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Tunable assembly of protein-microdomains in living vertebrate embryos

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Abstract

Subcellular events such as trafficking and signaling are regulated by self-assembled protein complexes inside the cell. The ability to rapidly and reversibly manipulate these protein complexes would likely enhance studies of their mechanisms and their roles in biological function and disease manifestation.^[1, 2] This manuscript reports that thermally-responsive elastin-like polypeptides (ELPs) linked to fluorescent proteins can regulate the self-assembly and disassembly of protein microdomains within the individual cells of zebrafish embryos. By exploring a library of fluorescent ELP proteins, this reports demonstrates that ELPs can co-assemble different fluorescent proteins inside of embryos. By tuning ELP length and sequence, fluorescent protein microdomains can be assembled at different temperatures, in varying sizes, or for desired periods of time. For the first time in a multicellular living embryo, these studies demonstrate that temperature-mediated ELP assembly can reversibly manipulate assembly of subcellular protein complexes, which may have applications in the study and manipulation of *in vivo* biological functions.

Graphical Abstract

A new temperature-dependent strategy to modulate protein assembly in a vertebrate embryo is developed using a thermo-responsive elastin-like polypeptide (ELP). By tuning ELP length and

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

sequence, protein microdomains can be induced to assemble at different temperatures, in varying sizes, or for different time periods inside of zebrafish embryos.

Thermo-responsive protein polymers

Keywords

thermo-responsive protein polymers; elastin-like polypeptides; zebrafish; protein assembly; genetically engineered protein microdomains

A major goal of synthetic biology is the design of biocompatible materials that can emulate the sub-micrometer reversibly assembled structures formed within cells.^[3–5] Subcellular structures (e.g. transcription machinery, the nuclear envelope, signaling complexes, endosomes) must assemble and disassemble in a regulated fashion for proper function. Since this fine tuned reversible assembly is so important for cell biology, technologies that can undergo reversible self-assembly inside of cells may yield greater control and understanding of intracellular pathways and their consequent roles in physiology and disease.

In previous work our group has shown that thermally responsive elastin-like polypeptides (ELPs) can be fused to effector proteins to reversibly assemble fluorescent cytosolic protein microdomains and to reversibly inhibit clathrin mediated endocytosis.^[6–8] ELPs are biocompatible, biodegradable polypeptides consisting of a pentameric repeat of $(VPGXG)_n$, where X determines the ELP hydrophobicity and n determines the molecular weight.^[9, 10] ELPs abruptly phase separate into protein-rich domains in response to heating, whereby they form a secondary aqueous phase known as a coacervate. Individual coacervates are typically several hundred nanometers to several micrometers in diameter. This phase separation is a thermodynamically reversible process, and ELP coacervates quickly resolubilize into bulk water upon cooling. The temperature of self-assembly, termed the transition temperature (T_l), is specified by the hydrophobicity of the guest residue and the number of repeats. When fused to an effector protein, such as GFP or clathrin light chain, this temperature sensitive behavior is retained, producing a temperature sensitive mutant that rapidly cycles between self-assembly and disassembly in response to temperature, enabling the reversible control of

protein clustering. While this system has been proven robust in cell culture, it remains unknown whether the same strategy will work in a multicellular organism.

Zebrafish (*Danio rerio*) is a small freshwater fish that is commonly used for the study of vertebrate developmental biology.^[11, 12] Several features make zebrafish an attractive model organism, including low cost of husbandry, high reproductive rates, and the *ex vivo* development of optically clear embryos. Zebrafish also exhibit a cellular and developmental biology which more closely mimics mammalian systems than commonly used invertebrate models, such as *C. elegans* or *Drosophila melanogaster*.^[13–15] Importantly, zebrafish can tolerate a reasonable range of temperatures found in their native environments, allowing specimens to be maintained above and below the T_i of optimized ELP fusion proteins.^[16]

This communication shows that GFP-ELPs (GFP cloned to amino-terminus of ELP) can be easily expressed in zebrafish, where they undergo temperature-dependent self-assembly and disassembly of protein microdomains (Figure 1). Moreover, by tuning the ELP sequence it is possible to design microdomains that form at different temperatures, have distinct sizes, or remain assembled for extended durations. These findings suggest that ELPs are a potentially powerful strategy for controlled protein assembly and disassembly within zebrafish.

A library of GFP-ELPs were first expressed and characterized within zebrafish embryos (Table 1, Figure 1a, c and S1). While there does appear to be a band of free GFP in the GFP-ELP injected embryos, this level is ~10% of the total integrated density. Since these GFP fragments lack an ELP, they are not expected to significantly participate in setting the phase behavior of intact GFP-ELPs; furthermore, their relative abundance is subject to the nonlinearity inherent in the development of a Western Blot. Since the large majority of the fusion remains intact, it is unlikely that cleaved GFP will contribute significantly to interpretation of fluorescence imaging studies. The optimal temperature used for zebrafish husbandry is 28.5 °C; however, larvae can survive for at least several days at temperatures ranging from 21 to 33 °C.^[17, 18] Therefore, GFP-V96 was selected for this study as its *in vitro* T_i is within this range (Figure 1d). To visualize the temperature-dependent microdomain assembly, a live fish embryo injected with 400 pg mRNAs encoding GFP-V96 was imaged at late blastula stages (4~5 hours post fertilization, hpf) while the temperature was increased from 20 to 40 °C (Figure 1e). The late blastula stage was selected because sufficient GFP became visible to image single cells using confocal laser scanning microscopy. At 20 °C, below the T_b , GFP-V96 remains soluble throughout the embryo (Supplementary Video). Within 5 min of ramping the temperature, microdomain formation was observed in every single cell. Once ELP coacervates have formed within the cytosol, their size and number remain constant over short durations. This may occur because the protein-rich phases are separated from each other by high concentrations of other cytosolic macromolecules. This differs from how purified ELPs concentrate into a single, continuous phase based on their high density with respect to buffer, which is accelerated using centrifugation. Immediately after heating, the fluorescent microdomains within the embryo were completely resolubilized by cooling to 15 °C. These results demonstrate the efficient temperature-mediated self-assembly and disassembly of proteins within the single cells of a zebrafish embryo. Short durations of heating (~5 min) suggest the high temporal resolution of this system.

Signaling and trafficking events inside living cells require the co-assembly of protein complexes composed of distinct proteins. Accordingly, we examined whether ELPs fused with different fluorescent proteins may co-assemble when induced to phase separate (Figure 2a). mRNAs encoding GFP-V96 and RFP-V96 (Table S1) were co-injected into single-cell zebrafish embryos. After 4 to 5 hours, blastula embryos were incubated above and below the T_i of V96, fixed, and then imaged. Figure 2b reveals that at 23 °C, both RFP-V96 and GFP-V96 remain soluble within the single cells of the embryo. However, at 30 °C both constructs form distinctive microdomains which readily colocalize with each other. While we cannot rule out the possibility that RFP-V96 has a different phase behavior than GFP-V96, the images clearly suggest that selection of an identical ELP tag, V96, leads to direct co-assembly above the T_i . This result is consistent with our previous *in vitro* results and serves as proof-of-principle that ELP mediated self-assembly can be used to co-assemble different proteins within a multicellular organism. However, we note two limitations with this proof-of-principle study. First, in Figure 2b there appears to be a preponderance of signal inside the nucleus compared with the cytosol. This difference in signal intensity may be an artifact of fixation, as it not nearly as apparent in live embryos (Figure 1e, Supplementary Video). However, future studies should determine the subcellular location of these or other ELP fusion proteins. This could be achieved through whole mount immunofluorescence to stain different organelles, by probing ELP fusions with secondary antibodies, or through the use of transgenic zebrafish lines which express tissue or organelle specific reporters. The second limitation is that the relative concentrations of GFP-V96 and RFP-V96 were not determined. The proper formation of many intracellular complexes (e.g. a signaling complex, an endosome, transcription machinery) depends on a proportional relationship between their various protein components. Accordingly, to properly self-assemble such a complex using ELP fusion proteins may require a means to calibrate their relative concentrations *in vivo*. To address this, our next experiments reveal that ELP protein levels can in fact be controlled by adjusting mRNA injection amount.

Prior studies have shown that the *in vitro* T_i can be fine tuned by varying ELP length and concentration.^[19] To examine this effect *in vivo*, GFP-V60, which contains 60 repeats of VPGVG, was compared with GFP-V96. GFP alone served as a non-switchable negative control. Given that mRNA injection amount was the only significant predictor of protein expression level (Figure S4a, Table S2), fish embryos were injected with equimolar amounts of mRNA (GFP:150 pg, GFP-V60: 300 pg, GFP-V96: 400 pg) and incubated at various temperatures for 30 mins prior to overnight fixation at the same temperature. At 23 °C, nearly all GFP-V96 remains soluble, while at temperatures above 28 °C there is extensive GFP-V96 self-assembly (Figure 3a, c, d and S3). In contrast, fish embryos injected with GFP-V60 mRNA did not show any microdomain assembly until heated up to 38 °C, suggesting that the *in vivo* T_i increased with decreased polymer length. In addition, the effect of ELP concentration on the T_i was examined by injecting different amounts of mRNAs (80 pg, 400 pg, 2000 pg) encoding GFP-V96. The change of *in vitro* T_i as a function of ELP concentration was modeled in Figure 1d, 3e and Table 1, showing that a 10-fold decrease of GFP-V96 concentration increased T_i by 3.4 °C with a log normal relationship between ELP concentration and T_i . Similarly, the *in vivo* T_i increased by 7.2 °C with a 10-fold decrease in the mRNA dose (Figure 3b, f). While the degree of concentration-

dependence differs slightly, both purified and cytosolic GFP-V96 follow the same log-linear relationship between ELP amount and T_t . In addition, the size and irregularity of the GFP-V96 puncta appear to increase with increased mRNA concentration (Figure 3b). This phenomenon is most likely due to a dramatic increase in intracellular GFP-ELP amount, and is consistent with our supplementary data which reveals that GFP-V96 integrated density increases with injected mRNA amount (Figure S4b). These results suggest that mRNA injection amount can be adjusted to control protein levels, transition temperature, and perhaps coacervate size. To further explore these possibilities, future studies should confirm *in vivo* ELP expression levels through a combination of fluorescent measurements and biochemical measurements taken from lysed whole embryos.

Having demonstrated the temperature-dependent tunability of ELP-mediated microdomain assembly in zebrafish embryos, we next explored the duration of assembly for mRNA-encoded ELPs. Since mRNA is injected into the embryo immediately after fertilization, the levels of mRNA are expected to decrease during the development. Eventually, this will reduce protein expression too low to promote assembly at a physiological temperature. Based on this, fusions with phase transition temperatures below 28 °C (Figure 1d) GFP-V96 and GFP-SI were compared. GFP-SI has a similar molecular weight and exhibits similar phase separation behavior as GFP-V96 (Figure 1c, 4a, S3); however, it transitions at lower temperature (Figure 1d). *In vivo* both GFP-SI and GFP-V96 exhibit significant microdomain assembly at physiological temperatures at late blastula stages (4~5 hpf) (Figure 4b, S5), with GFP-SI forming microdomains (median area = 0.146 μm^2) slightly smaller than GFP-V96 (median area = 0.341 μm^2) (Figure 4d). Once fish developed to prim-5 (24 hpf) or long-pec (48 hpf) stages, microdomain assembly at 28 °C was readily observed in fish expressing GFP-SI, but not GFP-V96 (Figure 4c, e). These results are consistent with GFP-SI having a lower *in vivo* T_t than GFP-V96. They also suggest that by selecting constructs with low transition temperatures, microdomain assembly can be retained in zebrafish embryos over periods of at least days despite the loss and dilution of the encoding mRNA. However, we note that the variability in particle number was quite high for the 24 hpf GFP-SI embryos (max = 729, min = 83) (Figure 4e, S6). In our studies, we noticed that each embryo displayed an intrinsic ability to translate injected mRNA into protein. This is likely due to the high genetic variation within zebrafish, which exhibit greater genetic diversity, even within the same strain, than other inbred vertebrate model systems.^[20–22] This biological variability is further compounded by the great technical difficulty associated with microinjecting each single celled embryo with the same amount of mRNA. Future studies should aim to mitigate these sources of biological and technical variability.

Finally, all constructs were compared for their effect on embryo survival at 48 hours, which show that ELP-mediated phase separation alone induces no significant loss of viability (Figure 4f). In addition, none of the ELP fusions produced gross developmental changes (Figure S7), which suggests that ELP fusions are well tolerated in zebrafish embryos.

In summary, for the first time we report a temperature-dependent strategy for controlled protein assembly in a vertebrate embryo. This approach is rapid, reversible, and highly tunable. In addition, it is biocompatible and can be used to assemble one or more protein species together inside the single cells of a multicellular organism. In future work, this

approach may aid developmental biology studies by allowing tunable assembly of functional proteins inside of zebrafish embryos.

Experimental Section

Zebrafish husbandry and care:

Zebrafish were raised and maintained at 28.5 °C in a circulating system according to standard protocols that are in accordance with Children's Hospital Los Angeles IACUC animal care protocol.^[23] Wild type AB fish were set up for breeding the day before to obtain embryos for microinjection.

Plasmid construction:

GFP-ELPs in mammalian expression vector pcDNA3.1 were cloned as previously described.^[7] To engineer GFP-ELPs for bacterial expression, GFP gene was amplified using PCR and inserted to the N terminus of ELPs in pET25b (+) vectors using the BseRI digestion site. The sequences were verified by DNA sequencing (Retrogen, San Diego, CA).

mRNA preparation and microinjection:

To linearize the plasmids before in vitro transcription, the GFP plasmid was cut with XbaI and the GFP-ELP plasmids were cut with EcoRI. Linearized GFP and GFP-ELP plasmids were then transcribed into full length capped mRNAs with the T7 mMESSAGE mMACHINE Ultra Transcription Kit (AM1345, Life technologies, Carlsbad, CA) using the manufacturer's protocol. This mRNA was then purified with the MEGAclear™ Transcription Clean-Up Kit (AM1908, Life technologies, Carlsbad, CA). We followed this kit's protocol with one major caveat: in the final step we eluted mRNA from the silica filters using room temperature elution buffer, instead of heated elution buffer. This is because the high temperatures suggested in the protocol degraded the mRNA. mRNA degradation was assessed by running aliquots on a denaturing bleach gel.^[24] mRNA purity was determined by measuring 260/280 and 260/230 values on a NanoDrop™ 2000 (ND-2000, ThermoFisher SCIENTIFIC, Waltham, MA). mRNAs with 260/280 and 260/230 values near 2.0 were selected for microinjection. Purified in vitro transcribed mRNA was then injected into the cytoplasm of one cell stage zebrafish embryos following standard injection protocols.

ELP purification and physicochemical characterization:

pET25b (+) vectors encoding GFP-ELPs were transformed into BLR (DE3) *Escherichia coli* competent cells (Novagen Inc., Milwaukee, WI) for protein expression. Inverse transition cycling (ITC) was used to purify ELP samples from the bacteria lysates as previously published.^[25] To characterize the phase behaviors of GFP-ELPs, optical density (OD) at 310 nm was monitored using DU800 UV-Vis spectrometer while the temperature was ramped from 15 °C to 45 °C at a rate of 1 °C/min, and then plotted as a function of temperature. The maximum first derivative of the curve was defined as the transition temperature.

Immunoblotting:

Zebrafish embryos injected with GFP-ELPs were lysed with RIPA buffer containing protease/phosphatase inhibitor (#5872, Cell Signaling Technology, Danvers, MA) at 24 hpf and electronically separated on a PAgE EX 4–12% gradient gel (#59722, Lonza, Walkersville, MD). Proteins were then transferred onto a nitrocellulose membrane using iBlot2 dry blotting system (Life Technologies, Carlsbad, CA) and probed for GFP (ab290, 1:5000, Abcam, Cambridge, MA) or GAPDH (ab8245, 1:5000, Abcam, Cambridge, MA). An HRP-conjugated anti-rabbit secondary antibody (#7074, 1:5000, Cell Signaling Technology, Danvers, MA) was used for visualization.

Zebrafish imaging:

Embryos at early time points (4~5 hpf) were placed on a 35-mm glass bottom dish (MatTek, Ashland, MA) and imaged using a LSM800 confocal microscope (Carl Zeiss Microscopy, Thornwood, NY) with a 40× 1.3 NA oil objective. For live fish imaging, embryos were incubated on an HCS60 microscope hot and cold stage (Instec, Boulder, CO) attached to a mK2000 high precision temperature controller (Instec, Boulder, CO) and imaged using a Fluoview FV3000RS confocal microscope (Olympus, Waltham, MA) equipped with a 40× 1.25 NA oil objective. For fixed fish imaging, embryos were incubated at different temperatures and fixed overnight with 2% paraformaldehyde (Alfa Aesar, Tewksbury, MA) at the same incubation temperature before imaging. Fish at late time points (24, 48 hpf) were imaged live on a Leica MZFLIII Stereo/Dissection Microscope (Buffalo Grove, IL).

Image processing:

16 bit grayscale images with a resolution of 1024 by 1024 (4~5 hpf) or 1344 by 1024 (24 hpf and 48 hpf) were analyzed with FIJI (version 2.0.0).^[26] The FIJI macros used to calculate microdomain area and number can be found on github: <https://github.com/d-tear/Image-Analysis-Pipeline-for-Tunable-assembly-of-protein-microdomains-in-living-vertebrate-embryos->.

Statistical analysis:

Data presented are representative curves or mean $\pm$ 95% confidence interval. Statistical analysis was performed in R (version 3.4.2). Graphs were made with Prism 6. In depth details regarding statistical analysis are available in the supporting information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- [1]. Papapostolou D, Howorka S, Mol Biosyst 2009, 5, 723. [PubMed: 19562111]
- [2]. Ng DYW, Vill R, Wu Y, Koynov K, Tokura Y, Liu W, Sihler S, Kreyes A, Ritz S, Barth H, Ziener U, Weil T, Nat Commun 2017, 8, 1850. [PubMed: 29185444]
- [3]. Benner SA, Sismour AM, Nature Reviews Genetics 2005, 6, 533.
- [4]. Cameron DE, Bashor CJ, Collins JJ, Nature Reviews Microbiology 2014, 12, 381. [PubMed: 24686414]
- [5]. Rawls RL, Chemical & Engineering News 2000, 78, 49.
- [6]. Shi P, Lin YA, Pastuszka M, Cui H, Mackay JA, Adv Mater 2014, 26, 449. [PubMed: 24136711]
- [7]. Pastuszka MK, Janib SM, Weitzhandler I, Okamoto CT, Hamm-Alvarez S, Mackay JA, Biomacromolecules 2012, 13, 3439. [PubMed: 23088632]
- [8]. Pastuszka MK, Okamoto CT, Hamm-Alvarez SF, MacKay JA, Adv Funct Mater 2014, 24, 5340. [PubMed: 25419208]
- [9]. Shah M, Hsueh PY, Sun G, Chang HY, Janib SM, MacKay JA, Protein Sci 2012, 21, 743. [PubMed: 22434766]
- [10]. Despanie J, Dhandhukia JP, Hamm-Alvarez SF, MacKay JA, Journal of Controlled Release 2016, 240, 93. [PubMed: 26578439]
- [11]. Dooley K, Zon LI, Curr Opin Genet Dev 2000, 10, 252. [PubMed: 10826982]
- [12]. Lieschke GJ, Currie PD, Nat Rev Genet 2007, 8, 353. [PubMed: 17440532]
- [13]. Becker CG, Becker T, Restorative neurology and neuroscience 2008, 26, 71. [PubMed: 18820403]
- [14]. Mackay EW, Apschner A, Schulte-Merker S, BoneKEy reports 2013, 2.
- [15]. Bakkers J, Cardiovascular research 2011, 91, 279. [PubMed: 21602174]
- [16]. Lahiri K, Vallone D, Gondi SB, Santoriello C, Dickmeis T, Foulkes NS, PLoS Biol 2005, 3, e351. [PubMed: 16176122]
- [17]. Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF, Developmental dynamics 1995, 203, 253. [PubMed: 8589427]
- [18]. Avdesh A, Chen M, Martin-Iverson MT, Mondal A, Ong D, Rainey-Smith S, Taddei K, Lardelli M, Groth DM, Verdile G, Journal of visualized experiments: JoVE 2012.
- [19]. Janib SM, Pastuszka M, Aluri S, Folchman-Wagner Z, Hsueh P, Shi P, Lin Y, Cui H, Mackay J, Polymer chemistry 2014, 5, 1614. [PubMed: 24511327]
- [20]. Guryev V, Koudijs MJ, Berezikov E, Johnson SL, Plasterk RH, Van Eeden FJ, Cuppen E, Genome research 2006, 16, 491. [PubMed: 16533913]
- [21]. Balik-Meisner M, Truong L, Scholl EH, Tanguay RL, Reif DM, Mammalian Genome 2018, 29, 90. [PubMed: 29368091]
- [22]. Holden LA, Brown KH, Scientific reports 2018, 8, 4780. [PubMed: 29555936]
- [23]. Brand M, Granato M, Nüsslein-Volhard C, Zebrafish 2002, 261, 7.
- [24]. Aranda PS, LaJoie DM, Jorcyk CL, Electrophoresis 2012, 33, 366. [PubMed: 22222980]
- [25]. Meyer DE, Chilkoti A, Nat Biotechnol 1999, 17, 1112. [PubMed: 10545920]
- [26]. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez JY, White DJ, Hartenstein V, Eliceiri K, Tomancak P, Cardona A, Nat Methods 2012, 9, 676. [PubMed: 22743772]

Figure 1. ELP expression and microdomain assembly in a zebrafish embryo.

a) Genes encoding three ELPs (V60, V96, SI, Table 1) were fused to the carboxy-terminus of GFP gene for direct visualization. **b)** Schematic representation of reversible microdomain assembly in a zebrafish embryo. Below their T_t , GFP-ELPs are uniformly distributed inside the single cells of the embryo. Upon heat stimulation, the protein polymers reversibly phase separate and assemble microdomains. **c)** Expression of different GFP-ELPs in zebrafish embryos were confirmed using an anti-GFP antibody. **d)** The *in vitro* temperature-concentration phase diagrams for all purified GFP-ELPs follow a log-linear relationship (Table 1). **e)** ELP microdomain assembly in a zebrafish embryo is rapid and reversible. Live embryo imaging was conducted at late blastula stages (4~5 hpf) to visualize the temperature-

dependent microdomain assembly from 20 to 40 °C. These polypeptide microdomains can be rapidly resolubilized by decreasing the temperature to 15 °C. Images are taken from the same live embryo at indicated temperatures. The insets are magnified 3.5x with respect to each panel.

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Figure 2. ELPs can co-assemble different proteins *in vivo*.

a) Schematic of triggered co-assembly of fluorescent ELP fusions in a zebrafish embryo. GFP-V96 and RFP-V96 are soluble throughout the cytosol below T_t and co-assemble into mixed microdomains above the T_t . **b)** Confocal microscopy imaging of GFP-V96 and RFP-V96 in zebrafish embryos. Fish embryos were co-injected with mRNAs encoding GFP-V96 and RFP-V96 at one-cell stage and then incubated either below (23 °C) or above (30 °C) T_t until fixation at last blastula stages (4~5 hpf).

Figure 3. ELP self-assembly temperature can be tuned both *in vitro* and *in vivo*.

Temperature-dependent microdomain assembly was characterized by varying either **a)** molecular weight (400 pg mRNAs encoding GFP-V96 vs. 300 pg mRNAs encoding GFP-V60) or **b)** concentrations (80 pg, 400 pg, 2000 pg mRNAs encoding GFP-V96) of ELPs. Zebrafish embryos at late blastula stages (4~5 hpf) were incubated at different temperatures, fixed, and imaged using a Zeiss confocal microscope. *indicates microdomain assembly observed. **c)** Representative *in vitro* optical density profiles for GFP-V60 and GFP-V96 at 25 μ M as a function of temperature. **d)** Quantification of the *in vivo* particle number per field as a function of temperature for GFP-V60 and GFP-V96. Mean $\pm$ 95% confidence interval ($n=3\sim20$). **e)** Representative *in vitro* optical density profiles for GFP-V96 at various concentrations (5 μ M, 25 μ M, 75 μ M) as a function of temperature. **f)** Quantification of the *in vivo* particle number per field as a function of temperature for embryos injected with different amounts (80pg, 400pg, 2000pg) of mRNAs encoding GFP-V96. Mean $\pm$ 95% confidence interval ($n=3\sim16$).

Figure 4. ELPs can be tuned for short- or long-term microdomain assembly.

a) Representative *in vitro* optical density profile (left) and DLS measurement (right) for GFP-SI and GFP-V96 at 25 μM as a function of temperature. **b)** Short-term microdomain assembly at physiological temperatures was observed in both GFP-SI and GFP-V96. Fish embryos injected with 1000 pg of mRNAs encoding either GFP-SI or GFP-V96 were fixed at late blastula stages (4~5 hpf) and imaged using a confocal microscope. **c)** Long term microdomain assembly at physiological temperatures was observed in GFP-SI, but not GFP-V96. Live fish embryos expressing GFP-SI or GFP-V96 were visualized using a dissecting scope at 24 hpf. **d)** Quantification of microdomain areas inside of fixed embryos at late blastula stages (4~5 hpf). GFP-SI (23 embryos, 6692 total particles, median area = 0.146 μm^2). GFP-V96 (25 embryos, 12078 total particles, median area = 0.316 μm^2). p value for

difference in median particle area = $2.6e-07$. **e)** Quantification of particle number per fish at 24 and 48 hpf, showing that GFP-SI assembled significantly more particles than GFP-V96 at longer time periods. Mean $\pm$ 95% confidence interval. 24 hpf: GFP-SI n = 5, GFP-V96 n = 6, two-sided t-test p value = 0.03. 48 hpf: GFP-SI n = 3, GFP-V96 n = 3, two-sided t-test p value = 0.009. **f)** Survival rate of zebrafish embryos with different treatments at 48 hpf. Mean $\pm$ 95% confidence interval (n=3, 20 to 50 fish embryos injected with 150~1000 pg mRNAs were evaluated within in each experiment).

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Table 1.

Nomenclature, amino acid sequence and phase behavior of expressed proteins.

Protein Label	Amino acid sequence ^a	MW [kD] ^b	Purity [%] ^c	T_t [°C] in PBS ^d	T_t [°C] in zebrafish ^e	Intercept, b [°C] ^f	Slope, m [°C] [$\log_{10}(\mu\text{M})$] ⁻¹
GFP	GFP	30.0	NA	NA	NA	NA	NA
GFP-V60	GFP -(VPGVG) ₆₀ Y	52.4	98.9	36	38	43.8 [42.7 to 44.8]	5.3 [4.5 to 6.2]
GFP-V96	GFP -(VPGVG) ₉₆ Y	67.1	98.2	30	28	35.4 [34.8 to 36.1]	3.4 [2.8 to 4.0]
GFP-SI	GFP -(VPGSG) ₄₈ (VPGIG) ₄₈ Y	67.2	96.0	22	23	27.0 [26.3 to 27.6]	3.4 [2.8 to 3.9]

Not applicable (NA)

^{a)} **GFP** sequence available in the supplementary Table S1.^{b)} Estimated molecular weight from open reading frame confirmed by western blot (Figure 1c).^{c)} Estimated from the copper stained SDS-PAGE (Figure S2).^{d)} Transition temperature of purified GFP-ELPs suspended in phosphate buffered saline at 25 μM .^{e)} Observed transition temperature in zebrafish embryos at late blastula stages (molar amount of mRNA equal to 400pg GFP-V96 mRNA injected at one-cell stage) using fixed-zebrafish imaging.^{f)} The Intercept b and slope m , were derived from the log-linear regression analysis for transition temperature *vs.* concentration (Figure 1d) fit to the equation $T_t = b - m \log_{10}[C_{\text{ELP}}]$. Data represent the mean [95% confidence interval].