained a 720 bp product named RaVC (ratiometric pHluorin, Venus, Citrine) that preserved mutations which confer pHluorin with its pH sensitivity and had been partially codon optimized (Supplementary Table 2 and Supplementary Fig. 2). A point mutation at amino acid site 206 was introduced. GFP-A206K was constructed by site-directed mutagenesis, using the QuickChange XL Site-Directed Mutagenesis Kit following the manufacturer's protocol (Stratagene) using the following primers: sense 5' cctgcacacacaatctaagctttcgaaagatc and antisense 5' gggatctttcgaaagcttagattgtgtgtgca.

The gene obtained was cloned into the pBlueScript plasmid (Stratagene) using the blunt end *Eco*RV restriction site and transformed into *Escherichia coli* DH5α for plasmid propagation. Sequencing of RaVC confirmed the expected DNA sequence. The RaVC construct was inserted into the bacterial expression plasmid pRSET (Invitrogen) using *Bam*HI and *Eco*RI restriction sites and was then expressed in *Escherichia coli* BL21(DE3) cells.

Supplementary Table 1. Primers used for PCR amplifications and PCR ligations. Underlined bases indicate where primers anneal to template. Red marks label the start and stop codon. The green fluorescent proteins Citrine, Venus and pHluorin were used as templates.

template	primers: forward and reverse	base position	amino acid position
Citrine	GGATCCGCCACCATGGTGAGCAAGGGCGAGG ACCATAAGAGAAGGTGGTCACGAGG	1-195	1-65
pHluorin	GTGACCACCTTCTCTTATGGTGTTCAATG GAAGTCGTGCCGTTTCATATGATCTG	196-243	66-81
Citrine	CATATGAAACGGCACGACTTCTTCAAGTC GTTTCCATCATCCTTGAAGTCGATGCCC	244-396	82-132
pHluorin	CGACTTCAAGGATGATGGAAACATTATTCTTG CTCGATGTTGTGGTGAACTTGAAAG	397-507	133-169
Venus	CAAGTTCACCACAACATCGAGGACG TTGTGTGTGCAGGTAGTGGTTGTCGG	508-606	170-202
pHluorin	ACCCACTACCTGCACACACAATCTG GAATTCTTATTTGTATAGTTCATC	607-720	203-240

Supplementary Table 2. Codon usage *Aspergillus niger* and the number of codon triplets in pHluorin and RaVC.

Amino acids	Codon triplet	Codon usage frequency *A. niger* [%]	pHluorin-R [amino acids number]	RaVC [amino acids number]	Amino acids	Codon triplet	Codon usage frequency *A. niger* [%]	pHluorin-R [amino acids number]	RaVC [amino acids number]
Ala	GCA	12	3	**1**	Thr	ACA	11	6	**3**
	GCC	43	2	**5**		ACC	50	3	**13**
	GCG	16				ACG	17		
	GCT	29	4	**3**		ACT	21	7	
Arg	CGA	8			Val	GTA	5	2	**2**
	CGC	36		**1**		GTC	45	6	**3**
	CGG	15	1	**3**		GTG	28	1	**11**
	CGT	27	1			GTT	22	8	**2**
	AGA	7	4	**2**	His	CAC	66	7	**10**
	AGG	7				CAT	34	5	**2**
Asn	AAC	76	7	**10**	Gln	CAA	24	7	**4**
	AAT	24	4	**1**		CAG	76	1	**4**
Lys	AAA	16	14	**6**	Gly	GGA	18	8	**2**
	AAG	84	5	**13**		GGC	40	4	**17**
Asp	GAC	58	6	**13**		GGG	9	3	**2**
	GAT	42	13	**6**		GGT	34	8	**2**
Glu	GAA	29	12	**4**	Leu	CTA	5	3	**1**
	GAG	71	4	**12**		CTC	29		**2**
Cys	TGC	67	2	**2**		CTG	33	1	**11**
	TGT	33				CTT	15	10	**3**
Trp	TGG	100	1	**1**		TTA	2	3	
Ser	TCA	6	1	**1**		TTG	16	2	**2**
	TCC	30		**2**	Phe	TTC	75	4	**10**
	TCG	16	1	**1**		TTT	25	10	**4**
	TCT	17	3	**2**	Pro	CCA	12	6	**1**
	AGC	21		**2**		CCC	42	2	**9**
	AGT	10	3			CCG	21		
Ile	ATA	4	1			CCT	25	2	
	ATC	66	3	**9**	Met	ATG	100	6	**6**
	ATT	30	7	**2**	STOP	TAA	39	1	**1**
Try	TAC	73	7	**9**		TAG	31		
	TAT	27	4	**2**		TGA	30		

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            1 2
gfp-1       M-GKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWPT
pHluorin    M-SKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWPT
Venus       MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWPT
Citrine     MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWPT
RaVC        MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWPT
            60
gfp-1       LVTTFSYGVQCFSRYPDHMKRHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTL
pHluorin    LVTTFSYGVQCFSRYPDHMKRHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTL
Venus       LVTTFGYGLQCFARYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTL
Citrine     LVTTFGYGLMCFARYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTL
RaVC        LVTTFSYGVQCFSRYPDHMKRHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTL
            120
gfp-1       VNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNGTKVNFKIRHNIEDGSVQLA
pHluorin    VNRIELKGIDFKDDGNILGHKLEYNYNEHLVYIMADKQKNGTKAIFQVHHNIEDGGVQLA
Venus       VNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNGIKVNFKIRHNIEDGGVQLA
Citrine     VNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNGIKVNFKIRHNIEDGSVQLA
RaVC        VNRIELKGIDFKDDGNILGHKLEYNYNEHLVYIMADKQKNGTKAIFQVHHNIEDGGVQLA
            180
gfp-1       DHYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMVLLEFVTAAGITHGMDELYK
pHluorin    DHYQQNTPIGDGPVLLPDNHYLHTQSALSKDPNEKRDHMVFLEFVTAAGITHGMDELYK
Venus       DHYQQNTPIGDGPVLLPDNHYLSYQSALSKDPNEKRDHMVLLEFVTAAGITLGMDELYK
Citrine     DHYQQNTPIGDGPVLLPDNHYLSYQSALSKDPNEKRDHMVLLEFVTAAGITLGMDELYK
RaVC        DHYQQNTPIGDGPVLLPDNHYLHTQSKLSKDPNEKRDHMVFLEFVTAAGITHGMDELYK
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Supplementary FIG. 1. Alignment of gfp-1, pHluorin, Venus, Citrine and RaVC.

Amino acids marked in gray were used to construct RaVC; yellow indicates mutations characteristic for pHluorin; red indicates point mutation known to prevent di- and oligo-merization of GFP.

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          Met Val Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu Val Glu Leu Asp     20
pHluorin  ATG --- AGT AAA GGA GAA GAA CTT TTC ACT GGA GTT GTC CCA ATT CTT GTT GAA TTA GAT     57
RaVC      ATG GTG AGC AAG GGC GAG GAG CTG TTC ACC GGG GTG GTG CCC ATC CTG GTC GAG CTG GAC     60

          Gly Asp Val Asn Gly His Lys Phe Thr Val Thr Gly Glu Gly Glu Gly Asp Ala Thr Tyr     40
pHluorin  GGT GAT GTT AAT GGG CAC AAA TTT TCT GTC AGT GGA GAG GGT GAA GGT GAT GCA ACA TAC
RaVC      GGC GAC GTA AAC GGC CAC AAG TTC AGC GTG TCC GGC GAG GGC GAG GGC GAT GCC ACC TAC    120

          Gly Lys Leu Thr Leu Lys Phe Ile Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr     60
pHluorin  GGA AAA CTT ACC CTT AAA TTT ATT TGC ACT ACT GGA AAA CTA CCT GTT CCA TGG CCA ACA
RaVC      GGC AAG CTG ACC CTG AAG TTC ATC TGC ACC ACC GGC AAG CTG CCC GTG CCC TGG CCC ACC    180

          Leu Val Thr Thr Phe Ser Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys     80
pHluorin  CTT GTC ACT ACT TTC TCT TAT GGT GTT CAA TGC TTT TCA AGA TAC CCA GAT CAT ATG AAA
RaVC      CTC GTG ACC ACC TTC TCT TAT GGT GTT CAA TGC TTT TCA AGA TAC CCA GAT CAT ATG AAA    240

          Arg His Asp Phe Phe Lys Thr Ala Met Pro Glu Gly Tyr Val Gln Glu Arg Thr Ile Phe    100
pHluorin  CGG CAT GAC TTT TTC AAG AGT GCC ATG CCC GAA GGT TAT GTA CAG GAA AGA ACT ATA TTT
RaVC      CGG CAC GAC TTC TTC AAG TCC GCC ATG CCC GAA GGC TAC GTC CAG GAG CGC ACC ATC TTC    300

          Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu Val Lys Phe Glu Gly Asp Thr Leu    120
pHluorin  TTC AAA GAT GAC GGG AAC TAC AAG ACA CGT GCT GAA GTC AAG TTT GAA GGT GAT ACC CTT
RaVC      TTC AAG GAC GAC GGC AAC TAC AAG ACC CGC GCC GAG GTG AAG TTC GAG GGC GAC ACC CTG    360

          Val Asn Arg Ile Glu Leu Lys Gly Ile Asp Phe Lys Asp Asp Gly Asn Ile Leu Gly His    140
pHluorin  GTT AAT AGA ATC GAG TTA AAA GGT ATT GAT TTT AAA GAT GAT GGA AAC ATT CTT GGA CAC
RaVC      GTG AAC CGC ATC GAG CTG AAG GGC ATC GAC TTC AAG GAT GAT GGA AAC ATT CTT GGA CAC    420

          Lys Leu Glu Tyr Asn Tyr Asn Glu His Leu Val Tyr Ile Met Ala Asp Lys Gln Lys Asn    160
pHluorin  AAA TTG GAA TAC AAC TAT AAC GAG CAC TTG GTG TAC ATC ATG GCA GAC AAA CAA AAG AAT
RaVC      AAA TTG GAA TAC AAC TAT AAC GAG CAC TTG GTG TAC ATC ATG GCA GAC AAA CAA AAG AAT    480

          Gly Thr Lys Ala Ile Phe Gln Val His His Asn Ile Glu Asp Gly Gly Val Gln Leu Ala    180
pHluorin  GGT ACC AAA GCT ATC TTT CAA GTT CAC CAC AAC ATT GAA GAT GGA GGC GTT CAA CTA GCA
RaVC      GGT ACC AAA GCT ATC TTT CAA GTT CAC CAC AAC ATC GAG GAC GGC GGC GTG CAG CTC GCC    540

          Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly Pro Val Leu Leu Pro Asp Asn His    200
pHluorin  GAC CAT TAT CAA CAA AAT ACT CCA ATT GGC GAT GGC CCT GTC CTT TTA CCA GAC AAC CAT
RaVC      GAC CAC TAC CAG CAG AAC ACC CCC ATC GGC GAC GGC CCC GTG CTG CTG CCC GAC AAC CAC    600

          Tyr Leu His Thr Gln Ser Asn Leu Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val    220
pHluorin  TAC CTG CAC ACA CAA TCT GCC CTT TCG AAA GAT CCC AAC GAA AAG AGA GAC CAC ATG GTC
RaVC      TAC CTG CAC ACA CAA TCT AAG CTT TCG AAA GAT CCC AAC GAA AAG AGA GAC CAC ATG GTC    660

          Phe Leu Glu Phe Val Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu Leu Tyr Lys Och    240
pHluorin  TTT CTT GAG TTT GTA ACA GCT GCT GGG ATT ACA CAT GGC ATG GAT GAA CTA TAC AAA TAA
RaVC      TTT CTT GAG TTT GTA ACA GCT GCT GGG ATT ACA CAT GGC ATG GAT GAA CTA TAC AAA TAA    720
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Supplementary FIG. 2. Sequence alignment of pHluorin and RaVC.

The text marked in gray indicates codon triplet that are different in pHluorin and RaVC.