Resource competition in CRISPRa genetic circuits

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ABSTRACT

CRISPR-mediated gene activation (CRISPRa) allows concurrent regulation of transcription of many gene targets and has found widespread applications in genome-wide screening, bioproduction, and therapeutics. In principle, multiple gene targets can be regulated independently by choosing orthogonal scaffold RNA (scRNA); in practice, this is not the case due to competition for dCas9 and activator protein. While prior studies have extensively characterized competition effects in CRISPRi circuits such an investigation in CRISPRa circuits is still lacking. In this paper, we investigate the on-target and off-target effects of competition for the two resources during CRISPRa. We observe a biphasic response in the on-target response of a CRISPRa module, wherein increased expression of scRNA leads to target repression instead of activation. The addition of a competitor further leads to the repression of any other CRISPRa system, showing the lack of modularity of concurrent CRISPRa systems. These effects are significant even when both resources are expressed at their maximum tolerable limit. We finally show that competition can also be used to design the response of a CRISPRa module by reducing leakiness while keeping the same maximal activation. The results from this study can significantly affect the interpretation of data obtained when using multiple CRISPRi/a modules and can be used as a predictive tool to determine how the operation of any such module in a concurrent configuration is different from the one expected in isolation.

Keywords: CRISPR-mediated gene activation, Resource competition, Coupling

CRISPR has revolutionized the fields of genetic engineering and has transformed our ability to edit or regulate the genome of several organisms[1]. In a CRISPR Cas9 system, the Cas9 protein binds to a guide RNA, that helps locate the target gene to be modified with high specificity. In its native context[2,3], this acts as an adaptive immune system where the Cas9 protein cleaves the DNA of any virus infecting the bacteria[4]. DNA modification through the CRISPR Cas9 system, commonly known as the genetic scissors, has moved from its original function of protecting the bacteria to genomic manipulations. Genomic manipulations were performed using purified Cas9, trans-activating CRISPR RNA[5], and CRISPR guide RNAs to finally reprogram Cas9 to target a specified site of choosing[6–9].

CRISPR has then expanded towards synthetic control of gene expression using catalytically-inactive denatured Cas9 (dCas9)[10]. Guide RNAs recruit dCas9 protein to target specific genes based on predictable Watson-Crick base pairing. This allows the design of multiple target-specific guide RNAs for orthogonal simultaneous regulation of multiple genes. During CRISPR-mediated gene interference (CRISPRi), dCas9 is recruited by the guide RNA to physically obstruct RNAP from binding at the promoter site of the target[10]. On the contrary, CRISPR-mediated gene activation (CRISPRa) involves an additional activator protein that recruits RNAP to the promoter site. The guide RNA used in CRISPRa is modified to include an additional hairpin loop for the activator protein recruitment and is referred to as the scaffold RNA (scRNA)[11,12].

The toxicity of dCas9 and RBP-AD resources at high concentrations[13] limits the extent to which their concentration can be increased. As a consequence, competition effects wherein expression of one guide RNA causes decreased repression of a different guide RNA has been observed in CRISPRi systems[14–16]. Mathematical models investigating competition have been developed also for CRISPRa systems, and have predicted that when dCas9 and a separate activator protein are used for activation, competition effects should be substantial[17]. By contrast, when dCas9 is modified to directly include an activator protein, then these effects are predicted to be not as prominent[18]. Followup experimental studies created a dCas9 controller to mitigate the effects of competition[15]. Here, we consider CRISPRa systems that use both dCas9 and an activator protein as developed in[11,12,19] and investigate the emergence of competition effects experimentally. This setup is particularly relevant since it allows the implementation of concurrent CRISPRi/a network, given that the activator protein is separate from dCas9. Our previous work[17] theoretically examined the competition during concurrent CRISPRa operation and found the existence of a double diamond reaction network structure as a consequence of competition for two shared resources. The double-diamond structure led to two key consequences in the system: on-target repression and significant coupling between theoretically orthogonal CRISPRa modules.

As predicted from theory, we observe an on-target biphasic response, where increasing the guide RNA levels leads to an initial increase followed by a decrease. We further examined the effect of competition on the orthogonality of two CRISPRa modules and observed significant unintended repression of a non-target gene with increased expression of an scRNA.

Modifications were then made in the scRNA and the scRNA binding site, to investigate individual resource competition effects and the role of a competitor target site (PAM site and the scRNA binding site).

1 System Description

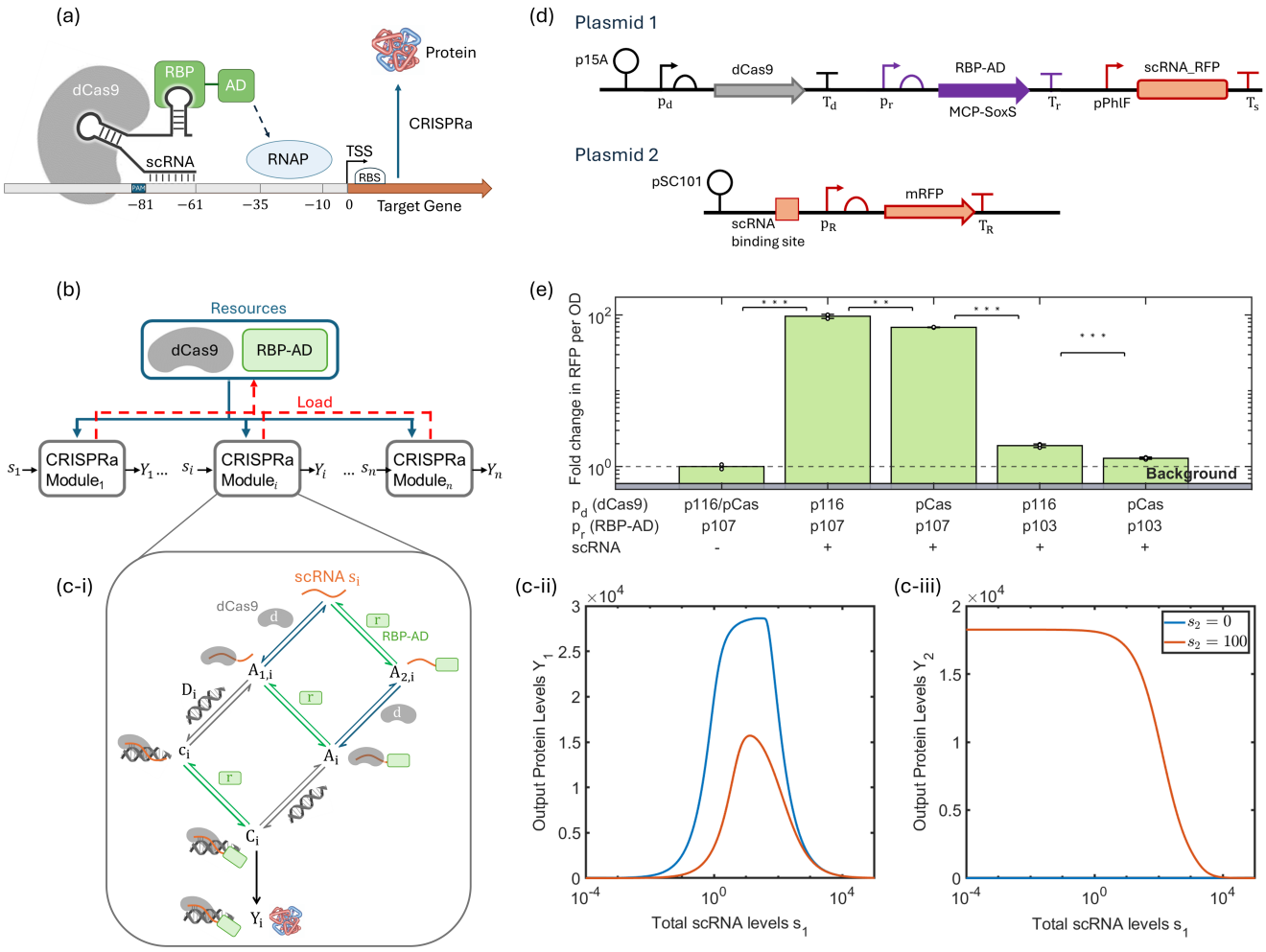

Figure 1. CRISPRa basic functionality and resource competition predicted by a model. (a) Schematic representation of the activation of a target gene through CRISPRa where the CRISPR complex consisting of dCas9, RBP-AD, and scRNA binds upstream of the transcription start site (TSS) of the target allowing activation. (b) Block diagram representation of concurrent CRISPRa modules with scRNA $\{s_1, s_2, ..., s_n\}$ activating the production of proteins $\{Y_1, Y_2, ..., Y_n\}$ using the shared dCas9 and RBP-AD resources. The loads on the resources by each module are shown in red. (c-i) Chemical reaction diagram of CRISPRa. It shows the pathways from the input scRNA (s_i) to the regulated output protein (Y_i) in a CRISPRa module. Double-sided arrows represent reversible binding reactions whereas the one-sided arrow from C_i to Y_i represents gene expression. The ordinary differential equation (ODE) model associated with the reactions in panel (c-i) predicts the (c-ii) biphasic on-target response and (c-iii) off-target repression. The on-target response is portrayed through the input-output response of Y_1 to its corresponding input s_1 in the presence ($s_2 = 100$) and absence ($s_2 = 0$) of the competitor for $n = 2$. The off-target response is portrayed through the variation of Y_2 with competitor s_1. (d) Genetic constructs of a CRISPRa system. The constituents of the CRISPR complex are produced by plasmid 1 and they activate the production of the red fluorescent protein (RFP) in plasmid 2. (e) Fold activation in RFP in the presence of CRISPRa for various combinations of promoters (dCas9 and RBP-AD). The levels of the resources are controlled by changing their respective promoter strengths. The grey-shaded region indicates the normalized background fluorescence of the parental Marionette *E. coli* strain.

CRISPRa is executed by designing short target-specific scaffold RNAs (scRNA) that recruit dCas9 and RNA-binding protein with activation domain (RBP-AD) to activate a target gene[11,12]. The scRNA used for CRISPRa consists of three distinct

regions: a 20 bp complementary region for target gene binding, a hairpin region for dCas9 protein binding, and an MS2 hairpin for RBP-AD binding. The RBP-AD unit consists of the MS2 coat protein (MCP) fused with SoxS(R93A/S101A) that binds to the MS2 hairpin of the scRNA. Figure 1(a) shows the binding of the scRNA to the complementary region located at the -81 to -61 site upstream of the target gene. An 'AGG' protospacer adjacent motif (PAM site) is designed to be located upstream of the scRNA binding site on the DNA. The activator protein recruits RNAP to the minimal promoter in the region -35 to -10, activating the expression of the target gene.

The sequence information for the CRISPRa target site is encoded on the 20bp complementary region on the scRNA. Therefore, the design of multiple scRNAs with varying 20 bp complementary regions enables concurrent orthogonal CRISPRa modules. Specifically, designing distinct scRNAs $\{s_1, s_2, ..., s_n\}$ targeting multiple genes $\{D_1, D_2, ..., D_n\}$ theoretically allows concurrent regulation of multiple proteins $\{Y_1, Y_2, ..., Y_n\}$, as shown in Fig. 1(b). In such a scenario, both dCas9 and RBP-AD are shared resources among these CRISPRa modules. The sequestration of these resources by a CRISPRa module can in principle decrease the availability of the free resources to other modules in the system, leading to unwanted coupling among theoretically independent modules (Fig. 1b,c).

Next, we review a chemical reaction network-based model (Fig. 1c-i) that we previously published[17]. As mentioned before, each CRISPRa module consists of an input scRNA (s_i) that regulates the production of the output protein, Y_i (Fig. 1b). The scRNA binds either with the free dCas9 (d) in the system forming $A_{1,i}$ or the free RBP-AD resource (r) in the system forming $A_{2,i}$. Note, the scRNA must recruit dCas9 before binding with the target gene. The formed complex $A_{1,i}$, having recruited dCas9, may bind with the target gene (D_i) to form c_i, or with RBP-AD to form the CRISPR complex A_i. This is followed by the binding with RBP-AD and the target gene, respectively, to form the transcriptionally active complex C_i. On the other hand, the complex $A_{2,i}$ must combine with dCas9 forming A_i followed by the combination with the target gene to form C_i. The transcriptionally active complex will then undergo transcription and translation producing the output protein Y_i. The pathways from the input s_i to its regulated output Y_i, therefore, can be portrayed as a double-diamond reaction network as shown in Fig. 1(c-i).

Numerical simulations of the on-target and off-target response of two concurrent CRISPRa modules are presented in Fig. 1(c-ii, c-iii). The input/output response of Y_1 to its input s_1 without a competitor ($s_2 = 0$), shows a biphasic response (Fig. 1c-ii), where the protein level increases for low levels of scRNA and decreases for high levels of scRNA. Such a behavior is referred to as self-sequestration or squelching[20]. Using the model, we infer that the biphasic response occurs due to the sequestration of resources by the intermediate complexes in the top half of the double diamond ($A_{1,1}$, $A_{2,1}$ and c_1) that leads to a decrease in the production of A_1, C_1 and therefore Y_1. This highlights the presence of internal competition for resources between scRNAs and intermediate complexes in the CRISPRa module. Introducing a competitor in the system reduces the overall production of Y_1. In the off-target response of the system captured through the variation of Y_2 with its competitor input s_1 for a constant s_2 (Fig. 1c-iii), we observe that protein levels decrease significantly at high s_1 showing the coupling due to competition between the two CRISPRa modules.

To investigate competition in CRISPRa experimentally, we designed a first set of constructs to recapitulate CRISPRa functionality (Fig. 1d). Plasmid 1 consists of dCas9, RBP-AD, and scRNA_RFP (scRNA designed to target the RFP gene), while Plasmid 2 consists of an RFP target gene with the scRNA binding site. These plasmids are co-transformed to the Marionette *E. coli* cell strains[21].

We confirm the functionality of the CRISPRa design by computing the fold change in output protein concentration levels in the presence of CRISPRa (Fig. 1e). The protein levels are normalized with respect to the averaged basal RFP levels (in the absence of the scRNA). We observe a 100-fold change in the output level when the promoters of dCas9 (p_d as BBa_J23116, represented as p116) and RBP-AD (p_r as BBa_J23107, represented as p107) are set to produce their respective maximum tolerable limits[13,22]. Furthermore, we observe a slight decrease in the fold activation after changing the promoter of dCas9 to the endogenous Sp.pCas9 promoter (represented as pCas). Also, a drastic drop in the fold activation is observed when the promoter of RBP-AD is changed to BBa_J23103 (represented as p103).

2 On target CRISPRa response: Emergence of self-sequestration

In this section, we investigate the on-target response of an isolated CRISPRa module, i.e. $n = 1$, with specific emphasis on the model-predicted biphasic response. Towards this, we co-transform the plasmids shown in Fig. 2(a) with a DAPG inducible scRNA into the parental cell strain. Other CRISPRa constituents, dCas9 and RBP-AD, are constitutively expressed in a p15A vector and the target gene is on a low-copy plasmid.

Figure 2(b) shows the induction curve of CRISPR-activated RFP with increasing levels of DAPG in the system. We observe that increasing the DAPG levels increases the output protein in the system for both low and high levels of dCas9. From hereon, low levels of dCas9 correspond to the pCas promoter whereas the high levels correspond to the p116 promoter from Fig. 1(e).

Increasing DAPG further does not lead to a corresponding increase in scRNA due to the saturation of the promoter activity[21]. Hence, to obtain increasing levels of scRNA in the system, the genetic construct is modified to Fig. 2(c). Here, there are two

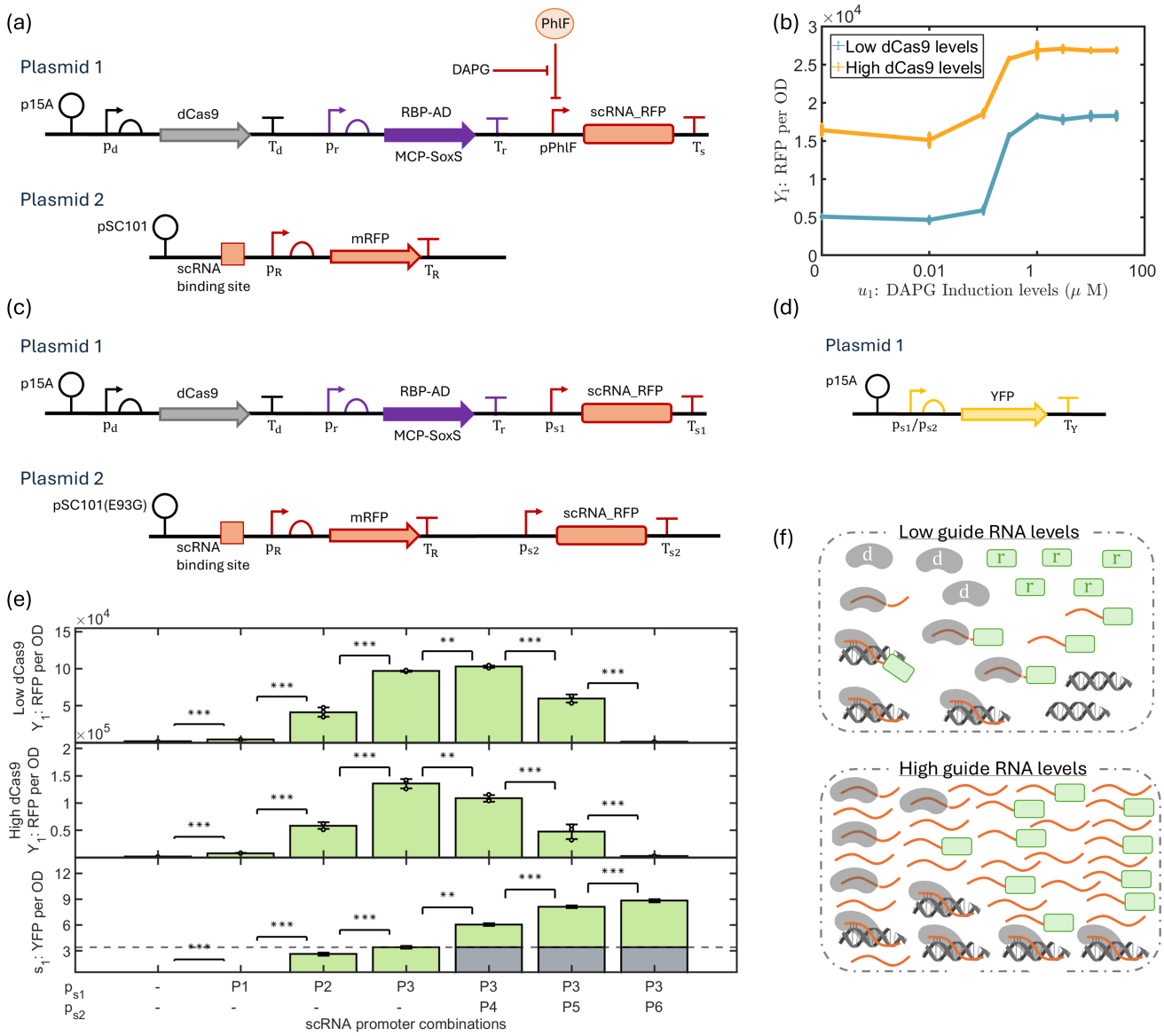

Figure 2. Biphasic on-target response of an isolated CRISPRa module. (a) The genetic construct to observe the isolated activation of RFP by the corresponding input scRNA varied through the induction of DAPG. The transcription factor PhlF is produced endogenously by the parental Mar. *E. coli* strain[21]. (b) Effect of expressing the scRNA on the production of the output protein for different levels of dCas9 in the system. dCas9 levels are dictated by using a weaker (pCas) or stronger (p116) promoter. (c) Genetic construct to observe biphasic response where the scRNA_RFP is progressively increased by increasing the strengths of promoters, p_{s1} and p_{s2}. (d) Genetic construct used to verify the promoter strength of the scRNAs. (e) Output protein levels (RFP) produced for increasing levels of scRNA in the system for low and high dCas9 levels showing the biphasic response of the system. YFP shows the relative increase in the production of scRNA_RFP while using the combinations of promoters implemented in (c) through the construct shown in (d). (f) Schematic representation of the relative abundance of resources and complexes for (top panel) low scRNA levels and (bottom panel) high scRNA levels highlighting the role of competition in the emergence of the biphasic response. Specifically, when scRNA is at high levels, all dCas9 and RBP-AD will be bound to one scRNA and the likelihood of two of them being bound to the same scRNA decreases.

scRNA cassettes distributed among the two plasmids, with scRNA on plasmid 1 having the promoter p_{s1} and the scRNA on plasmid 2 having the promoter p_{s2}. The promoter strength of the scRNA is varied for both cassettes to obtain increasing scRNA levels in the system. The strength of the promoters (with either $\{p_{s1}$ or $p_{s2}\}$) were verified using the YFP construct shown in Fig. 2(d). The bottom panel in Fig. 2(e) shows the variation of YFP levels with the promoter combinations.

From Fig. 2(e), we observe that increasing scRNA in the system shows a biphasic behavior in the on-target response as predicted by the model. The behavior is observed for both low and high levels of dCas9 in the system. At low levels of scRNA, the presence of free resources in the system leads to an increase in the production of the protein as the scRNA level is increased (top panel of Fig. 2f). Further increase in the scRNA levels in the system leads to a complete depletion of free resources leading to the sequestration of resources by the intermediate complexes (Fig. 1c-i). Therefore at high scRNA levels (bottom panel of Fig. 2f), we observe an abundance of complexes formed by the scRNA with either of the two resources and the complete lack of complexes with both resources. This causes the output protein levels to drop resulting in the biphasic response of the system.

3 Off-target response: Coupling between two orthogonal CRISPRa modules

Having investigated the effects of competition on the on-target response of an isolated CRISPRa module, we now look at the off-target response in a concurrent pair of CRISPRa modules ($n = 2$). Towards this, the genetic construct of Fig. 2(a) is modified to include an additional scRNA_GFP (scRNA that activates the production of GFP) and a target GFP site (Fig. 3a). scRNA_GFP is constitutively expressed whereas the scRNA_RFP has a DAPG-inducible promoter.

Figure 3(b) shows the variation in the output protein levels of both RFP (blue) and GFP (orange) as DAPG is increased. As expected, increasing the levels of DAPG increases the scRNA_RFP leading to the activation of RFP. On the other hand, increasing DAPG leads to a 46% decrease in the levels of GFP. This shows that for the same levels of scRNA_GFP in the system increasing an off-target scRNA_RFP decreases the output GFP levels, highlighting the coupling between the two modules. The coupling is caused due to both scRNAs (scRNA_RFP and scRNA_GFP) utilizing dCas9 and RBP-AD resources from a common shared resource pool (Fig. 3c).

Looking at coupling from another perspective, Figure 3(d) shows the variation of RFP and GFP levels with increasing levels of scRNA_GFP. Both protein levels are normalized with respect to their levels in the absence of the competitor scRNA_GFP. For low DAPG levels, we observe that increasing the scRNA_GFP leads to an approximately 6-fold increase in the levels of GFP, whereas the coupling due to competition reduces the RFP by 85%. For high DAPG levels, the fold-activation in GFP drops to almost half the initial fold-change, i.e. from an approximate 6-fold activation to 3-fold activation for the high competitor scRNA_GFP levels. The reduction in RFP due to coupling is decreased to approximately 34%.

So far, competition for dCas9 and RBP-AD resources leads to an approximately 95% decrease in the activation of the on-target gene due to self-sequestration at high scRNA levels in the absence of a competitor and to an 85% decrease in the expression of off-target genes. Therefore, competition for resources significantly affects the functionality of networks of CRISPRa modules.

4 Using competition as an investigation tool

Having analyzed the effects of competition in CRISPRa modules, in this section, we verify the existence of equally significant competition for both dCas9 and RBP-AD. Towards this, we take advantage of the competition effects to examine competition for individual resources in the CRISPRa module. Specifically, modifications are made in the competitor scRNA (in this case, scRNA_GFP) such that even though the competitor protein is not expressed (no GFP activation), inferences can be obtained by observing the RFP module. To this end, we design the scRNA_GFP to selectively sequester the resources leading to competition for individual resources (Fig. 4a).

To elaborate, the competitor scRNA consists of three distinct regions as shown in Fig. 4(b), the target gene (DNA), dCas9, and RBP-AD binding sites. Modifications are made in the scRNA_GFP to implement four different cases. In the first case (Fig. 4b,c I), scRNA_GFP is the perfect competitor used in Fig. 3, which binds with both dCas9 and RBP-AD. Next, the dCas9 binding site on the scRNA_GFP is scrambled to obtain the second case (Fig. 4b,c II), which competes for the RBP-AD resource alone. Note that such an scRNA cannot bind with the target gene due to the absence of dCas9. On the other hand, scrambling the RBP-AD binding site on the scRNA_GFP produces the third case (Fig. 4b,c III), which competes for the dCas9 resource alone. Such a CRISPRa module can combine with dCas9 and further combine with the target gene. Finally, the fourth case corresponds to the complete absence of scRNA_GFP leading to no competition for resources between the two modules (Fig. 4b,c IV).

Figure 4(d) shows the output RFP levels for the four cases of scRNA_GFP. For high dCas9 (right panel), irrespective of the levels of DAPG in the system, we observe that the RFP levels with no competitor scRNA are the highest whereas the levels with competition for both resources are the lowest. The RFP levels when subject to competition for only one of the resources always stay between the aforementioned extremes. This shows that both dCas9 and RBP-AD resources are being competed for in the system and the competition effects are not due to limited quantities of a single resource. To elaborate, comparing the blue bars with the orange ones, we observe that adding a competitor that sequesters RBP-AD leads to a reduction in RFP showing the existence of competition for RBP-AD. Similarly, comparing the blue bars with the yellow ones, we observe that adding a competitor that sequesters dCas9 also leads to a reduction in RFP showing the existence of competition for dCas9.

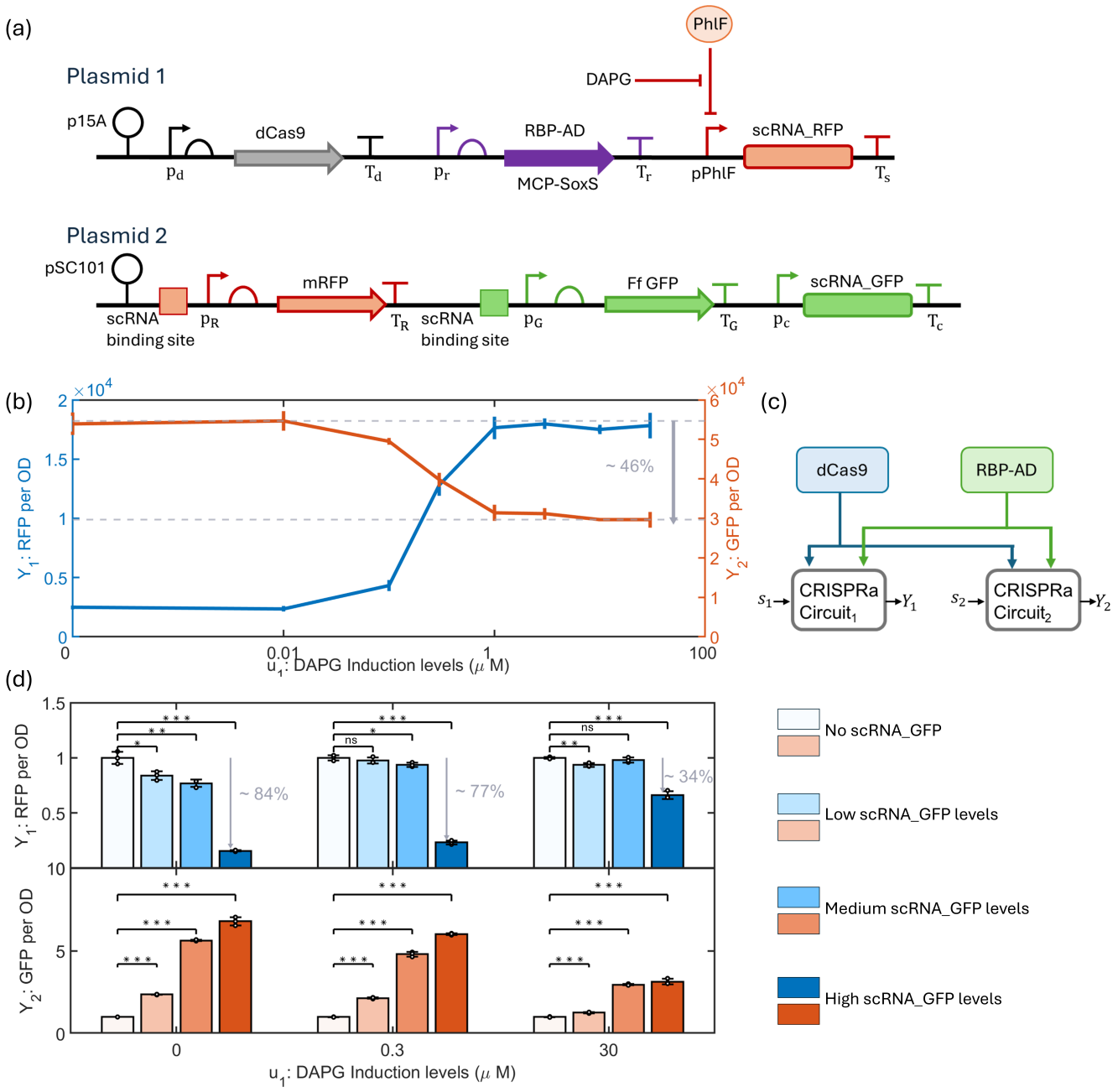

Figure 3. Coupling between two independent CRISPRa modules through resource competition. (a) Genetic construct to investigate the effect of competitor CRISPRa module (scRNA_GFP activating the production of GFP) in the activation of mRFP through CRISPRa. (b) On-target response of RFP and off-target response of GFP with increasing DAPG levels for a fixed promoter strength for scRNA_GFP. (c) Block diagram representation of the resources, dCas9 and RBP-AD, being shared by the two CRISPRa modules. (d) Bar chart representation for the fold change in RFP and GFP in the presence of increasing levels of competitors in the module. The charts are normalized using the protein levels obtained in the absence of scRNA_GFP. The concentration levels of scRNA_GFP are varied by using low (BBa_J23116), medium (LacUV5), and high (pTrc) promoter strengths[23].

However, this is not the case for low dCas9. The output values for no scRNA_GFP (blue bar) are at a similar level as the one with RBP-AD competition (orange bar) (in Fig.4(d) left panel). This indicates that dCas9 is the limiting factor in the system.

Furthermore, increasing DAPG in the system causes a shift in the relative sequestration of resources in the system. From Fig. 4(d), at low DAPG levels competition for RBP-AD leads to more decrease in RFP when compared to competition for

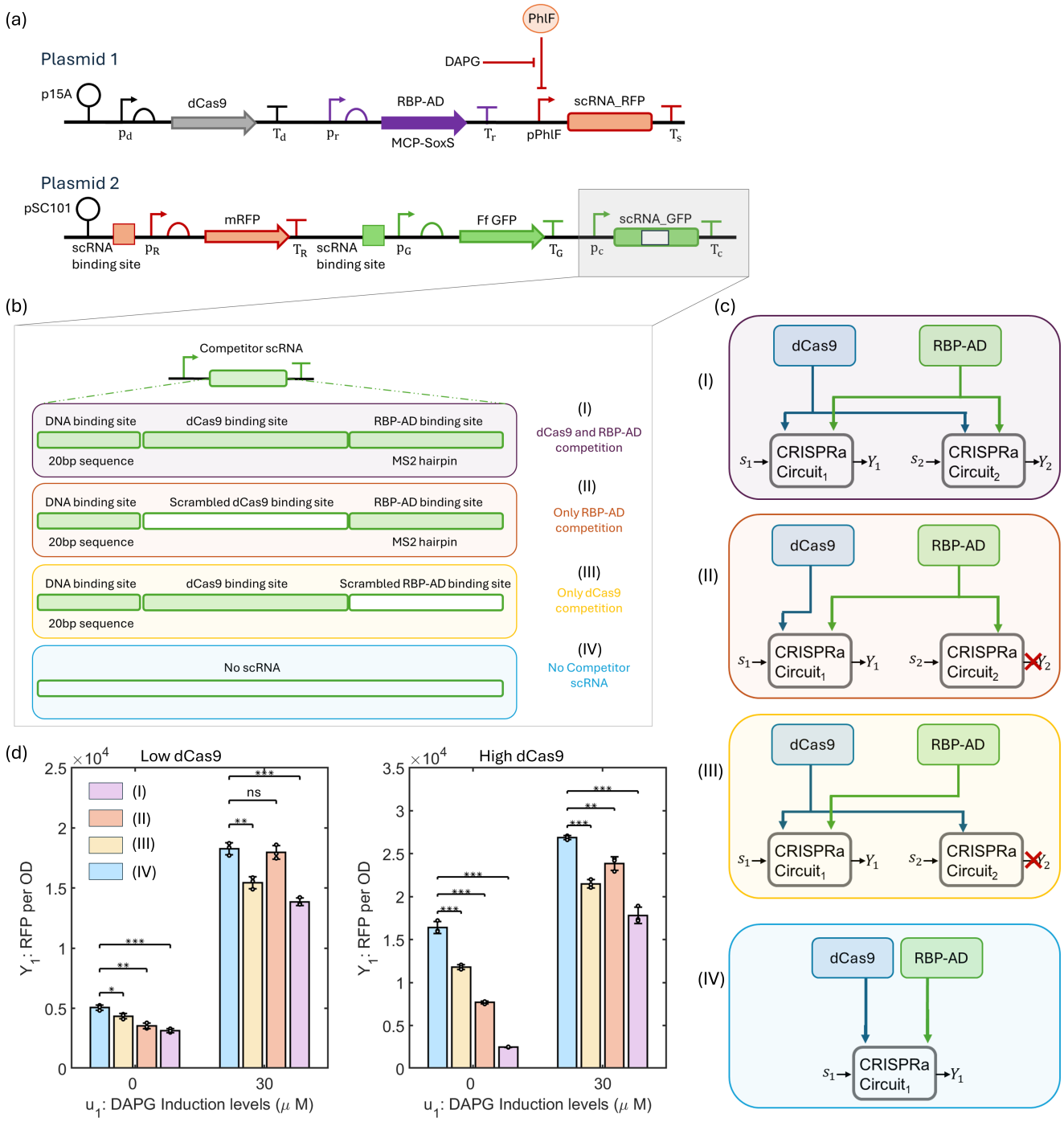

Figure 4. Designing competitor scRNA to enable selective resource sharing (a) Genetic constructs for two concurrent CRISPRa modules with scRNA_RFP activating RFP and scRNA_GFP modified for selective sequestration of resources. (b) Modifications made in the scRNA_GFP to investigate the effect of competition for each resource individually. Four cases of scRNA_GFP implemented correspond to competition for (I) both dCas9 and RBP-AD, (II) only RBP-AD, (III) only dCas9, and (IV) none of the resources. Although the modifications made on the scRNA_GFP would impede the activation of GFP, coupling between the two CRISPRa modules allows us to study the effects by examining the response in the RFP signal. (c) Block diagram representation of selective competition for resources by the two CRISPRa modules for the four scRNA_GFP implementations. (d) On-target response of RFP in the presence of the four competitor scRNA_GFP implementations for various DAPG and dCas9 levels in the system.

dCas9 (orange bars are lower than yellow bars), whereas, at high DAPG levels, competition for dCas9 leads to more decrease in RFP when compared to competition for RBP-AD (yellow bars are lower than orange bars). Such a shift is due to the presence of an additional DNA-bound complex for the scRNA in case III with dCas9 binding, which adds stability to the complex which will be discussed in detail in the next section.

5 Shaping the response of a CRISPRa module by designing competitor RNA

In this section, we examine the target site requirements for CRISPRa, specifically the existence of a PAM site and the 20 bp complementarity to the scRNA, and their effects on the competition for resources. Towards this end, we design the scRNA binding site on the CRISPRa module for GFP with various modifications and examine the response in the RFP levels (Fig. 5a). The target site for scRNA_GFP is modified to engender four different cases (Fig. 5b). The first case consists of a perfect target site with both the PAM site and the 20 bp complementarity requirements being met. The second and third case consists of no PAM site and no 20 bp complementarity, respectively, thereby removing the existence of a target gene. Finally, we compare the three cases with the fourth case with no competitor scRNA_GFP.

Figure 5(c) shows the response in the RFP levels for the four cases discussed above. We observe that for low DAPG levels, the presence of competitor scRNA leads to a significant decrease (approximately 85%) in RFP irrespective of the presence of a target site for the competitor. This is not observed at high DAPG levels. For high DAPG levels, the presence of a competitor that binds to a target gene exhibits lower RFP levels when compared to the presence of a competitor without target sites. This shows that the existence of a target site stabilizes the binding of resources to the scRNA. This causes the sequestration of resources in the presence of the target site to be higher when compared to the absence of a target site.

Furthermore, we also observe that for the case with no competitor scRNA, the RFP levels show a 1.5-fold activation with increasing DAPG levels. The low fold-activation is attributed to the leaky nature of DAPG leading to significant expression of scRNA_RFP at zero induction, thereby reducing the effectiveness of CRISPRa. The addition of a competitor scRNA without a target site can lead to an increase in the fold activation from 1.5 to 10, with minimal decrease in the maximum activation level (protein levels at high induction show approximately 5% decrease). Therefore, the addition of a competitor scRNA without a target site can be used as an effective strategy to shape the on-target response to reduce leakiness.

6 Methods

6.1 Bacterial strain construction and manipulation

Plasmids used in this study were cloned using standard molecular biology protocols. The bacterial strain *E. coli* NEB10B (NEB, C3019I) grown in LB Broth Lennox (240230) with appropriate antibiotics were used for genetic circuit construction. The sequence-verified plasmids are co-transformed or retransformed to *E. coli* Marionette strain[21] for all presented results. The experiment's growth medium is an M9 minimal medium composed of M9 salts (1X), 0.4% glucose (SIGMA-ALDRICH, 49159), 0.2% casamino acid (VWR, TS61204-5000), and 1mM thiamine hydrochloride (SIGMA-ALDRICH, T4625-25G) supplemented by appropriate antibiotics. The final concentrations of antibiotics, ampicillin (SIGMA-ALDRICH, A0166-5G) and kanamycin (SIGMA-ALDRICH, K1377-5G) are 100, and 50 ug mL^{-1}, respectively. 2,4-Diacetylphloroglucinol or DAPG (Cayman Chemical, 16345) is used as the inducer.

6.2 Genetic circuit construction

The genetic circuit construction was based on Gibson assembly methods using pCK005.6 and pJF143.J3 from Addgene[11] as the backbone. DNA fragments to be assembled were amplified by PCR using Phusion High-Fidelity PCR Master Mix with GC Buffer (NEB, M0532S). The PCR mix underwent gel electrophoresis for purification. Zymoclean Gel DNA Recovery Kit (Zymo Research, D4002) was used for gel extraction. Later, the plasmid is assembled with Gibson assembly protocol using NEBuilder HiFi DNA Assembly Master Mix (NEB, E2621S). Assembled DNA was transformed into competent cells. Overnight cultures of singular colonies are grown in LB at 37°C. Plasmid DNA is then extracted from the overnight cultured using the plasmid miniprep-classic kit (Zymo Research, D4015). DNA sequencing is outsourced and is performed by Primordium Labs.

6.3 Plate reader experiments

The glycerol stocks (stored at -80°C) are used to prepare overnight cultures. These cultures are grown in M9 media supplemented with appropriate antibiotics at 37 ^{o}C, shaking at 220 rpm in a horizontal orbiting shaker for 6-8 hours in 15 mL culture tubes (VWR, 60818-667). These overnight cultures are diluted to an optical density at 600nm (OD600) measurement of 0.01 in 200 uL growth medium per well in a transparent 96-well plate with a flat bottom (FalconR 96-Well Cell Culture Plates, Corning, 15705-066, VWR) with appropriate antibiotics and inducers. The 96-well plate was incubated at 30°C in a Tecan Infinite 200 PRO microplate reader in static condition and was shaken at a fast speed for 5 seconds right before OD and fluorescence measurements. The sampling interval was 5 min. Excitation and emission wavelengths to monitor RFP fluorescence were

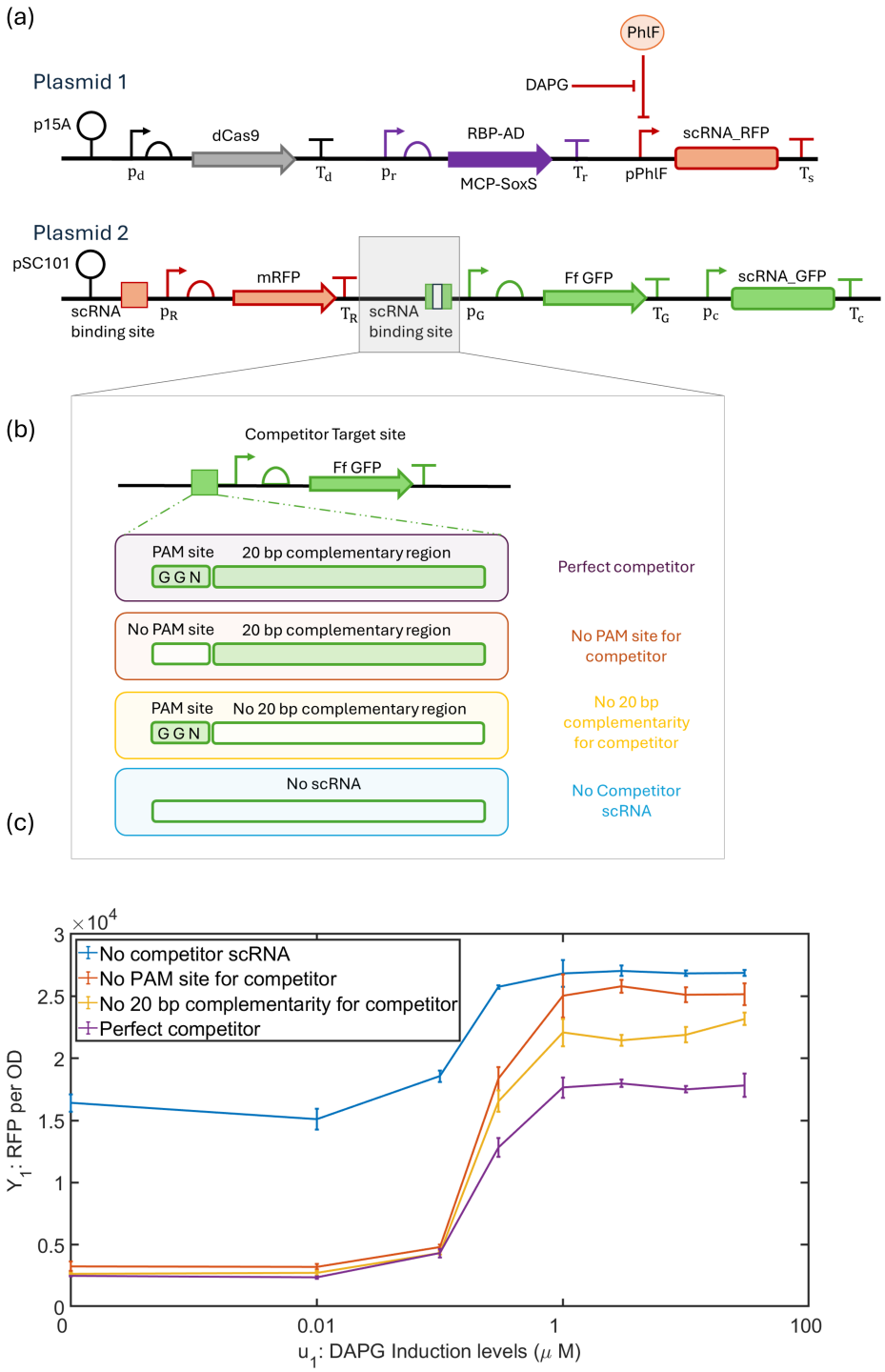

Figure 5. Shaping the response by designing the competitor RNA interactions. (a) Genetic construct for two concurrent CRISPRa modules with scRNA_RFP activating mRFP and scRNA_GFP activating GFP with modification made on the scRNA_GFP binding site. (b) The modification made in the scRNA_GFP binding site to investigate the effect of the target site of the competitor. Four cases of scRNA_GFP binding site implemented correspond to (I) a perfect binding site with both 20 bp complementarity and a PAM site, (II) no PAM site, (III) no 20 bp complementarity, and (IV) no competitor scRNA_GFP. (c) On-target response of RFP in the presence of the four competitor scRNA_GFP binding site implementations for increasing DAPG levels in the system.

584 and 619 nm, and for GFP fluorescence were 485 and 530 nm, respectively. To ensure continued exponential growth, cell cultures were diluted with fresh growth medium (with antibiotics and inducers) to OD600 of 0.01 when OD600 approaches 0.08 at the end of one batch. Multiple batches were conducted until gene expression reached a steady state. The reported steady-state fluorescent values are normalized with the OD600 and are reported when each culture is at an OD600 of 0.06 in the final batch.

6.4 Statistics and reproducibility

Statistical significance was calculated using two-tailed two sample t-tests with the MATLAB Statistics and Machine Learning Toolbox. To ensure reproducibility, experiments were performed using 3 biological replicates.

7 Conclusion

To summarize, we studied the effect of competition during CRISPRa, where dCas9 and RBP-AD are shared among multiple scRNAs. The competition for these resources can give rise to two major consequences: biphasic on-target response and coupling between CRISPRa modules. In an isolated CRISPRa network, we observed a biphasic response with an increase in the output protein levels at low scRNA levels and a decrease at high scRNA levels. In a system with a concurrent pair of CRISPRa modules, we observed unintended repression with the expression of a competitor scRNA. We further confirmed the existence of equally significant competition for both resources. We finally propose using the addition of a competitor scRNA without a target site as a strategy to reduce the leakiness and reshape the response of a system.

8 Acknowledgements

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