1 # Lyophilized cell-free systems display tolerance to organic solvent
2 # exposure

3 Supplemental Information

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16 Supplementary Information
17
18 **Supplementary Results and Discussion**
19 *Lyophilizing larger batches of CFPS reaction mixture and measuring water content*
20 Larger 250 µL scale batches of PANOx-SP CFPS reaction were lyophilized for 48 hours to achieve
21 sufficient dryness to allow milling into a powder (Supplementary Figure S5A). Lyophilization for
22 only 20 hours resulted in a viscid cake that could not be milled and was difficult handle, though
23 still demonstrated normal CFPS activity when rehydrated (data not shown). After 48-hr drying,
24 the dry weight of the powder stabilized at approximately 76 mg per 1 mL of aqueous CFPS
25 solution. Thermogravimetric analysis (TGA) is another more sensitive technique to quantify the
26 amount of water remaining in the sample and only requires around 5 mg of sample for the
27 measurement. TGA measurements indicate that 250 µL samples lyophilized for 48 hours were
28 between 1.2% and 3% water by weight (Supplementary Figure S5B). Endpoint GFP productivity
29 was the same after lyophilization and exposure to acetone compared to a freshly prepared
30 CFPS reaction (Supplementary Figure S5C).
31
32 *Correlation of Solvent Properties and CFPS productivity*
33 Correlation analysis comparing GFP productivity to each of several parameters describing the
34 physical and chemical properties of solvents is summarized in Supplementary Figures S2 and S3,
35 and Supplementary Tables S2 and S3. This analysis sheds light on the trends in solvent
36 characteristics that affect CFPS activity. Supplementary Figure S2 depicts correlation plots for
37 samples where complete CFPS reactions were exposed to solvents. Significant Pearson
38 correlation coefficients were found comparing productivity and solvent boiling point, dielectric
39 constant, Hansen polar parameter, Hansen hydrogen bond parameter, and the octanol/water
40 partition coefficient (log Kow). However, when lysate alone was exposed to organic solvents
41 (Supplementary Figure S3), there were no clear trends between productivity and solvent
42 properties except for a slight but significant relationship to the Hansen dispersion parameter.
43 For complete reaction exposures, decreased solvent hydrogen bonding propensity or increase
44 hydrophobicity correlates with improved productivity, suggesting solvents that participate in
45 molecular interactions similar to water could cause a greater extent of deactivation likely by an
46 increased ability to strip water molecules crucial to protein function. Some polar solvents like
47 ethanol also have a greater tendency to absorb water from the air, which could introduce
48 sufficient concentrations of water to allow protein unfolding or hydrolysis of molecules in the
49 resource mix. There appears to be a cutoff value for solvent boiling point. Two solvents with a
50 high boiling point, DMF and DMSO, caused inhibition of GFP productivity. This could be an
51 indication that trace amounts of these less-volatile solvents are not sufficiently removed by
52 evaporation.
53
54 *Solvent evaporation at ambient conditions*
55 A reduced solvent screen was also implemented without applying a vacuum to learn how
56 evaporation at ambient conditions could affect solvent compatibility. Acetonitrile, acetone,
57 methanol and ethanol were tested on complete lyophilized CFPS reactions. The results are

58 depicted in Figure 2B. GFP productivity is not significantly different for the conditions tested as
59 compared to samples dried with a vacuum.
60
61 *Acetonitrile washes to remove DMSO do not improve exposure outcomes*
62 Since DMSO has a relatively high boiling point of 189°C, it is not clear that evaporation with or
63 without application of a vacuum would be sufficient to remove the solvent from the system in
64 the time allotted before rehydration. It is possible that DMSO causes inactivation of the
65 reaction by protein unfolding or resource extraction, or inhibition is caused by residual DMSO
66 left behind during the rehydration step. To ascertain the real cause, the removal of DMSO was
67 improved by washing with a more volatile solvent. In this test, lyophilized CFPS components
68 were suspended in DMSO, and most of the DMSO was removed by aspiration as before. Then,
69 acetonitrile was used to wash the insoluble CFPS components twice, followed by removal of the
70 acetonitrile by aspiration and application of a vacuum. The activity of GFP expression upon
71 rehydration following this treatment is depicted in Supplementary Figure S4. The acetonitrile
72 washes did not improve the activity of CFPS following DMSO exposure, suggesting that DMSO
73 likely decreases activity by disabling some component of the CFPS during the exposure rather
74 than by residual solvent impacting the reaction.
75
76
77 **Supplementary Materials and Methods**
78 *PANOx-SP lysate preparation*
79 *E. coli* BL21 DE3 star bacteria were pre-cultured from frozen stock in 100 mL 2xYPTG media in a
80 500 mL shake flask at 37°C, 250 rpm for 16 hours. 5 mL of the pre-culture was used to seed 1 L
81 of 2xYPTG media in a 2 L baffled shake flask that was shaken at 37°C, 250 rpm for
82 approximately 3.25 hours, or until optical density at 600 nm reaches 3.0. Cells were then
83 pelleted by centrifugation at 5000xg for 15 minutes and washed four times with S30A buffer
84 (10 mM Tris acetate pH 8.2, 14 mM magnesium acetate, 60 mM potassium acetate, 2 mM DTT).
85 The wet weight of the pellet was then measured before the pellet was flash frozen in liquid
86 nitrogen and stored at -80°C until further processing. For lysis, cell pellets were re-suspended in
87 cold S30A buffer at 1 mL buffer per 1 g wet cell mass, then lysed by sonication on ice in 1.5 mL
88 aliquots (Qsonica Q500, 20% amplitude, 40 seconds on, 59 seconds off, 540 J total energy). 4.5
89 µL 1 M DTT is added to each tube after sonication. Lysates were then centrifuged at 12,000xg
90 for 10 minutes at 4°C and the supernatant was collected, aliquoted, flash frozen in liquid
91 nitrogen, and stored at -80°C until reaction assembly.
92
93 *3-PGA lysate preparation*
94 *E. coli* BL21 Rosetta2 bacteria were pre-cultured 7 hours at 37°C, 250 rpm from a colony in 3 mL
95 2xYPT media in a 14 mL culture tube. 30 µL of the pre-culture were then transferred to
96 inoculate a “midi-culture” in 60 mL 2xYPT media in a 500 mL Erlenmeyer flask and was
97 incubated 7 hours at 37°C, 250 rpm. Then, four 750 mL 2xYPT final cultures in 2.5 L baffled
98 flasks are each inoculated with 7.5 mL of the midi-culture. Flasks are incubated with shaking at
99 37°C, 250 rpm for three hours or when optical density at 600 nm reaches 1.5-1.6. Cells were

then pelleted at 5000xg for 10 minutes and washed four times with S30A* buffer (50 mM Tris pH 8.2, 14 mM magnesium glutamate, 60 mM potassium glutamate, 2 mM DTT). Note this S30A* buffer differs from the PANOx-SP S30A buffer by using magnesium and potassium salts with glutamate counter ion. The wet weight of the cell mass is measured, then diluted by a factor of 2.05mL per gram with S30A* buffer. A M-110P microfluidizer set to 16,000 psi is used to lyse the cells (Microfluidics, Westwood, MA). The lysate is centrifuged at 18000 rpm, 20 min, 4°C. The supernatant is pooled, then incubated 90 minutes at 37°C in a run-off reaction. Then, the lysate is centrifuged again at 20,000 rpm, 10 min, 2°C. The lysate supernatant is dialyzed for 1 hour at 4°C against 950 mL S30B buffer (14 mM magnesium glutamate, 150 mM potassium glutamate, 2M Tris pH 8.2, 1 mM DTT) before a last centrifugation at 20,000 rpm, 10 min, 2°C. The treated lysate supernatant is aliquoted, flash frozen in liquid nitrogen, and stored at -80°C until reaction assembly.

CFPS Reagent preparation

For PANOx-SP, a pre-mix solution was assembled containing magnesium glutamate, potassium glutamate, ammonium glutamate, ATP, GTP, CTP, UTP, folinic acid, tRNAs, each of the 20 canonical amino acids, phosphoenolpyruvate (PEP), nicotinamide adenine dinucleotide (NAD), coenzyme A (CoA), spermidine, putrescine, oxalic acid, and HEPES buffer (pH 7.4). Concentrations of each component in the final reaction are listed in Table S4. This premix stock solution was aliquoted, flash frozen in liquid nitrogen, and stored at -80°C until use in CFPS reaction assembly. In addition, leftover stock solutions of each component were flash frozen alone and stored at -80°C for use in the CFPS component screen experiment. Three PANOx-SP ingredients not included in the premix are stored separately at -20°C until reaction assembly: purified T7 RNA polymerase (RNAP), murine RNase inhibitor (New England Biolabs), and purified plasmid DNA.

The reagents added to 3-PGA CFPS reaction are somewhat different than PANOx-SP. The pre-mix solution for 3-PGA contains ATP, GTP, CTP, UTP, folinic acid, tRNAs, NAD, CoA, Spermidine, HEPES buffer (pH 8), cyclic adenosine monophosphate (cAMP), 3-phosphoglyceric acid (3-PGA), and dithiothreitol (DTT). Concentrations of each component in the final reaction are listed in Table S4. This premix is also aliquoted, flash frozen in liquid nitrogen, and stored at -80°C. A stock solution of all 20 amino acids are aliquoted and stored separately at -80°C. Other component stock solutions are stored at -20°C: potassium glutamate, magnesium glutamate, polyethylene glycol (PEG), and T7 RNAP. A maltodextrin solution is prepared fresh before reaction assembly.

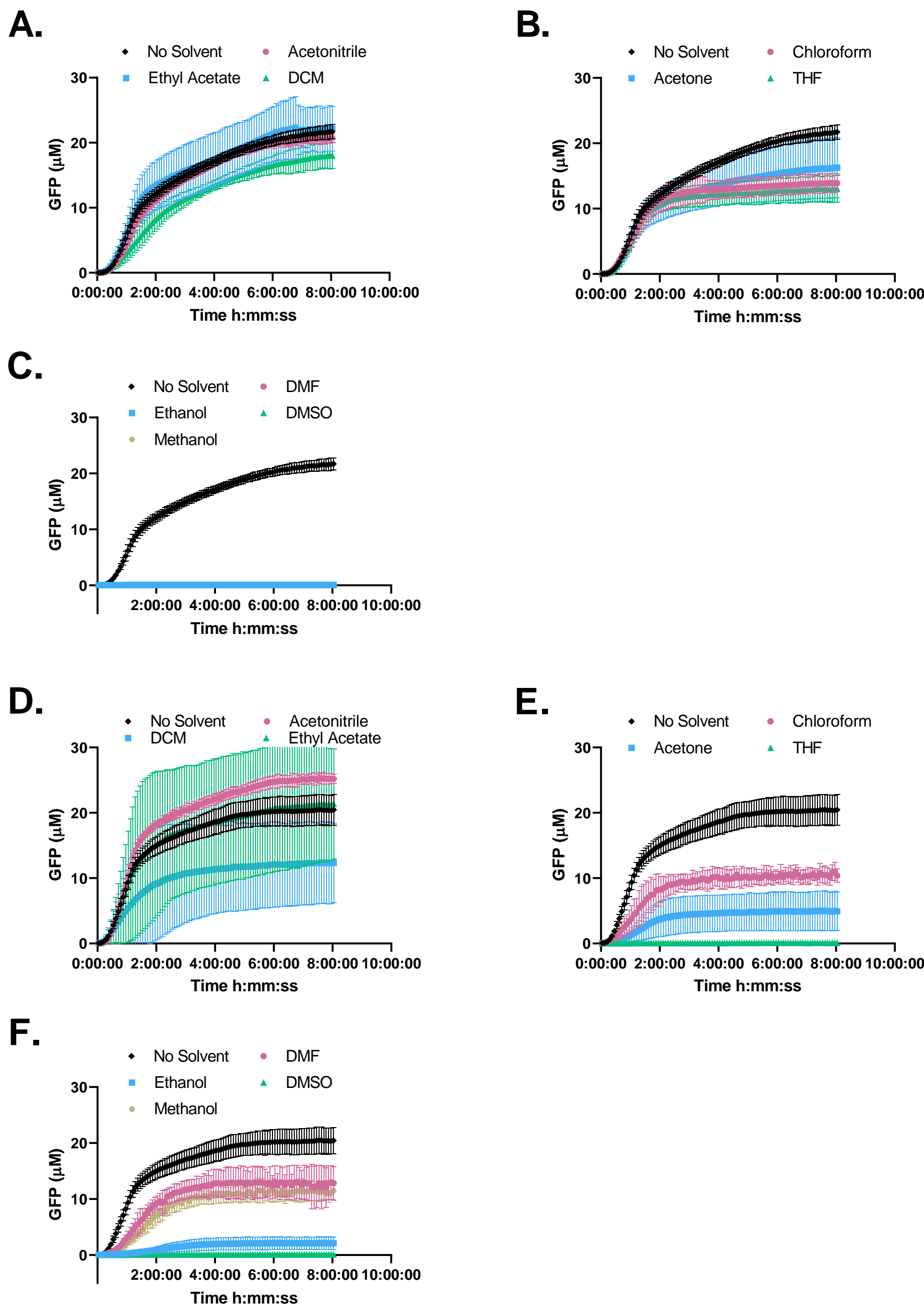
Lyophilization of CFPS reactions

To lyophilize CFPS reactions in a microplate, the 96 well plate is dipped in liquid nitrogen and transferred to a shelf-type lyophilizer (SP Scientific, VirTis Wizard 2.0) with initial temperature set to -40°C. When the lyophilizer chamber is sealed, the vacuum is initiated to reach approximately 200-300 mTorr. Then, primary drying is initiated with a shelf temperature of -20°C and maintained for four hours. The Secondary drying step changes the shelf temperature to 15°C, and is maintained overnight or approximately 17 hours. Microplate samples removed

143 from the lyophilizer were immediately treated with solvent as indicated and rehydrated to
144 monitor protein synthesis activity.
145
146 *Acetonitrile wash after DMSO exposure*
147 Acetonitrile washes after DMSO exposure were tested for improved solvent removal. After
148 DMSO exposure as described above, the insoluble reaction material is washed twice with 100
149 µL washes of acetonitrile, which are removed by aspiration followed by evaporation under a
150 vacuum for 20 minutes. Reactions were rehydrated as described.
151
152 *Preparation, acetone treatment, and analysis of larger scale CFPS powder*
153 Larger 250 µL scale PANOx-SP CFPS reactions were assembled on ice in 1.5-mL Eppendorf
154 tubes. For larger scale reactions, we pre-expressed T7 RNA polymerase in the E. coli culture
155 used to make cell extract rather than adding T7 RNAP purified separately. Also, we omitted the
156 RNAse inhibitor ingredient. These cost-saving measures did not majorly impact productivity in
157 solvent tolerance experiments. The tubes were flash frozen in liquid nitrogen and transferred to
158 a FreeZone 4.5 Liter benchtop manifold lyophilizer set to 0.08 mBar with internal refrigeration
159 at -105°C. Samples were lyophilized for 48 hrs. The dry weight of the sample and tube were
160 measured, and the weight of the tube alone was subtracted to find the dry weight of the CFPS
161 mixture. The lyophilized CFPS cake was easily ground to a powder using a pipet tip. This powder
162 was weighed into aliquots for TGA analysis. To compare acetone tolerance to fresh reactions, a
163 250 µL CFPS mixture was lyophilized and milled as above, submerged in 200 µL of acetone, and
164 rehydrated in 250 µL of nuclease-free water. 5 uL aliquots of the rehydrated solution were
165 distributed into clear, v-bottom 96 well plates to track GFP fluorescence using a SpectraMax M5
166 Microplate Reader. Reactions were carried out over the course of 4 hours at 30°C. Fluorescence
167 signal was monitored with an ex/em of 490/515 nm with gain set to 'low'. GFP signal in relative
168 fluorescence units (RFU) was converted to µM GFP using fluorescence measurements of a
169 purified GFP standard at the same settings on the microplate reader.
170
171 *PANOx-SP CFPS component screen*
172 CFPS components were screened to identify which components of the lysate-based PANOx-SP
173 CFPS method could contribute to protect lyophilized lysate during acetone exposure. The
174 component stock solutions tested are DNA, T7 RNAP, RNAse inhibitor, 10xSS, HEPES, 15xMM,
175 20AA, PEP, NAD, CoA, oxalic acid, putrescine, and spermidine. 10xSS refers to a solution of
176 magnesium, potassium, and ammonium glutamate. "15xMM" is a solution of ATP, GTP, UTP,
177 CTP, folinic acid, and tRNA. "20AA" is a solution containing all 20 canonical amino acids. Control
178 reactions were labeled "None", referring to PANOx-SP cell extract without any additional
179 ingredients, and "Complete," a complete PANOx-SP CFPS reaction mixture. Each component
180 stock solution was stored frozen separately until reaction assembly.
181
182 To assemble reactions, each component listed above was first combined with lysate and water
183 to reach a volume of 15 µL per well in the microplate. The plate was flash frozen in liquid

184 nitrogen and lyophilized as described above. 100 µL acetone was added to solvent wells for one
185 hour while control wells were left untreated. A polypropylene mat was used as before to
186 prevent solvent evaporation. Following acetone treatment, the majority of the solvent was
187 removed by aspiration with care not to disturb the insoluble pellet. The remaining solvent was
188 allowed to evaporate at room temperature in a fume hood (without application of a vacuum).
189 Then, all reactions apart from the “complete” reaction controls were rehydrated with a mixture
190 of water and all components of a complete CFPS reaction excluding the lysate (pre-mix, T7
191 RNAP, RNase inhibitor, and DNA stock solutions). “complete” control wells were rehydrated
192 with water. This experimental setup results in a final concentration two times that of a normal
193 reaction for the component being tested. For example, in the wells testing the DNA component,
194 DNA is at a 1x6.4 nM concentration prior to lyophilization, but at a 2x6.4 nM concentration
195 after rehydration with water and CFPS reagents. Acetone treatment results are normalized to
196 the no-solvent control to isolate the effect of each added component on acetone sensitivity of
197 the lysate.
198
199

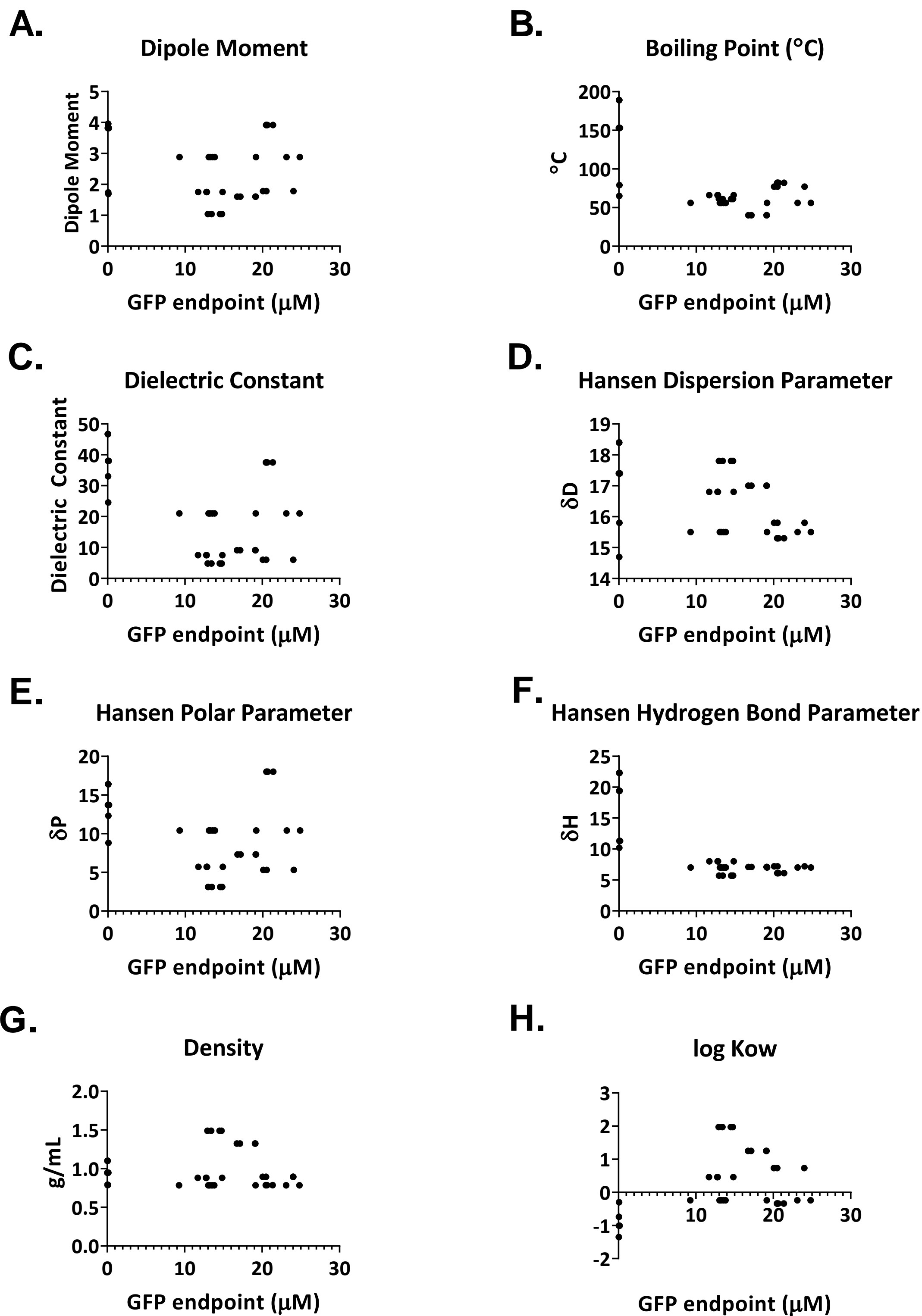
200 Supplementary Figures



201

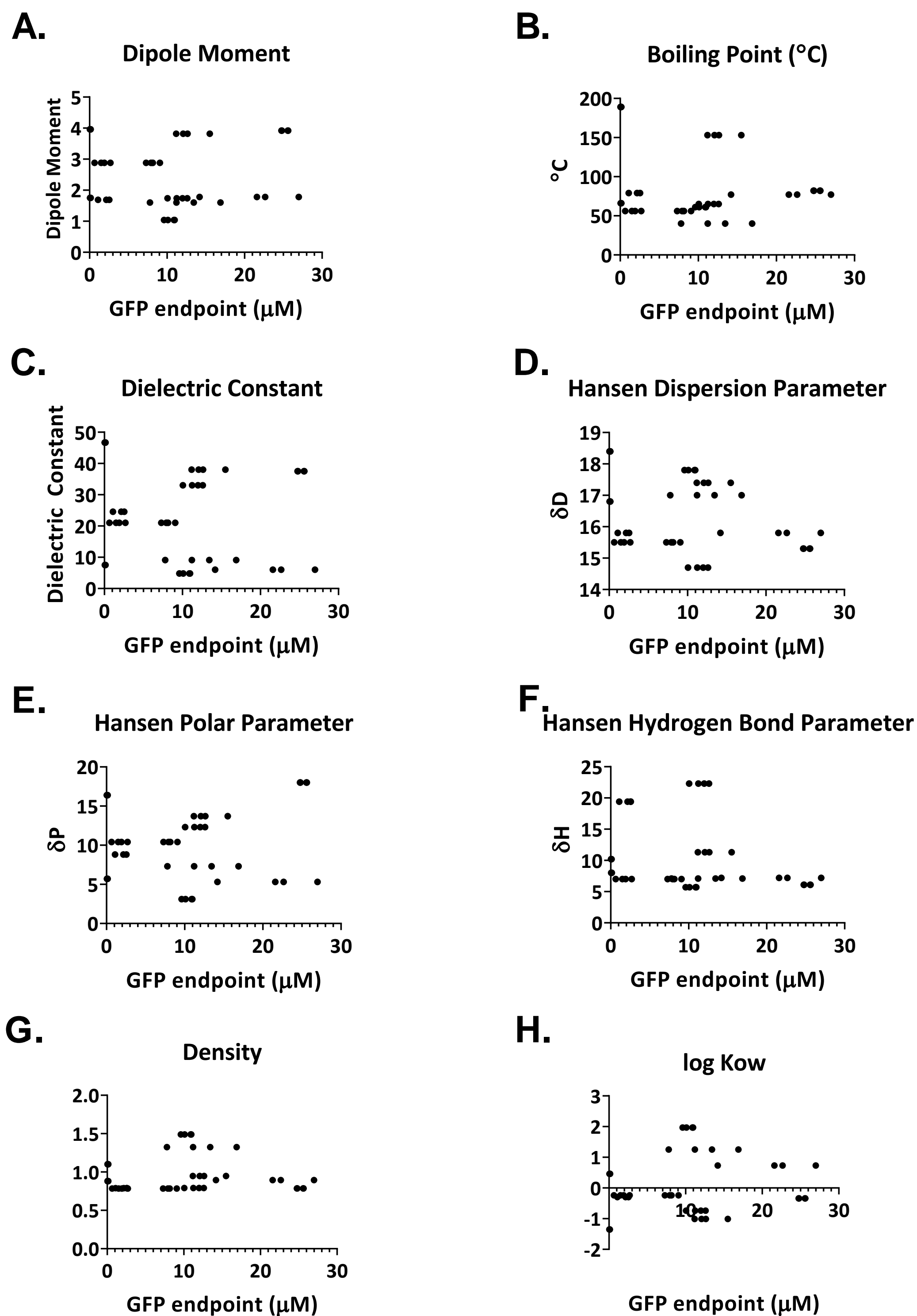
202 **Figure S1** Kinetics plots of solvent screen with endpoints represented in Figure 2 of the main
203 text. Complete reactions exposed to solvent (a-c), cell lysate only exposed to solvent (d-f).

204



205

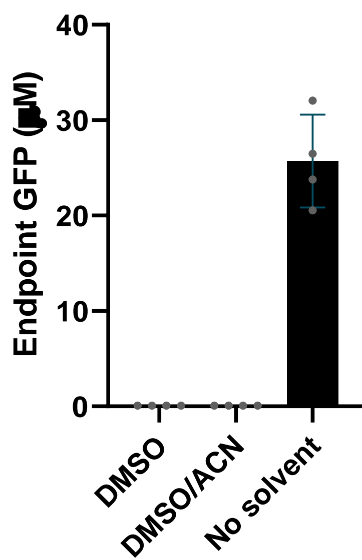
206 **Figure S2** Correlation plots comparing endpoint GFP productivity to the physical property
207 parameters of the organic solvents used. Lyophilized, complete CFPS reactions were exposed to
208 solvent, then rehydrated with water.



209

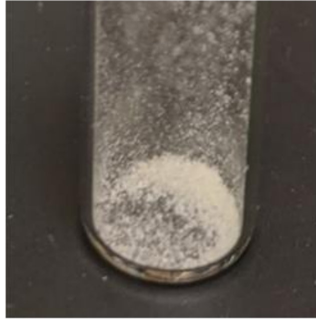
210 **Figure S3** Correlation plots comparing endpoint GFP productivity of solvent-exposed lysate to
211 the physical property parameters of the organic solvents used. Lyophilized lysate was exposed
212 to solvent alone, then complemented with the other ingredients of a complete CFPS reaction
213 upon rehydration.

214

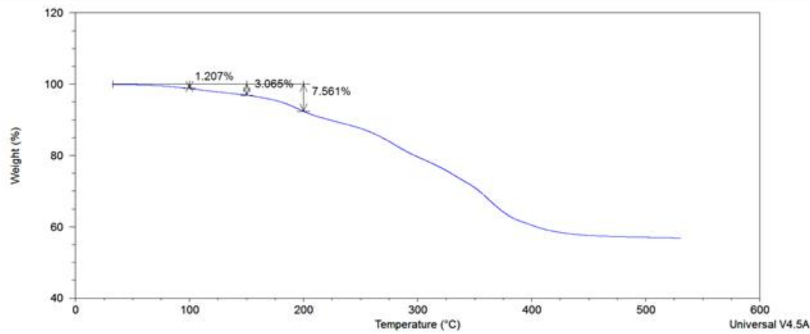


215
216 **Figure S4** Washing with acetonitrile to remove DMSO. Each bar across the x-axis is labeled with
217 the solvent treatment. "DMSO/ACN" refers to samples treated first with DMSO, then washed
218 with acetonitrile.
219
220

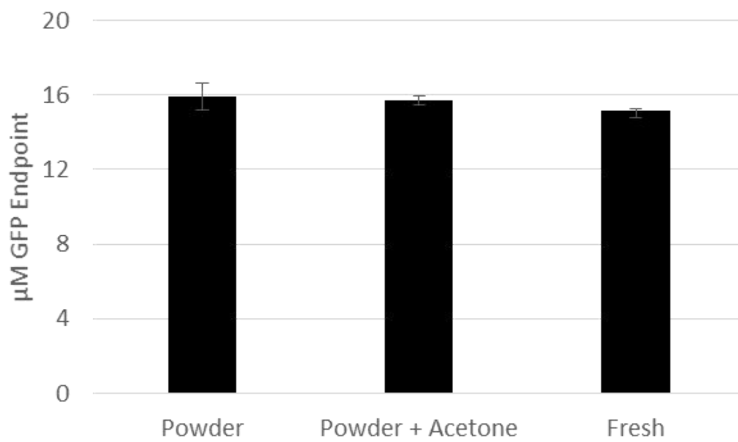
A.



B.



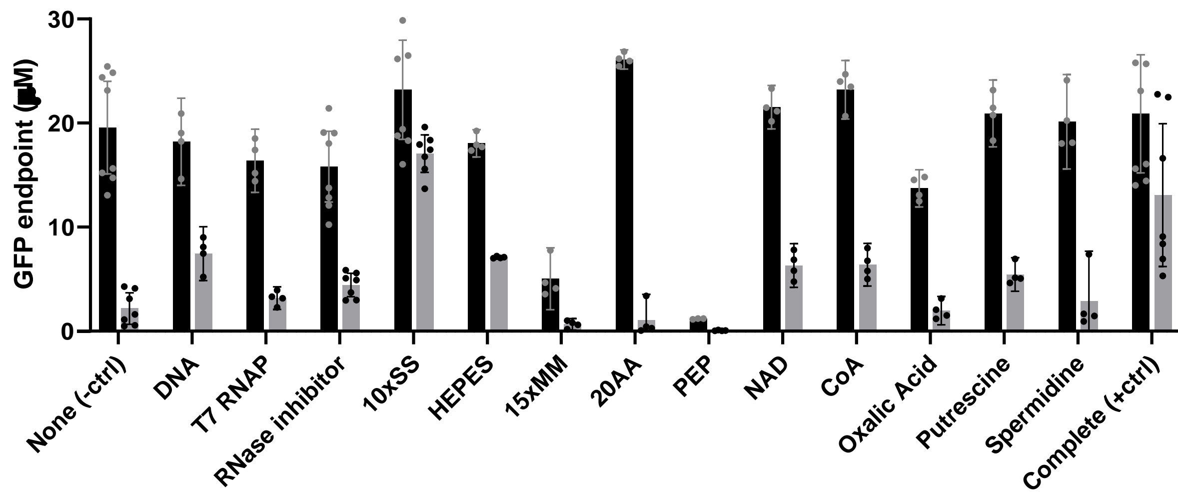
C.



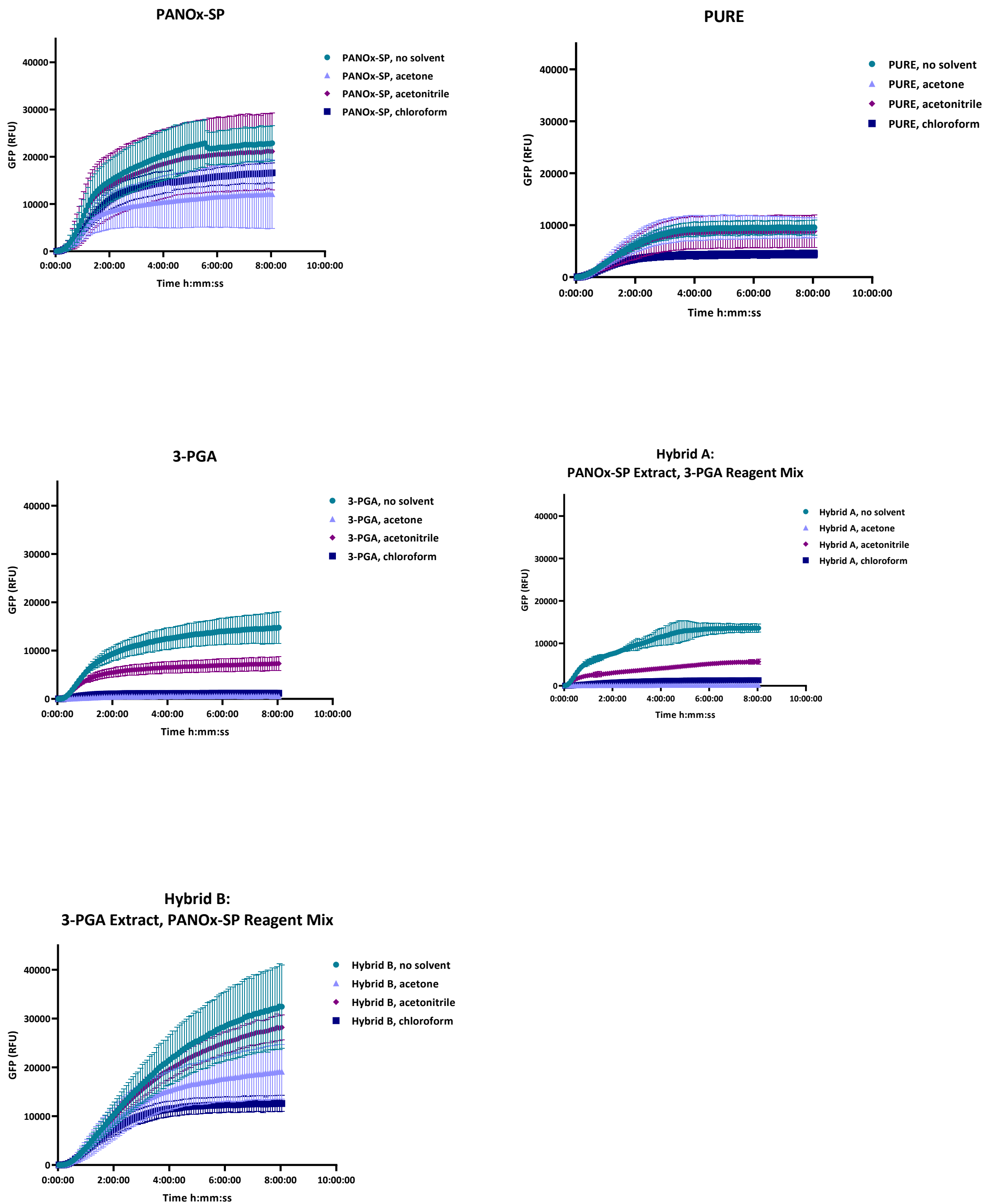
221

222 **Figure S5** CFPS powder after 48 hr lyophilization (a). TGA analysis of the powder (b) where
223 water is the first volatile component and makes up 1.2-3% of the initial weight. Endpoint GFP
224 concentrations after rehydration of CFPS powder and incubation for 8 hours, with and without
225 acetone treatment (c). Non-lyophilized control reaction is labeled "fresh."

226



227
228
229 **Figure S6** Non-normalized PANOx-SP CFPS component screen for acetone tolerance. Black bars
230 are results from a control without solvent exposure. Grey bars refer to a 1 hr acetone exposure.
231 Acetone is evaporated under ambient conditions. Each set of bars is labeled with the CFPS
232 ingredient used to supplement the lysate sample at drying. The negative control labeled "None"
233 is lysate without any supplemented additive. The positive control labeled "Complete" includes
234 all CFPS ingredients.
235



236

237 **Figure S7** Kinetics plots for different CFPS recipes exposed to four solvent conditions. Plots
238 correspond to Figure 4 endpoint data in the main text.

239

240

241

242 Supplementary Tables

243 **Table S1** Solvent properties and source information

Solvent	Vendor	Catalog No.	Solvent Group	Dipole Moment	Boiling Point (°C)	Dielectric Constant	Hansen Dispersion Parameter	Hansen Polar Parameter	Hansen Hydrogen Bond Parameter	Density	Log(Kow)
Acetone	Millipore -Sigma	270725	Polar aprotic	2.88	56	21	15.5	10.4	7.0	0.7845	-0.240
Aceto-nitrile	Millipore -Sigma	360457	Polar aprotic	3.92	82	37.5	15.3	18	6.1	0.7860	-0.340
Ethyl Acetate	Acros	42368-0040	Polar aprotic	1.78	77	6.02	15.8	5.3	7.2	0.8940	0.730
Dimethyl-formamide	Millipore -Sigma	186317	Polar aprotic	3.82	153	38	17.4	13.7	11.3	0.9480	-1.010
Dichloro-methane	Millipore -Sigma	D65100 -4L	Non-polar	1.60	40	9.1	17.0	7.3	7.1	1.3250	1.250
Tetrahydro -furan	Millipore -Sigma	34865-100mL	Polar aprotic	1.75	66	7.5	16.8	5.7	8	0.8800	0.460
Ethanol	Millipore -Sigma	493511 -4L	Polar protic	1.69	79	24.55	15.8	8.8	19.4	0.7893	-0.300
Methanol	Fisher	A411-4	Polar protic	1.74	65	33	14.7	12.3	22.3	0.7910	-0.740
Chloroform	Fisher	C6060-1	Non-polar	1.04	61	4.81	17.8	3.1	5.7	1.4900	1.970
DMSO	Sigma	D2650	Polar aprotic	3.96	189	46.7	18.4	16.4	10.2	1.1000	-1.350

244

245

246 **Table S2** Correlation statistics for productivity of solvent-exposed CFPS reactions

GFP endpoint (µM) Complete reaction exposure	**vs. Dipole Moment**	**vs. Boiling Point**	**vs. Dielectric Constant**	**vs. Hansen Dispersion**	**vs. Hansen Polar**	**vs. Hansen Hydrogen Bond**	**vs. Density**	**vs. log(Kow)**
Pearson r								
r	-0.1556	-0.5766	-0.5830	-0.2311	-0.3196	-0.7478	0.08675	0.5907
95% confidence interval	-0.4325 to 0.1481	-0.7458 to -0.3375	-0.7501 to -0.3460	-0.4941 to 0.07063	-0.5631 to -0.02514	-0.8549 to -0.5796	-0.2157 to 0.3740	0.3653 to 0.7552
R squared	0.02423	0.3325	0.3399	0.05341	0.1022	0.5592	0.007526	0.3489
P value								
P (two-tailed)	0.3130	<0.0001	<0.0001	0.1312	0.0344	<0.0001	0.5755	<0.0001
P value summary	ns	****	****	ns	*	****	ns	****
Significant? (alpha = 0.05)	No	Yes	Yes	No	Yes	Yes	No	Yes
Number of XY Pairs	44	44	44	44	44	44	44	44

247

248

249 **Table S3** Correlation statistics for productivity of solvent-exposed *E. coli* lysate

GFP endpoint (µM) Complete reaction exposure	**vs. Dipole Moment**	**vs. Boiling Point**	**vs. Dielectric Constant**	**vs. Hansen Dispersion**	**vs. Hansen Polar**	**vs. Hansen Hydrogen Bond**	**vs. Density**	**vs. log(Kow)**
Pearson r								
r	0.09429	-0.1509	-0.02309	-0.3073	0.1437	-0.2137	-0.002955	0.1932
95% confidence interval	-0.2084 to 0.3805	-0.4286 to 0.1528	-0.3178 to 0.2757	-0.5537 to -0.01151	-0.1600 to 0.4226	-0.4801 to 0.08882	-0.2996 to 0.2942	-0.1100 to 0.4635
R squared	0.008891	0.02277	0.0005334	0.09446	0.02065	0.04566	8.730e-006	0.03732
P value								
P (two-tailed)	0.5426	0.3282	0.8817	0.0424	0.3521	0.1637	0.9848	0.2089
P value summary	ns	ns	ns	*	ns	ns	ns	ns
Significant? (alpha = 0.05)	No	No	No	Yes	No	No	No	No
Number of XY Pairs	44	44	44	44	44	44	44	44

250

251 **Table S4** Final concentrations of components in three different CFPS buffers

Component	PANOx-SP	3-PGA	PURE
Magnesium glutamate	12 mM	10 mM + 4.62 mM†	-
Potassium glutamate	130 mM	30 mM + 49.5 mM†	100 mM
Ammonium glutamate	10 mM	-	-
Magnesium acetate	3.7 mM*	-	13 mM
Potassium acetate	16.02 mM*	-	-
Tris acetate	2.67 mM (pH 8.2)*	1.65 mM (pH 8.2)†	-
HEPES	57 mM (pH 7.4)	50 mM (pH 8)	50 mM (pH 7.6)
ATP	1.2 mM	1.5 mM	2 mM
GTP	0.85 mM	1.5 mM	2 mM
CTP	0.85 mM	0.9 mM	1 mM
UTP	0.85 mM	0.9 mM	1 mM
Folinic acid	0.072 mM	0.068 mM	0.02 mM
tRNA	170.6 µg/mL	200 µg/mL	2.8 A_{260} units in 50 µL = 3.5 mg/mL
Alanine	2 mM	3 mM	0.3 mM
Arginine	2 mM	3 mM	0.3 mM
Histidine	2 mM	3 mM	0.3 mM
Lysine (monoHCl)	2 mM	3 mM	0.3 mM
Aspartic acid	2 mM	3 mM	0.3 mM
Glutamic acid	2 mM	3 mM	0.3 mM
Isoleucine	2 mM	3 mM	0.3 mM
Leucine	2 mM	3 mM	0.3 mM
Methionine	2 mM	3 mM	0.3 mM
Phenylalanine	2 mM	3 mM	0.3 mM
Tryptophan	2 mM	3 mM	0.3 mM
Tyrosine	2 mM	3 mM	0.3 mM
Valine	2 mM	3 mM	0.3 mM
Serine	2 mM	3 mM	0.3 mM
Threonine	2 mM	3 mM	0.3 mM
Asparagine	2 mM	3 mM	0.3 mM
Glutamine	2 mM	3 mM	0.3 mM
Cysteine	2 mM	3 mM	0.3 mM
Glycine	2 mM	3 mM	0.3 mM
Proline	2 mM	3 mM	0.3 mM
PEP	33 mM	-	-

NAD	0.33 mM	0.33 mM	-
CoA	0.27 mM	0.26 mM	-
Spermidine	1.5 mM	1 mM	2 mM
Putrescine	1 mM	-	-
Oxalic acid	4 mM	-	-
T7 RNA polymerase	100 µg/mL	100 µg/mL	10 µg/mL
Plasmid DNA	6.4 nM	6.4 nM	6.4 nM
RNase Inhibitor	0.8 U/µL	-	-
Cell extract	26.7% v/v*	33% v/v†	-
Maltodextrin	-	30 mM	-
PEG	-	1.5 % w/v	-
cAMP	-	0.75 mM	-
3-PGA	-	30 mM	-
DTT	0.8 mM*	1 mM+0.33mM†	1 mM
Creatine Phosphate	-	-	20 mM

252 * Items in Style I recipe added as part of the cell extract solution

253 † Items in Style II recipe added as part of the cell extract solution

254