

**INVESTIGATION OF STOCHASTICITY IN GENE EXPRESSION IN RESPONSE TO
OSMOTIC STRESS**

By

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INVESTIGATION OF STOCHASTICITY IN GENE EXPRESSION IN RESPONSE TO OSMOTIC STRESS

Abstract

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Stochasticity in gene expression of a synthetic gene network in *E.coli* in response to osmotic stress has been characterized by fluorescence microscopy. A synthetic gene network consisting of three genes in a cascade that are sequentially downregulated and reported by CFP and YFP, and fourth independent gene reported by RFP has been subjected to osmotic stress in the range of 0.3 – 130 atmospheres exerted by NaCl and sucrose (0 – 3 M). The fluorescence intensities and self-correlation parameters for *cfp*, *yfp*, and *rfp* gene expression, and intrinsic, and extrinsic noise parameters for *cfp-yfp*, *cfp-rfp*, and *yfp-rfp* gene pairs determined from the respective protein expression exhibit a combined repression and activation dependence on osmotic stress. Stochasticity in gene expression is almost entirely due to intrinsic noise and decreases in the order *cfp-yfp* > *cfp-rfp* > *yfp-rfp*. This result is surprising given the gene construct where the expression of RFP protein should be independent of CFP and YFP proteins, whose genes repress each other. Under osmotic stress, interaction between coupled and uncoupled genes are activated or repressed to varying levels as a function of pressure contributing to stochasticity in the expression of the genes.

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Dedication

This dissertation is dedicated to my father, Balandaram Nathan who is my pillar of support and the reason I came this far. Thank you for everything.

CHAPTER 1

INTRODUCTION

Stochasticity in gene expression

For an isogenic population of cells grown in homogeneous environments, phenotypic variation has been observed. Examples include, difference in coat pattern and behavior in genetically identical cats, and slight differences in fingerprints in genetically identical twins (1, 2). This variation is caused by the randomness or stochasticity in gene expression that is inherent to any given cell. This stochasticity is brought about by fluctuations in the transcriptional and translational processes during gene expression. Transcription is controlled by the various regulatory proteins that bind upstream to a promoter region or influence the binding of other regulatory molecules at the promoter to initiate transcription. The rate of transcription, and hence translation, depend greatly on the concentration of these regulatory proteins and their ability to form the appropriate transcriptionally competent complexes (3-5). Also in cells, DNAs, mRNAs, and proteins are usually found in very low numbers and any fluctuation in reaction rates can have a pronounced effect on the expression of a gene causing it to be switched on or completely switched off. This is probably why protein copy number varies from cell to cell within isogenic populations (6).

It is important to study the stochasticity of gene expression because fluctuations in protein concentrations can interfere with intra cellular signaling properties, which can in turn affect cellular regulation (7). Examples of how stochasticity plays a role in cell fate is seen in the entry into the state of competence for a population of cells in *Bacillus Subtilis* (B. Subtilis), the generation of alternative color vision in the retina of *Drosophila Melanogaster* and entry into persister state for certain populations of *E.Coli*, all of which are completely random processes (8, 9).

Noise in synthetic networks

A method to study stochasticity is to measure noise levels during gene expression. Random fluctuations during gene expressions generate signals or sounds termed noise. Noise is a measure of the levels of variation in gene expression of an isogenic population of cells, regardless of the source. Noise (n_{tot}) generated in cells can be broken down into two components known as intrinsic noise (n_{int}) and extrinsic noise (n_{ext}) (10). Intrinsic noise arises from the fluctuations in reaction events of transcription and translation of a gene, which for a given population of identical cells, will vary from cell to cell. In this case, the stochasticity occurs locally to the gene sequence and the properties of the protein it encodes. Extrinsic noise on the other hand arises from other molecules present in the cells such as RNA polymerases and ribosomes etc, which will also vary from time to time and from cell to cell because they are gene products as well, but will affect all copies of the gene equally in a given cell. This variation will additionally contribute to the expression of a specific gene of interest. (11, 12).

The use of genetically engineered synthetic networks and fluorescence based methods to monitor gene expression and mathematical modeling to calculate corresponding noise levels produced as a result in single cells has greatly advanced this area of study. For example the “repressilator” (13) a synthetically designed construct of three transcriptional repressors was used to show a significantly noticeable fluctuation in the oscillations of gene expression, demonstrating the stochastic effects of gene expression. In fact, work has been performed to show that autoregulatory negative feedback loops made of simple gene circuits can reduce the stochasticity in gene expression (14) and it was further shown that negative translational feedback has a greater efficacy at lowering stochasticity than negative transcriptional activity (15). This could be the in built mechanism of the cell to counteract the hindrance of stochasticity to faithful processing of cellular information.

Cellular responses to mechanical stress

We have studied at how stochasticity in gene expression responds to mechanical perturbations in the form of osmotic stress. Previous work in the field have included how fluctuations in gene expression vary with induced mutations at the ribosome binding sites (16) and with varying concentrations of inducers interacting with promoters of genes of interest (16, 17). No one has of yet looked at how these stochastic processes behave in presence of mechanical stress applied to the cell. We decided to use the bacterium *E.Coli* as our model system because it was a much simpler system compared to a eukaryotic model, and provides a basis for comparing our studies employing mechanical stress as perturbation with existing studies employing chemical perturbations. Prokaryotes such as *E.Coli* and *B. Subtilis*, have mechanosensitive channels like the MscL (Mechano sensitive channel large conductance) protein that are gated through changes in bilayer tension and respond to mechanical stress by transducing bilayer deformations into a biochemical process such as protein motion and in the process changing conductance by opening a large aqueous pore (18-20). Another case where mechanical stress affects cellular processes is seen in the increased adhesion of *E.Coli* to mannose coated surfaces in response to shear stress. Elevated levels of shear stress cause conformational changes in the fimbrial FimH protein causing *E.Coli* to adhere more tightly to mannose coated surfaces (21). Also when various types of bacteria were grown in stimulated microgravity environment, inhibition of secondary metabolism of certain processes was discovered (22). So clearly, mechanical stress influences gene regulation and expression at the cellular level.

Mechanical perturbation in the form of osmotic stress.

Early transcriptional alterations to genes in the *E.Coli* genome in response to osmotic stress were profiled using DNA microarrays and it was shown that the osmotic upshift caused a series of genes to increase expression and at the same time causing some genes to be down-regulated (23). This prompted us to look at how noise levels of gene expression varied when

subjected to osmotic stress. Sucrose and sodium chloride were used as a form of non-ionic and ionic osmotic stress inducers. Time-lapse experiments in *E. Coli* show that intrinsic noise occurs within the first 10 min. but extrinsic noise linger for about 40 min. (24). This directed us to exert osmotic stress to bacterial cells expressing our construct for a period of 60 min. and imaging them at 10 min intervals. We use the reporter gene network utilizing the lactose uptake network in *E.Coli* (pJM31, courtesy of Pedraza et al). The pJM31 synthetic network consists of four genes where three of the genes are monitored by different fluorescent proteins (CFP, YFP, and RFP). The first gene expressed will inhibit the second, and that in turn will inhibit the third gene. The fourth gene is independent of the cascade and is used to monitor other cellular processes in the cell. The *E. Coli* JM101 laboratory strain was transformed with this plasmid because of its unique property of having a strong *Lac I* gene that is constitutively transcribed.

The *long-term goal* is to understand the effect of mechanical forces on gene networks, and how it can be used to understand and manipulate gene expression. The *objective* of this research is to understand how external perturbations influence noise levels in gene networks in bacterial model systems. The *central hypothesis* of the research is that mechanical perturbations can cause noise fluctuations in gene networks and in the process enhance or repress gene expressions. The *rationale* that underlies this hypothesis is that bacterial cells respond to mechanical forces via mechanosensitive channels and also to external stimulations like chemical induction. Measuring noise in gene networks in response to external perturbations will provide a fundamental understanding of their stochasticity.

CHAPTER 2

MATERIALS AND METHODS

Materials and Methods

The plasmid pJM31 (17) contains all the components of the synthetic network to be studied except the lac repressor, Lac I. The JM101 strain of E.Coli (*supE thi-1 (lac-proAB) [F' traD36 proAB lacIqZ.M15]*) from *Stratagene*, was used to implement this network, because it has the entire lac operon deleted and has the lac I^q instead which produces the same lac I repressor, but has a stronger promoter. This network of genes works in a cascade whereby the first gene *lacI* is constitutively transcribed, producing the lactose repressor which down regulates the expression of the second gene *tetR* which in turn down regulates the expression of the third gene *yfp*. The fourth gene *rfp*, which is under the control of the lambda repressor promoter, is constitutively transcribed and is independent of the cascade of the other three genes.

Strains, Growth Conditions, and Media

JM31 *E. coli* cells were grown overnight in M9 minimal media supplemented with 1mM glucose and 50µg/ml of ampicillin. Cells were grown to an OD₆₀₀ ≈ 0.150 – 0.2 prior to stressing and imaging. Various concentrations of NaCl and Sucrose were added to cell solutions to induce osmotic stress. Cells were immobilized with poly-L-lysine (1%) on microscope slides and observed at room temperature. Image J is used to measure fluorescence intensity and the model proposed by Elowitz(10) has been used to analyze and interpret the data.

Data Acquisition and Analysis:

JM31 cells exposed to varying degrees of osmotic stress were imaged at 10 min. intervals for a period of 60 min. The Zeiss Axioimager D1 with an automated turret and an AxioCam MRm CCD camera was used to observe and image cells. Approximately 500 cells were imaged per sample.

Zeiss Automeasure Plus software was employed to measure fluorescence intensity of individual cells. The threshold intensity was set at 251 to only account for bacteria expressing all the three fluorescent proteins (RFP, CFP and YFP). Absorption, excitation, and emission spectra of cells exposed varying degrees of osmotic stress were obtained with the Shimadzu UV-1650 PC spectrophotometer and the RF-5301 PC fluorescence spectrometer respectively, to demonstrate that spectral characteristics of the cells at any given pressure do not vary with time.

Equations for Analysis:

Osmotic Stress is calculated using equation 1 where Π = osmotic stress in atmospheres, i = number of particles (2 for NaCl and 1 for sucrose), M = molality of NaCl or sucrose, R = gas constant ($0.083 \text{ L atm K}^{-1} \text{ mol}^{-1}$), and T = temperature in K (298 K in these studies).

$$\Pi = iMRT \quad (1)$$

The parameters to characterize stochasticity in the gene network due to osmotic stress are those defined by Elowitz (10). Self-Correlation η_{sc}^2 is given by equation 2.

$$\eta_{sc}^2 = \frac{\sigma}{\mu} \quad (2)$$

σ = standard deviation of the areas for a given protein for a population of bacteria

μ = average area for a given protein for all the bacteria in the frame

Intrinsic noise η_{int}^2 is given in equation 3 where g_i and g_j are the fluorescence intensities of i th and j th proteins ($i \neq j$; $i, j = 1, 2, \text{ or } 3$; 1 = CFP, 2 = YFP, and 3 = RFP), and $\langle g_i \rangle$ and $\langle g_j \rangle$ are the average intensities.

$$\eta_{\text{int}}^2 = \frac{\langle (g_i - g_j)^2 \rangle}{2\langle g_i \rangle \langle g_j \rangle} \quad (3)$$

Extrinsic Noise η_{ext}^2 is given by equation 4 where the intensities and their averages are as defined in equation 3.

$$\eta_{\text{ext}}^2 = \frac{\langle g_i g_j \rangle - \langle g_i \rangle \langle g_j \rangle}{\langle g_i \rangle \langle g_j \rangle} \quad (4)$$

Total Noise is defined by equation 5.

$$\eta_{\text{tot}}^2 = \frac{\langle g_i^2 + g_j^2 \rangle - 2\langle g_i \rangle \langle g_j \rangle}{2\langle g_i \rangle \langle g_j \rangle} \quad (5)$$

The fluorescence intensities of CFP, YFP, and RFP were corrected for the quantum yields and molar absorptivities by dividing the measured intensities by the product of these two constants also known as brightness. The brightness values for these proteins are given in Table 1 (25).

Table1. Spectral Properties of fluorescent proteins

Protein	Color	Excitation Maximum (nm)	Emission Maximum (nm)	Relative Quantum Yield	Extinction Coefficient ($M^{-1} cm^{-1}$)	Brightness
DsRed Express	red	554	586	0.44	33,800	14,870
ECFP	Blue	439	476	0.15	20,000	3,000
EYFP	Yellow	512	529	0.54	45,000	24,300

The osmotic pressure values for various concentrations of NaCl and sucrose calculated from equation 1 using the densities of NaCl and sucrose solutions are listed in Table 2.

Table 2. Densities and Osmotic pressures of NaCl and Sucrose solutions

NaCl/ Sucrose Concentration, M	NaCl density, g/mL	Sucrose density, g/mL	NaCl pressure, atmospheres	Sucrose pressure, atmospheres
0	0.99538	0.9995	0.68717	0.34217
0.1	0.99988	1.01223	5.34065	2.63773
0.18	1.00348	1.02242	9.46709	4.64583
0.26	1.00707	1.03261	13.30586	6.48842
0.33	1.01022	1.04152	16.80816	8.15153
0.83	1.03271	1.10519	40.11258	18.74099
1.15	1.04711	1.14594	54.36585	24.83848
1.43	1.0597	1.1816	66.45268	29.79867
1.7	1.07185	1.21598	78.209	34.46937
2	1.08534	1.25418	90.75497	39.26869
2.2	1.09434	1.27965	98.94584	42.30851
2.5	1.10783	1.31785	110.98634	46.64947
3	1.13032	1.38152	130.40555	53.34704

CHAPTER 3

RESULTS AND DISCUSSION

Prokaryotic and eukaryotic cells have characteristic cell mechanical properties and respond to mechanical stress such as osmotic stress (26, 27). Mechanosensitive channels (MscLs) in bacteria such as *E.coli* have been shown to mediate mechano-transduction resulting in upregulation and down regulation of genes (7, 16, 23, 28-33). Gene networks are characterized by stochasticity, which has been investigated by modeling with a view to understand autoregulatory, negative, and positive feedback loops (7, 16, 33). These studies have also employed chemical perturbations to gain insights into the gene networks and their stochasticity. Perturbation of gene networks by mechanical stress and study of resulting stochasticity would illuminate on the networks and the interactions between genes. While the influence of chemical perturbations on gene networks has been widely studied, that of mechanical stress on the stochasticity is not well characterized. We have investigated the influence of osmotic stress on a synthetic gene network in *E.coli* bacterial cells in terms of intrinsic and extrinsic contributions to stochasticity of the genes in the network. Our long-term goal is to understand mechanotransduction in prokaryotic and eukaryotic systems through gene stochasticity in response to mechanical stress.

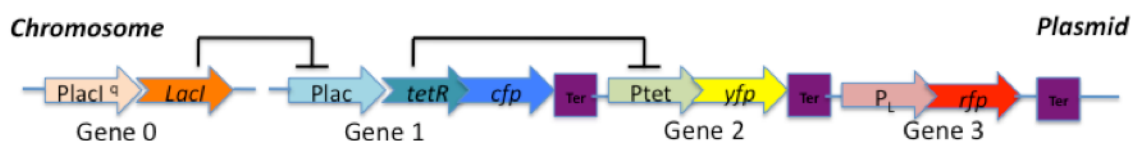


FIGURE 1. Synthetic gene network of 4 genes in *E.coli*.

We chose the synthetic gene network employed by Pedraza, Figure 1 to characterize the influence of osmotic stress on its stochasticity (17). The stochasticity of this network had been characterized employing chemical inducers (17), and thus provided an ideal system to compare our studies.

In this network pJM31 gene 0, *lacI* codes for lactose repressor, is constitutively transcribed and downregulates transcription of gene 1, *tetR*, which is bicistronically transcribed with *cfp*. The tetracycline repressor, which is the gene product of *tetR* downregulates gene 2 reported by *yfp*. Gene 3, reported by *rfp* is outside the cascade of gene 0, gene 1, and gene 2 and is under the control of the strong constitutive lambda repressor promoter P_L . The pJM31 plasmid (Fig. S1, Appendix) kindly provided by van Oudenaarden was transformed and expressed in *E.coli* strain JM101. Experimental details are provided under Materials and Methods Chapter 2, and extensive compilation of results is included in the Appendix. The bacteria were grown in M9 medium to an OD_{600} of 0.15 - 0.2 and subjected to various levels of osmotic stress employing NaCl and sucrose (0 – 3 M; NaCl osmotic stress range = 0.7 – 130 atm., sucrose osmotic stress range = 0.3 – 53 atm.; osmotic stress $\Pi = iMRT$, i = number of particles, 2 for NaCl and 1 for sucrose, M = molarity, R = gas constant, T = temperature, 298 K). The fluorescence intensities of CFP, YFP, and RFP in a population of bacteria (200 – 400) were measured with Zeiss Axioimager D1 by immobilizing the bacteria on 1 M poly-L-lysine on glass slides every 10 min. up to a maximum of 60 min. The intensity of each protein in a bacterium was determined with the Automeasure plus program from their fluorescence images (Fig. S2 contains representative data). The measured intensities were corrected for the differences in the quantum yield and molar absorptivities by dividing the intensities by the product of these two parameters, also defined as brightness (25). Representative data of fluorescence intensity distribution at 10 min. after addition of NaCl and sucrose are included in Supporting Information (Fig. S3 and S4).

The absorbance (Fig. S5) and fluorescence (Fig. S6) spectra of a population of *E.coli* expressing CFP, YFP, and RFP were recorded for the 60 min. at the same time as microscopy measurements. The absorbance and fluorescence spectra did not change with time for a given concentration of NaCl or sucrose. The absorbance spectra at high concentrations of NaCl and sucrose at or above 2 M exhibited an immediate decrease upon the addition of these reagents

with the decrease being greater for sucrose than for NaCl but remained constant over 60 min. The viscosity of sucrose changes by a factor of 1000 from 0 – 3M while that of NaCl changes by a factor of 1.5. The initial absorbance decrease at high concentrations of NaCl and sucrose could be due to osmotic stress and viscosity. The spectra at high concentrations also indicate that the response of the genes to osmotic stress is very fast. This is borne out by the plots of average CFP, YFP, and RFP fluorescence intensities of bacterial populations as a function of time at various osmotic pressures (Fig. S7 for NaCl; Fig. S8 for sucrose) which indicate that variation with time for a given osmotic stress is not very large.

The intensities of CFP, YFP, and RFP exhibit a bimodal behavior that is different for NaCl and sucrose. Fluorescence intensity (Fig. S7) in NaCl medium decreases up to a pressure of 60 atm. and increases up to the maximum pressure of 130 atm. except for YFP which shows a decrease through the entire range. The fluorescence intensity changes occur roughly over half the range of osmotic pressures in sucrose compared to NaCl decreasing from 0 – 30 atm. and increasing from 30 – 60 atm. for all three proteins (Fig. S8). The correlation of intensities of each protein with respect to the other is useful towards understanding the interactions of the genes and the intrinsic and extrinsic contributions to these interactions (Fig. S9 and S10). The stochasticity in the expressions of the genes in response to osmotic stress was characterized by the self-correlation (η_{sc}^2), intrinsic (η_{int}^2), and extrinsic (η_{ext}^2) parameters as defined by Elowitz (10, 24). The intrinsic and extrinsic parameters indicate the extent of intrinsic and extrinsic factors to stochasticity. The self-correlation parameter indicates the variation of fluorescence intensities and hence gene expression due to osmotic stress. The η_{sc}^2 values as seen for osmotic stress dependence with NaCl (Fig. S11) and sucrose (Fig. S12) exhibit bimodal behavior that could be either repression followed by activation or vice versa.

It is evident that the intrinsic contribution (Fig. S13 and S14) is much higher than the extrinsic contribution (Fig. S15 and S16) to stochasticity. Almost all of the contribution to

stochasticity is from intrinsic factors. It is also evident that the intrinsic contribution in NaCl is much higher than in sucrose. The intrinsic parameter η_{int}^2 invariably exhibits a bimodal behavior which occurs in the osmotic pressure range of 0 – 130 atm. in the case of NaCl and 0 – 60 atm. in the case of sucrose. The bimodal behavior of the fluorescence intensities and η_{sc}^2 , η_{int}^2 , and η_{ext}^2 can be rationalized by a combination of repression and activation of the interactions of the genes in response to osmotic stress. This can be mathematically expressed as a Hill type behavior, equation 6.

$$y = \frac{a_1 k_1^{n_1}}{k_1^{n_1} + x^{n_1}} + \frac{a_2 x^{n_2}}{k_2^{n_2} + x^{n_2}} \quad (6)$$

The first part of the equation accounts for repression and the second part activation by osmotic stress.

TABLE 3. Repression and activation parameters for η_{int}^2 as a function of osmotic stress

Gene pair	NaCl						Sucrose					
	Repression			Activation			Repression			Activation		
	a_1	k_1	n_1	a_2	k_2	n_2	a_1	k_1	n_1	a_2	k_2	n_2
<i>cfp-yfp</i>	9.4	22.2	1	34.3	78.4	9.2	9.9	9.5	0.6	11	30	3.9
<i>yfp-rfp</i>	1.6	24	3.5	12.7	87.4	4.6	1.3	10	3.2	3.5	35	1.8
<i>rfp-cfp</i>	2.1	20	0.97	1.9	90	1.5	2.2	8	1.8	2.6	33.1	1.4

The variable y represents fluorescence intensity, η_{sc}^2 , η_{int}^2 , or η_{ext}^2 . The constants a_1 and a_2 are the initial and final values for the repression and activation osmotic stress regions, k_1 and k_2 are the mid points of osmotic stress for the repression and activation regions where $y = a_1/2$ and $a_2/2$ respectively. The Hill type coefficients n_1 and n_2 indicate the extent to which osmotic stress influences the repression and activation of the gene expression and interaction respectively. The various parameters obtained for η_{int}^2 calculated 10 min. after the addition of NaCl and sucrose for pairs of genes is given in Table 3. The parameters were obtained by varying all parameters in the equation to obtain the best fit for the data. Finer refinement of the fit was

performed by keeping k_1 and k_2 constant and obtaining values of the other parameters. The values in Table 3 with standard deviations and similar parameters for fluorescence intensities and η_{sc}^2 are given in the Appendix (Tables S1, S2, and S3 respectively). The η_{int}^2 and CFP

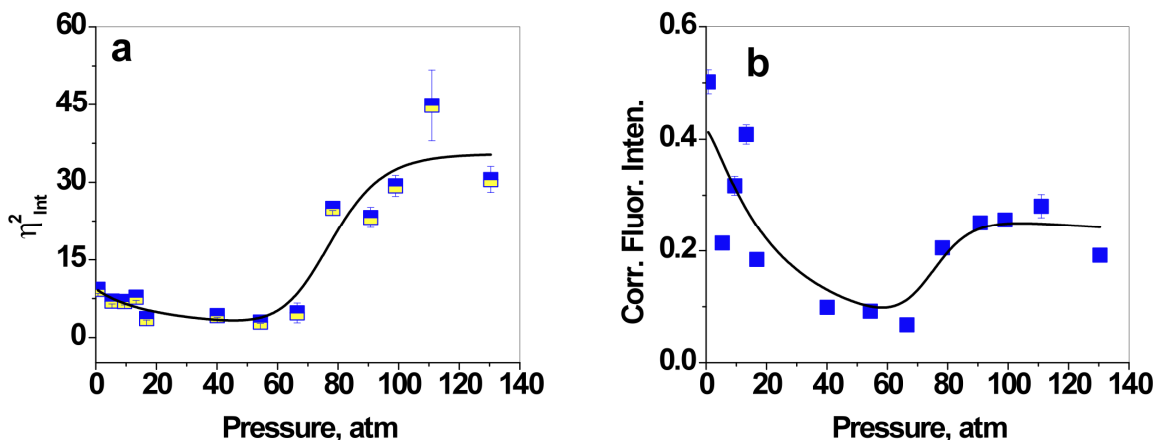


FIGURE 2. (a) Bimodal fit of η_{int}^2 for *cfp-yfp* vs. osmotic stress with NaCl. (b) Bimodal fit for CFP intensity vs. osmotic stress with NaCl.

intensity as a function of osmotic stress 10 min. after NaCl addition fitted to the bimodal equation 1 is displayed in Figures 2 (a) and (b). Table 1 indicates that repression and activation osmotic pressure mid points in sucrose medium are approximately half the values of osmotic pressure mid points in NaCl indicating that sucrose more efficiently represses and activates fluorescence intensities and gene interactions. The comparison of NaCl and sucrose induced osmotic stress on η_{int}^2 in the range of 0 – 60 atm. osmotic stress is shown in Figure S24 in the Appendix.

The n_1 values are relatively small for NaCl compared to n_2 values indicating a strong activation at high osmotic stress compared to weak repression at low osmotic stress. The n_1 values for sucrose osmotic stress are similar to NaCl but n_2 values are smaller than NaCl. Osmotic stress due to NaCl and sucrose have similar repression but NaCl has higher activation than sucrose despite the activation occurring at much higher osmotic pressures compared to sucrose. The gene 3 reported by RFP is outside the gene cascade in the network and yet it has

significant interactions with both CFP and YFP expressing genes 1 and 2 as indicated by the fluorescence intensity correlations and η_{int}^2 . This is in contrast to chemical induction in which these interactions are not significant (12). The genes 1 and 2 reported by CFP and YFP are strongly correlated under all conditions as expected. In this gene network osmotic stress induced contribution to stochasticity is mainly due to intrinsic factors.

A recent report in the literature indicates that the *LacI* repressor is downregulated at osmotic pressures greater than 16 atm. in NaCl as indicated by the increased activity of β -galactosidase in *E.coli* (16). Additional activation of this enzyme also occurred due to the upregulation of *Lac* operon in the chromosome. The *LacI* repressor in the chromosome in our study is most likely upregulated at low osmotic pressures resulting in reduced expression of CFP and downregulated at high osmotic pressures leading to increased expression of CFP in both NaCl and sucrose media. This is evident in Figure 2(b) from the variation of CFP intensity with increasing osmotic stress induced by NaCl. The repression and activation of gene 1 reported by CFP could be expected to have activation and repression influence respectively on gene 2 reported by YFP based on the gene network in Figure 1. In other words increased expression of CFP could be expected to result in reduced expression of YFP and vice versa. However, the expression of YFP is repressed in the entire range of osmotic pressures in NaCl and sucrose. Additionally the expression levels of RFP, which is not coupled to genes reported by CFP and YFP is also downregulated and upregulated in similar manner to CFP. These results indicate that osmotic stress impacts plasmid in addition to the chromosome in *E.coli*. The current studies have provided a new insight into mechanotransduction driven gene stochasticity. We are currently investigating this and other gene networks to gain a better understanding of the influence of mechanical stress on gene expression, interaction, and noise.

CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

Studies of the effect of osmotic stress on synthetic gene network in *E.coli* consisting of three cascading genes that downregulate the next gene in sequence and a fourth uncoupled gene lead to the following significant conclusions:

1. The intensities of the fluorescent reporter proteins CFP, YFP, and RFP exhibit a bimodal change with osmotic stress, decreasing at low osmotic stress and increasing at high osmotic stress.
2. The osmotic pressure range in which repression followed by activation of genes occurs is almost half for pressure induction with sucrose compared to NaCl. Sucrose is more effective in repressing and activating genes than NaCl. However, NaCl has a much larger impact on activation compared to sucrose while repression is essentially the same for both inducers.
3. The main contribution to the stochasticities of the genes is due to intrinsic factors. Extrinsic factor contribution is very minor.
4. The coupled genes reported by CFP and YFP interact in a bimodal manner with repression occurring at low osmotic stress and activation occurring at high osmotic stress.
5. The gene reported by RFP exhibits coupling with the genes reported by CFP and YFP. The order of coupling is *cfp-yfp>yfp-rfp>cfp-rfp*.
6. The bimodal behavior can be modeled as a sum of repression and activation with a Hill like function. The experimental data can be fitted well with such a function, which yields Hill type coefficient for the influence of osmotic stress on gene interaction.
7. Hill coefficient for repression is small for osmotic stress exerted by NaCl and sucrose. The Hill coefficient for activation with NaCl is higher than the activation by sucrose.

8. The *LacI* repressor in the chromosome is most likely upregulated at low osmotic pressures and downregulated at high osmotic pressures as indicated by CFP fluorescence intensity changes. The repression and activation of RFP and the repression of YFP at all osmotic stress values also indicate that osmotic stress impacts the plasmid containing the genes of the synthetic network. Essentially osmotic stress has an impact on the chromosome and plasmid.
9. Observed bimodal behavior is the result of osmotic stress on the chromosome and the synthetic gene network.
10. Effect of osmotic stress on the gene network is essentially all intrinsic in a similar manner to chemical induction

Future Directions

The current studies have provided a new insight into mechanotransduction driven gene stochasticity. Study of gene stochasticity stimulated by mechanical stress would provide a fundamental understanding of mechanotransduction in gene networks. The following studies building on the current studies will illuminate further on mechanotransduction in gene networks and the response of genes to mechanical stress:

1. The synthetic gene network subjected to compression force with an inert gas such as N₂ or Ar will complement osmotic stress studies. Compression force can be increased and decreased reversibility to determine if the effect of this force on gene expression and stochasticity is also reversible.
2. The network studied consisting of four genes of which three are in a cascade sequentially downregulating the next gene can be perturbed with chemical inducers such as IPTG and ATC. Perturbing the gene network with chemical inducers and mechanical stress simultaneously is fundamentally interesting and important.
3. A gene network where identical promoters (10) equidistant from the origin of replication express two different fluorescent reporter proteins will complement the current studies

with a more complex network. Such a system is useful for understanding bimodal behavior of activation and repression.

4. The effect of mechanical perturbation on comK regulatory network in *Bacillus Subtilis* (*B.Subtilis*) that regulates the transition of this bacterium into competence will be interesting to investigate. This form of genetic competence is highly regulated in *B.Subtilis* and the *comK* gene is the master regulator of this competence (34). This state of competence allows bacteria to use this transformability as a survival strategy in fluctuating and harsh environmental conditions to which they are subjected. This regulation has been found to be a stochastic process (35). This will characterize at how biochemical pathway in prokaryotes is influenced by mechanical stress.
5. Mechanical stress could be a significant factor in promoting cancer metastasis as cells from primary tumors when they migrate are subjected to a variety of mechanical forces. Such forces could be significantly contributing to stochasticity of mechanosensitive genes and as a result to metastasis at the gene, transcription, and translation stages.

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APPENDIX

Figure Captions

Figure S1 Gene network and pJM31 Plasmid construct.

Figure S2 Fluorescence Microscopy images of bacterial populations in a) 0.33 M NaCl (a1, a4, a7 – RFP at 0, 30 and 60 min., a2, a5, a8 – CFP at 0, 30 and 60 min., a3, a6, a9 –YFP at 0, 30, 60 min.), b) 0.83 M NaCl (b1, b4, b7 – RFP at 0, 30 and 60 min., b2, b5, b8 – CFP at 0, 30 and 60 min., b3, b6, b9 –YFP at 0, 30, 60 min), c) 0.33 M sucrose (c1, c4, c7 – RFP at 0, 30 and 60 min., c2, c5, c8 – CFP at 0, 30 and 60 min., c3, c6, c9 –YFP at 0, 30, 60 min) and d) 0.83 M sucrose (d1, d4, d7 – RFP at 0, 30 and 60 min., d2, d5, d8 – CFP at 0, 30 and 60 min., d3, d6, d9 –YFP at 0, 30, 60 min).

Figure S3 Fluorescence intensity distributions of a bacterial population in NaCl, a) RFP, 40 atm., b) CFP, 40 atm., c) YFP, 40 atm., d) RFP, 91 atm., e) CFP, 91 atm., f) YFP, 91 atm.

Figure S4 Fluorescence intensity distributions of a bacterial population in sucrose, a) RFP, 19 atm., b) CFP, 19 atm., c) YFP, 19 atm., d) RFP, 39 atm., e) CFP, 39 atm., f) YFP, 39 atm.

Figure S5 Absorption spectra of bulk JM31 in a) NaCl and b) sucrose.

Figure S6 CFP fluorescence excitation and emission spectra of bulk JM31 bacteria in a) 0.83 M NaCl, (40 atm.) and b) 3M, (53 atm.) sucrose.

Figure S7 Fluorescence intensity with increasing osmotic pressure in NaCl at 0 – 60 min., a) RFP, b) CFP and c) YFP. 0 min. - ■, 10 min - ●, 20 min - ▲, 30 min - ▼, 40 min. - ◆, 50 min. - ►, 60 min - ◆.

Figure S8 Fluorescence intensity with increasing osmotic pressure in sucrose at 0 – 60 min., a) RFP, b) CFP and c) YFP. 0 min. - ■, 10 min - ●, 20 min - ▲, 30 min - ▼, 40 min. - ◆, 50 min. - ►, 60 min - ◆.

Figure S9 Fluorescence intensity correlation in NaCl between a) CFP-YFP, 40 atm., b) CFP-RFP, 40 atm., c) RFP-YFP, 40 atm., d) CFP-YFP, 91 atm., e) CFP-RFP, 91 atm., f) RFP-YFP, 91 atm.

Figure S10 Fluorescence intensity correlation in sucrose between a) CFP-YFP, 19 atm., b) CFP-RFP, 19 atm., c) RFP-YFP, 19 atm., d) CFP-YFP, 39 atm., e) CFP-RFP, 39 atm., f) RFP-YFP, 39 atm.

Figure S11 Self-correlation (η_{sc}^2) with increasing osmotic pressure in NaCl at 0 – 60 min. of a) RFP, b) CFP and c) YFP. 0 min - ■, 10 min - ●, 20 min - ▲, 30 min - ▼, 40 min. - ◀, 50 min. - ▶, 60 min. - ◆.

Figure S12 Self-correlation (η_{sc}^2) with increasing osmotic pressure in sucrose at 0 – 60 min. of a) RFP, b) CFP and c) YFP. 0 min. - ■, 10 min - ●, 20 min - ▲, 30 min - ▼, 40 min. - ◀, 50 min. - ▶, 60 min - ◆.

Figure S13 Intrinsic noise (η_{int}^2) with increasing osmotic pressure in NaCl at 0 – 60 min. of a) CFP-RFP, b) CFP-YFP and c) RFP-YFP. 0 min - ■, 10 min - ●, 20 min - ▲, 30 min - ▼, 40 min. - ◀, 50 min. - ▶, 60 min. - ◆.

Figure S14 Intrinsic noise (η_{int}^2) with increasing osmotic pressure in sucrose at 0 – 60 min. of a) CFP-RFP, b) CFP-YFP and c) RFP-YFP. 0 min - ■, 10 min - ●, 20 min - ▲, 30 min - ▼, 40 min. - ◀, 50 min. - ▶, 60 min. - ◆.

Figure S15 Extrinsic noise (η_{ext}^2) with increasing osmotic pressure in NaCl at 0 – 60 min. of a) CFP-RFP, b) CFP-YFP and c) RFP-YFP. 0 min - ■, 10 min - ●, 20 min - ▲, 30 min - ▼, 40 min. - ◀, 50 min. - ▶, 60 min - ◆.

Figure S16 Extrinsic noise (η_{ext}^2) with increasing osmotic pressure in sucrose at 0 – 60 min. of a) CFP-RFP, b) CFP-YFP and c) RFP-YFP. 0 min - ■, 10 min - ●, 20 min - ▲, 30 min - ▼, 40 min. - ◀, 50 min. - ▶, 60 min - ◆.

Figure S17 Total noise (η_{tot}^2) with increasing osmotic pressure in NaCl at 0 – 60 min. of a) CFP-RFP, b) CFP-YFP and c) RFP-YFP. 0 min - ■, 10 min - ●, 20 min - ▲, 30 min - ▼, 40 min. - ◀, 50 min. - ▶, 60 min. - ◆.

Figure S18 Total noise (η_{tot}^2) with increasing osmotic pressure in sucrose at 0 – 60 min. of a) CFP-RFP, b) CFP-YFP and c) RFP-YFP. 0 min - ■, 10 min - ●, 20 min - ▲, 30 min - ▼, 40 min. - ◀, 50 min. - ▶, 60 min. - ◆.

Figure S19 Bimodal fits of fluorescence intensity with increasing osmotic pressure in NaCl of a) RFP ■, b) CFP ■ and c) YFP ■.

Figure S20 Bimodal fits of fluorescence intensity with increasing osmotic pressure in sucrose of a) RFP ●, b) CFP ● and c) YFP ●.

Figure S21 Bimodal fits of η_{int}^2 with increasing osmotic pressure in NaCl of a) CFP-YFP ■, b) RFP-YFP ■.

Figure S22 Bimodal fits of η_{int}^2 with increasing osmotic pressure in sucrose of a) CFP-YFP ●, b) RFP-YFP ●.

Figure S23 Bimodal fits of η_{sc}^2 with increasing osmotic pressure of a) CFP ■ in NaCl and b) RFP ● in Sucrose







Figure S24 Comparison of intrinsic noise (η_{int}^2) for osmotic stress induced by NaCl and sucrose over 0-54 atm. a) YFP-CFP- (NaCl)  (Sucrose) b) RFP-YFP  (NaCl),  (sucrose) c) CFP-RFP  (NaCl) and  (sucrose)

Figure S1

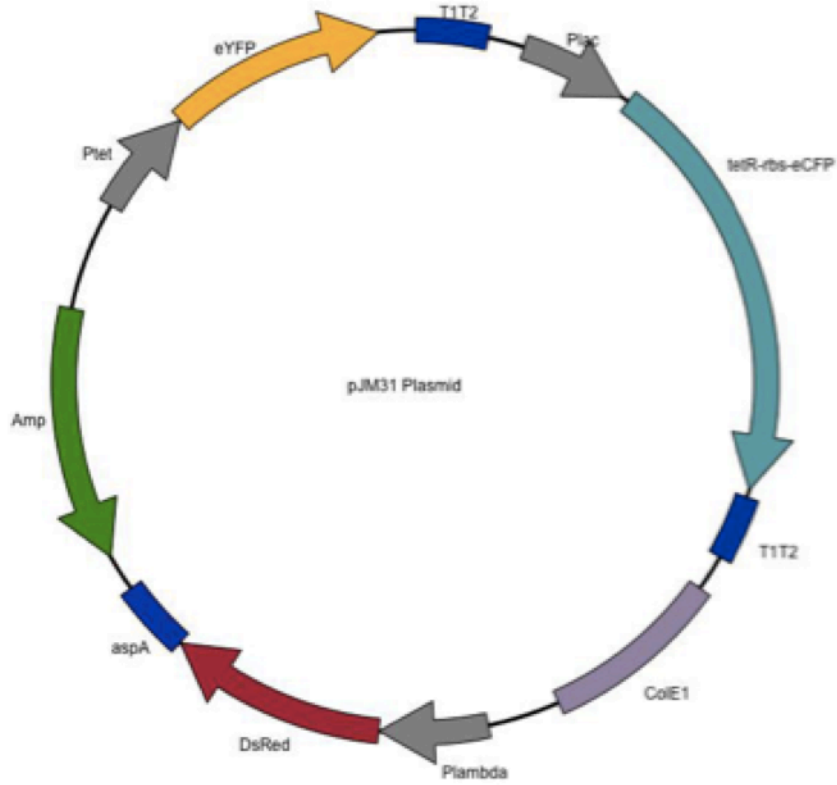
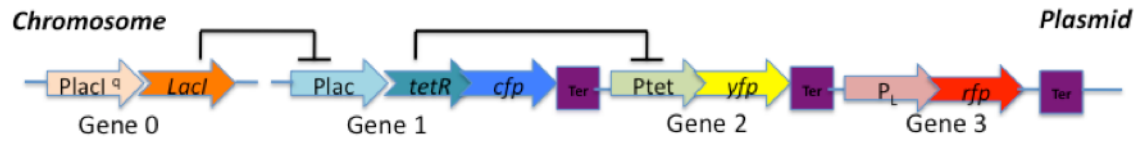


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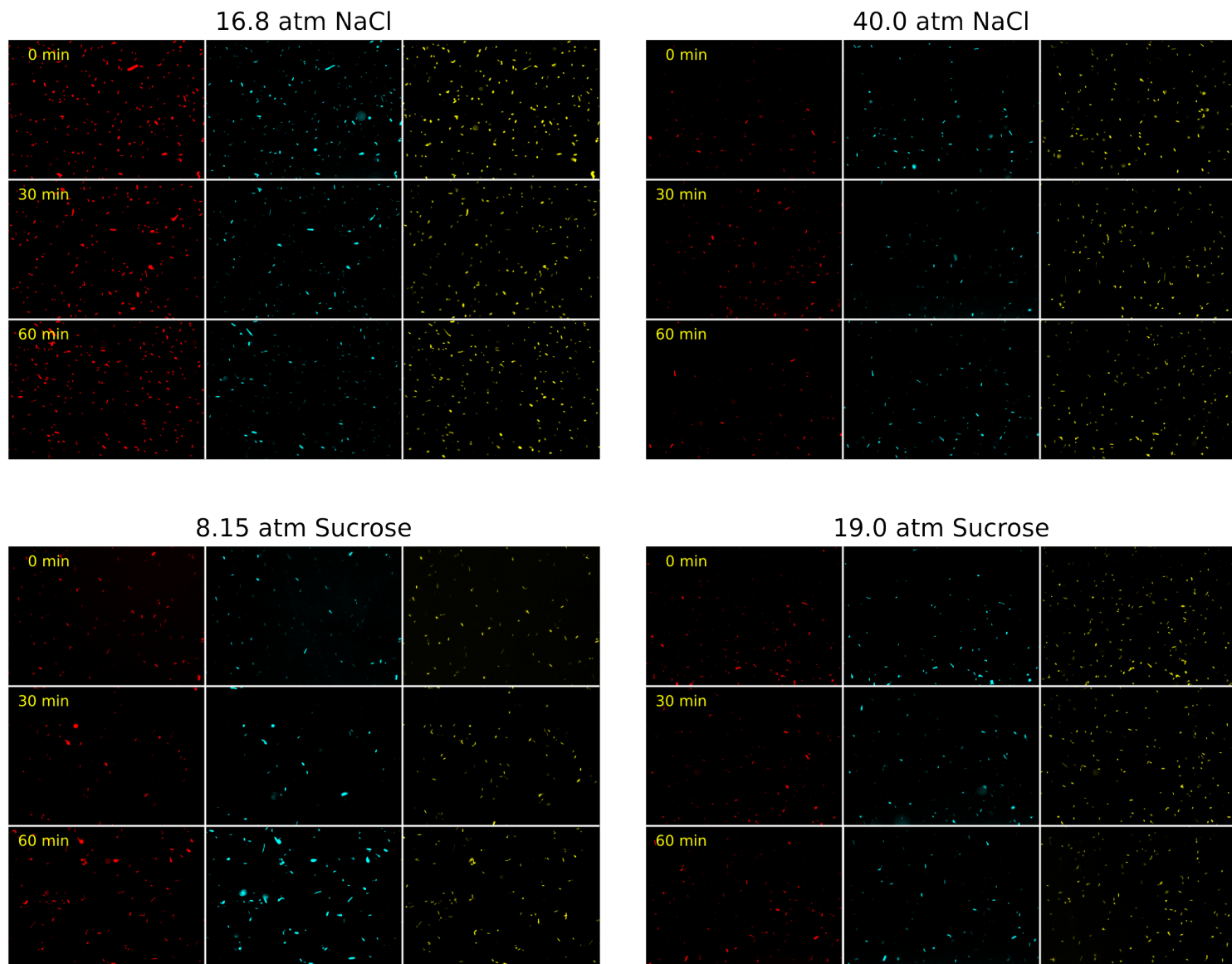


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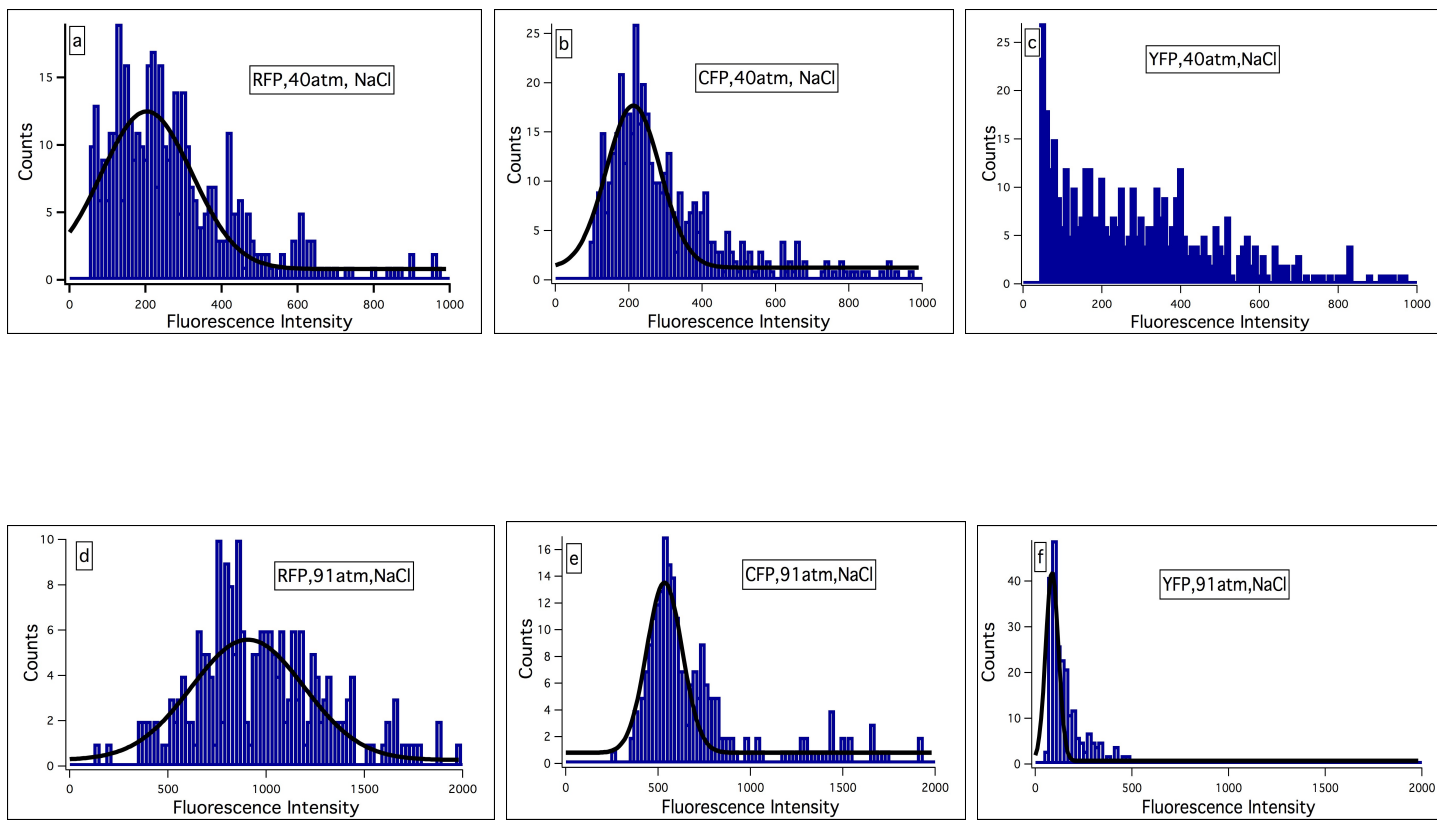


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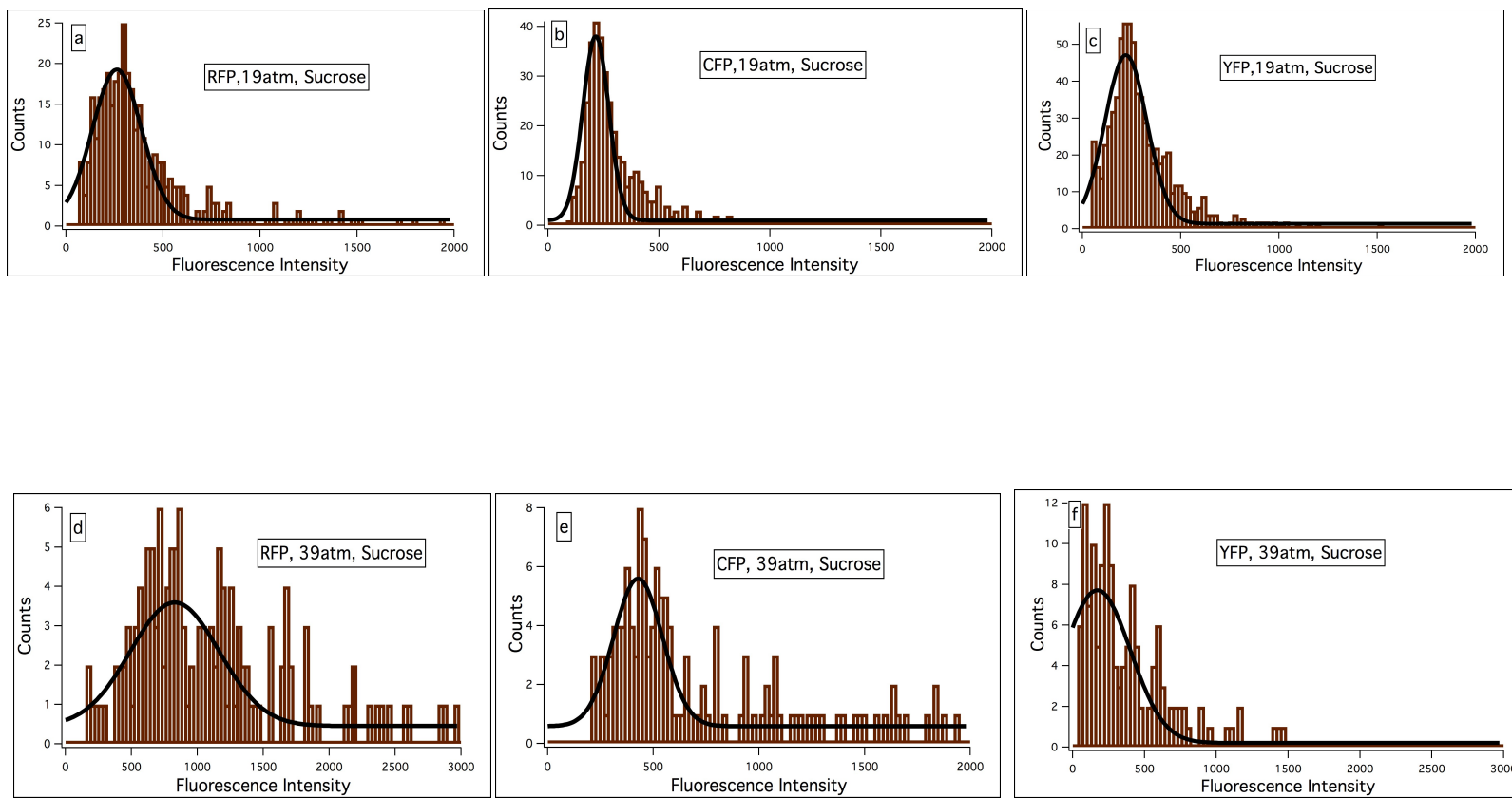


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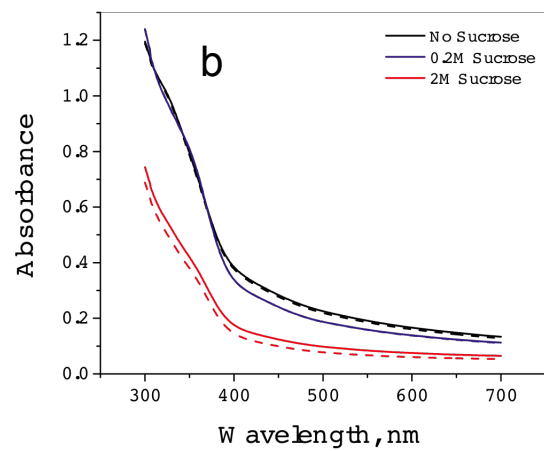
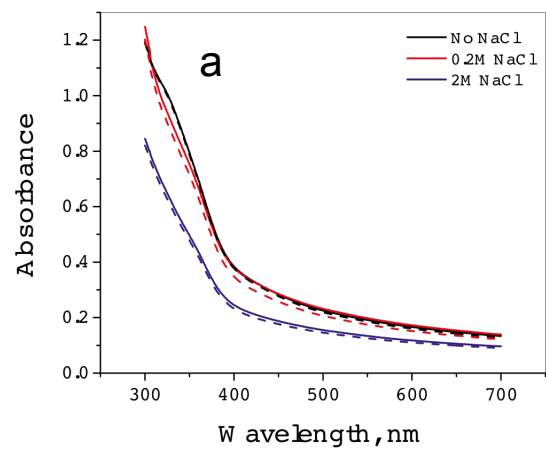


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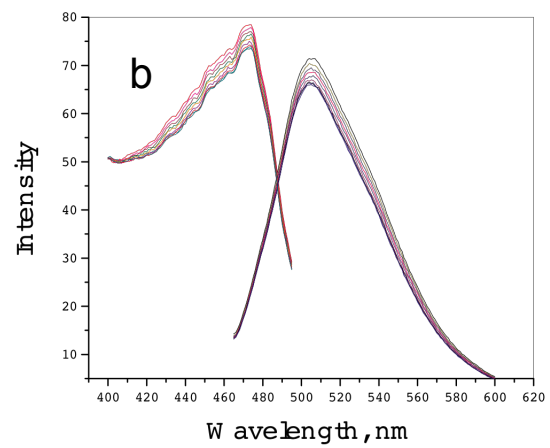
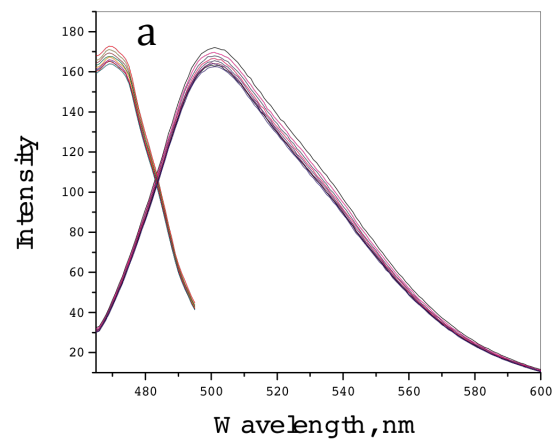


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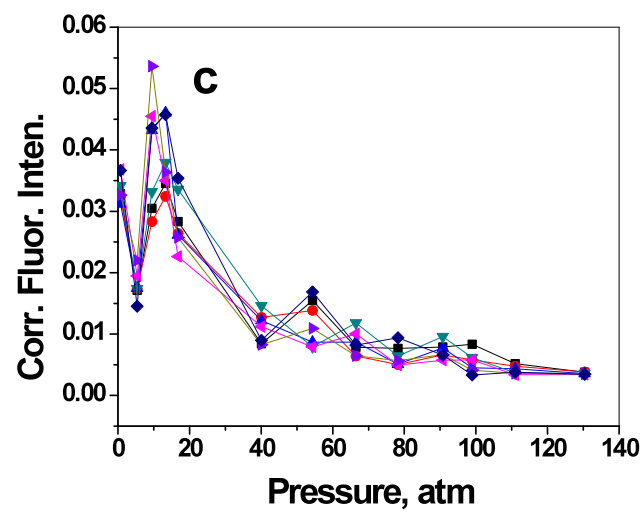
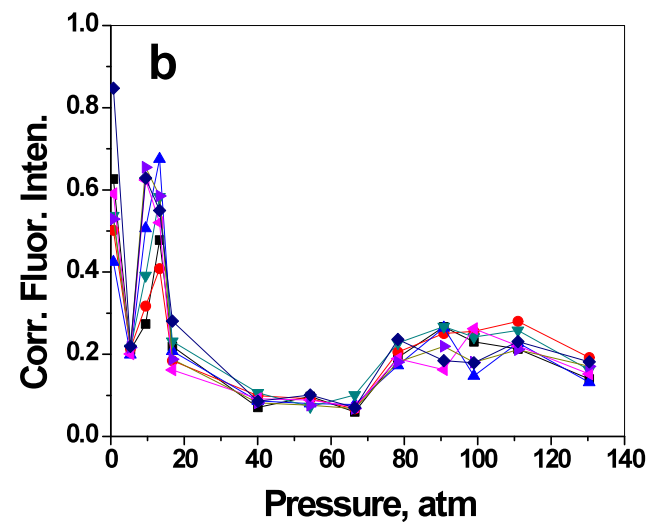
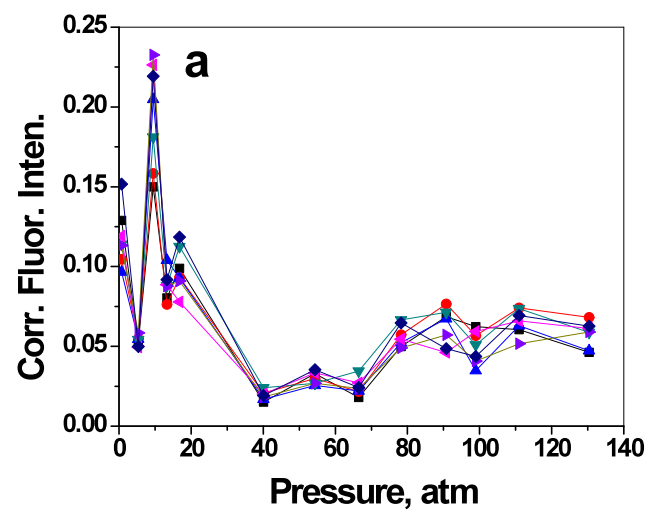


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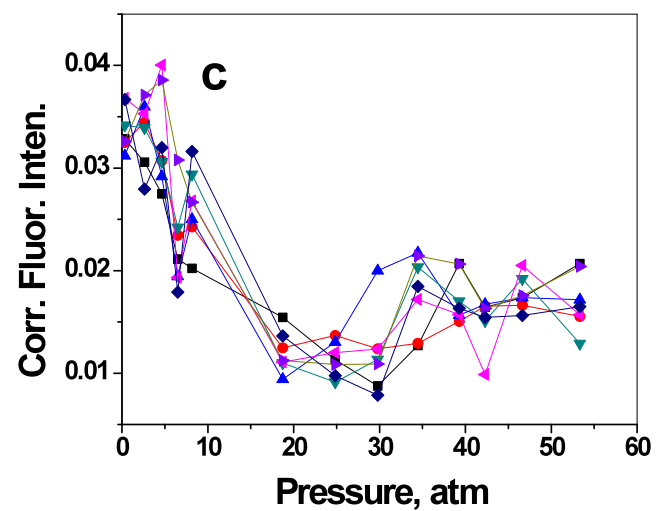
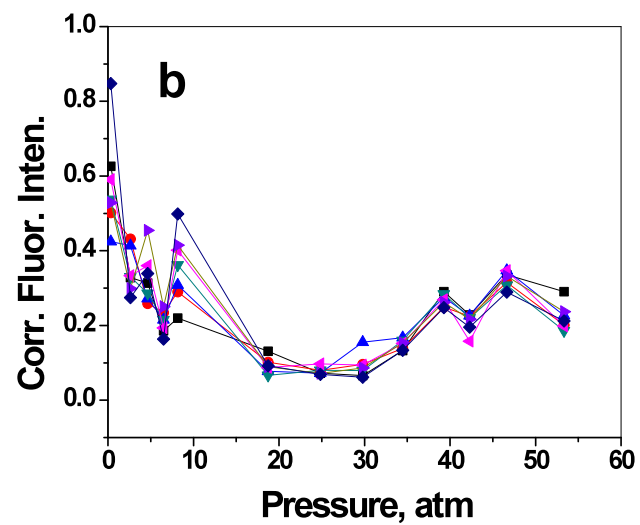
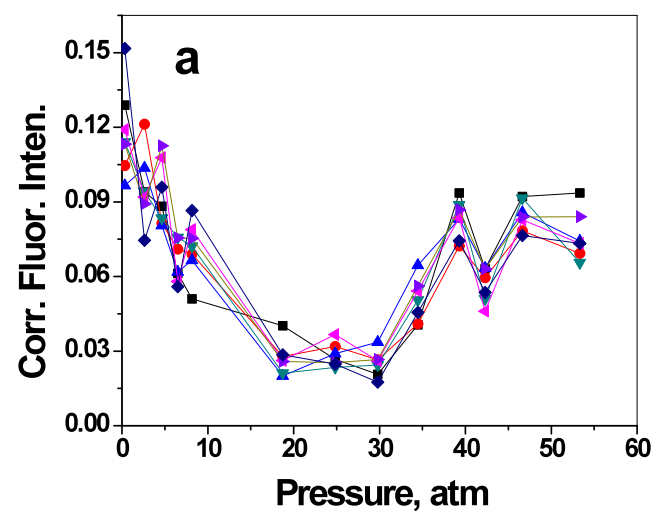


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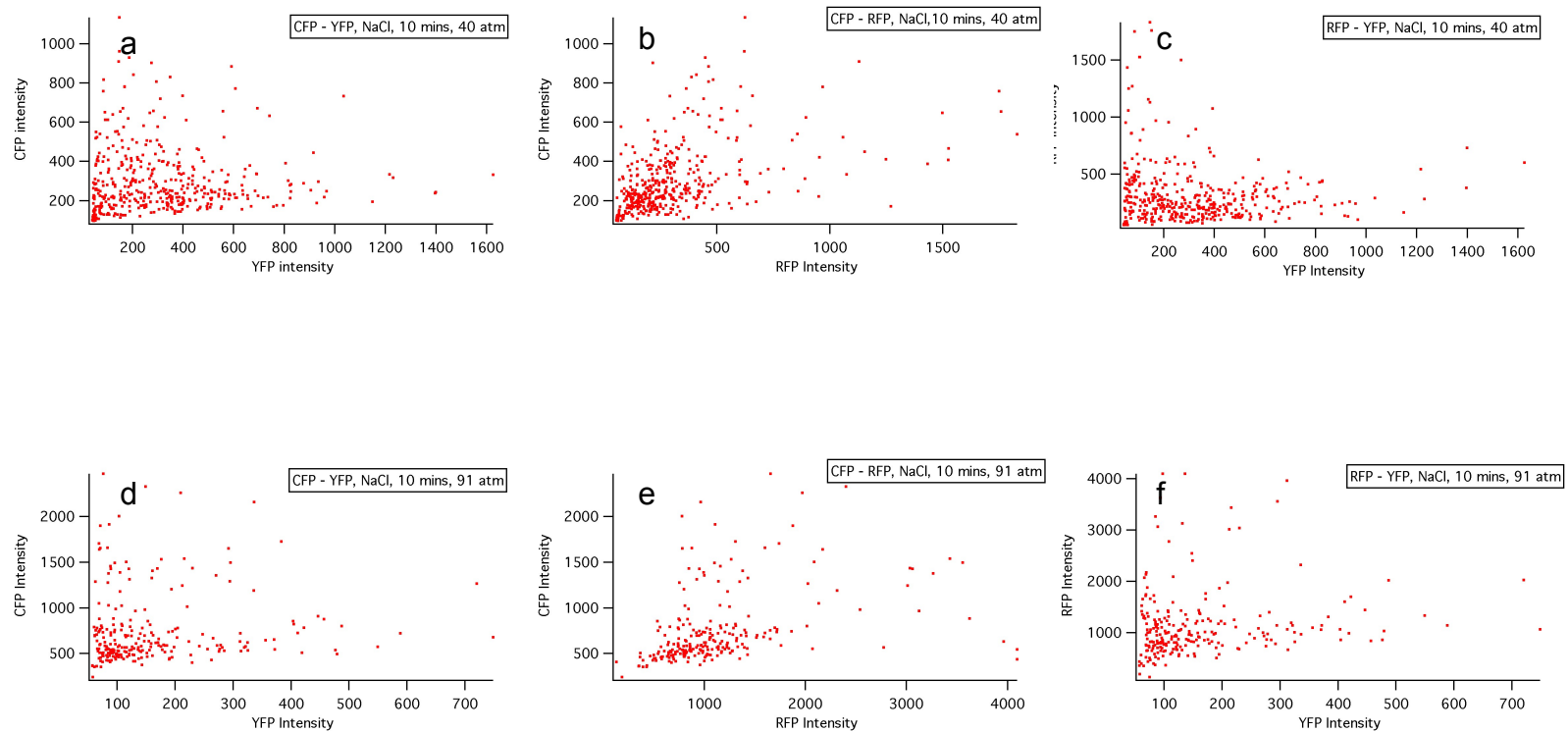


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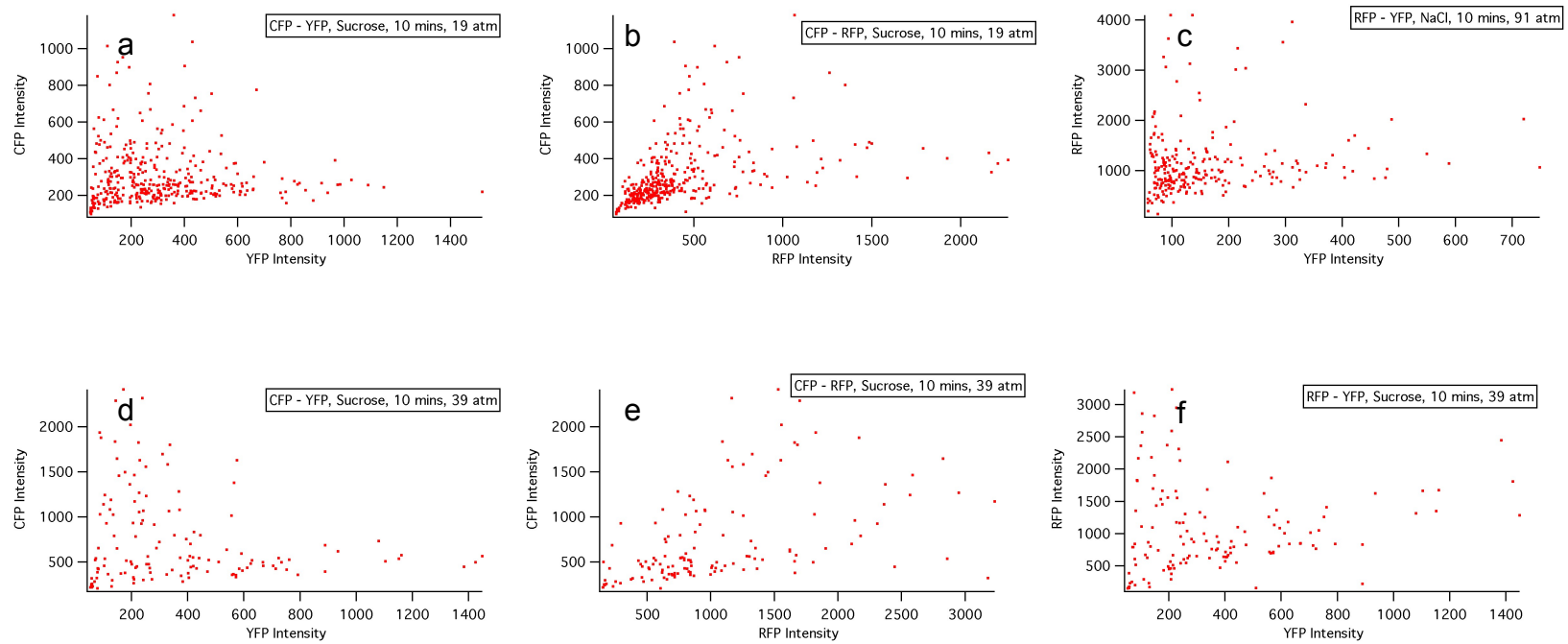


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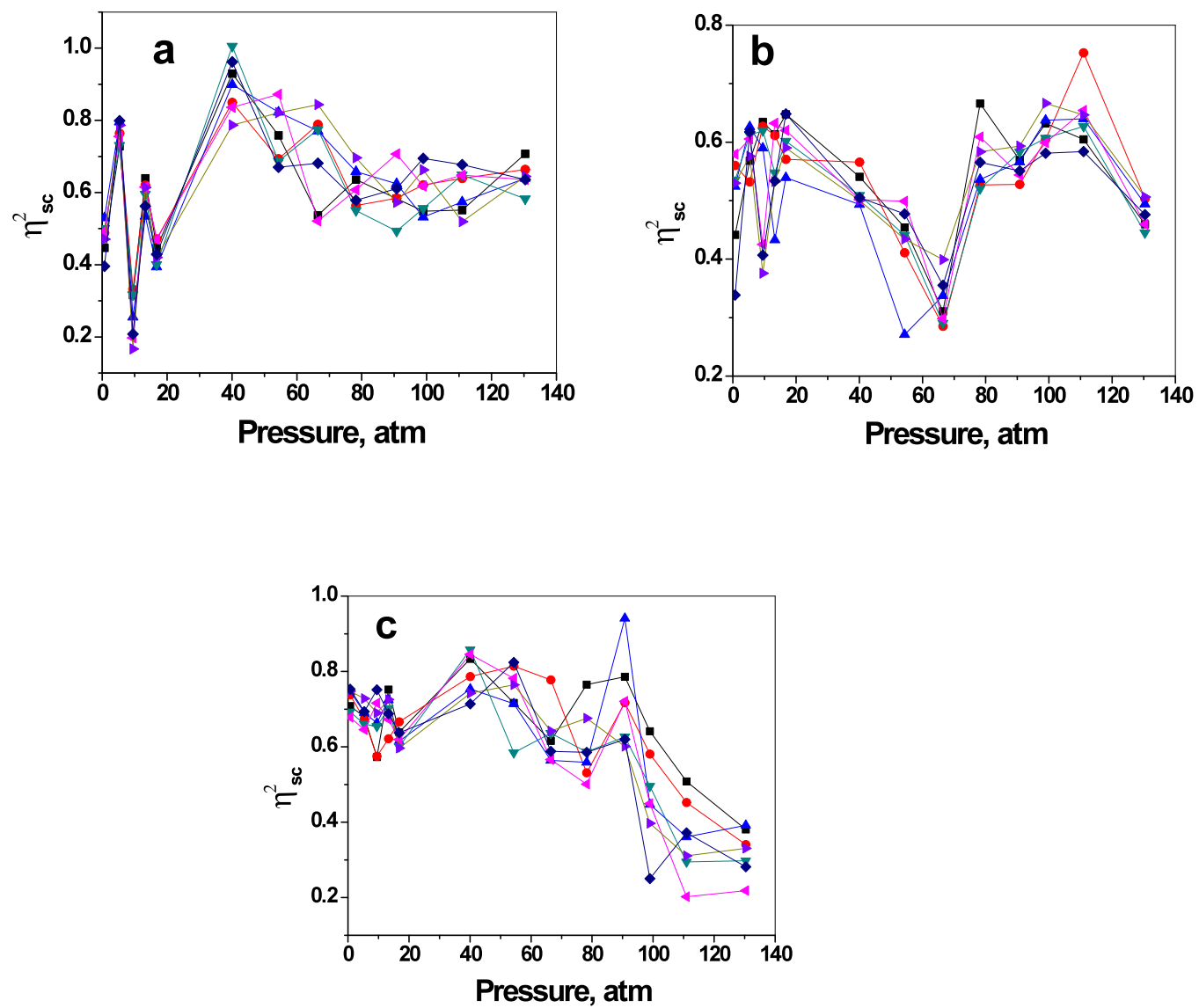


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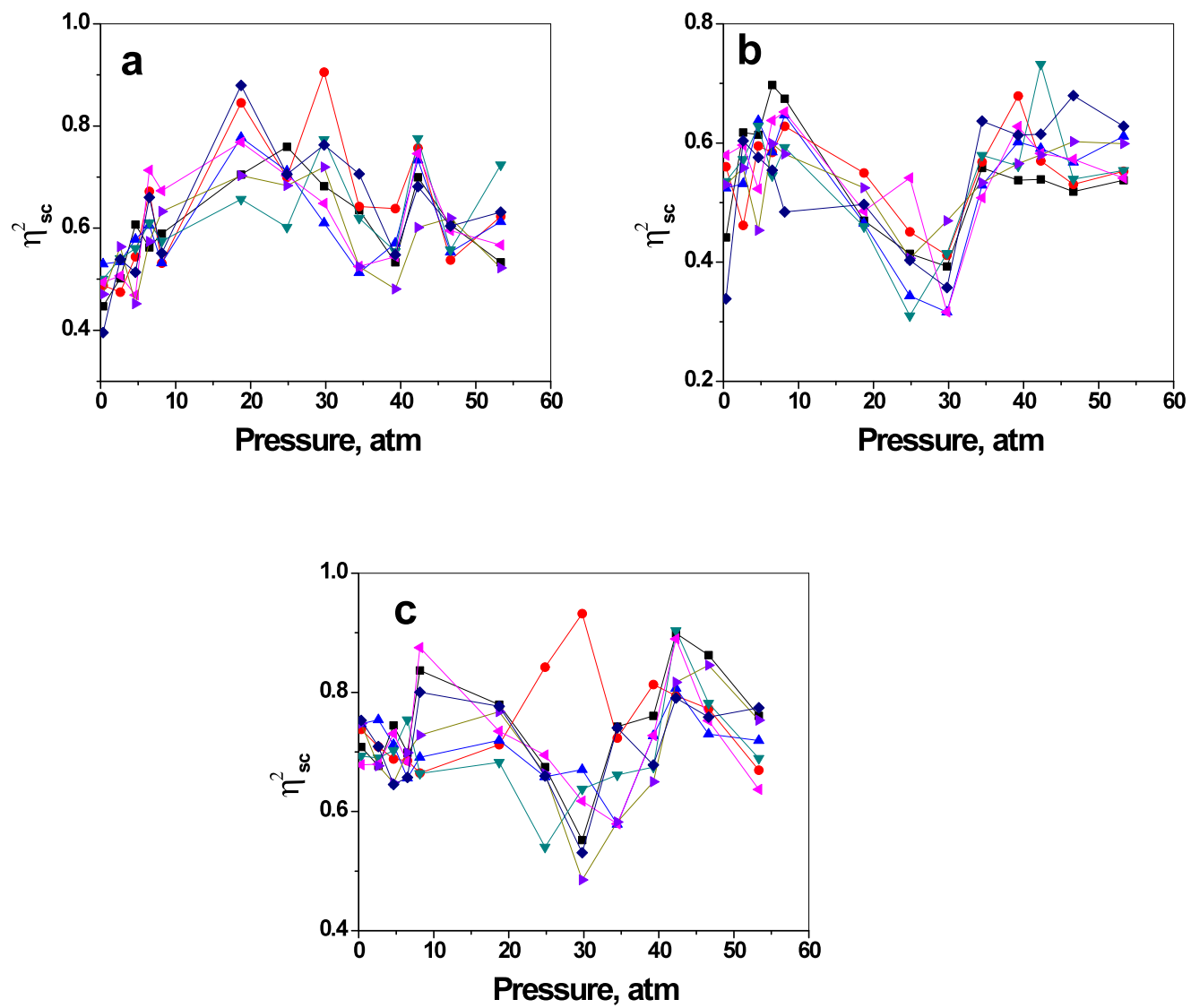


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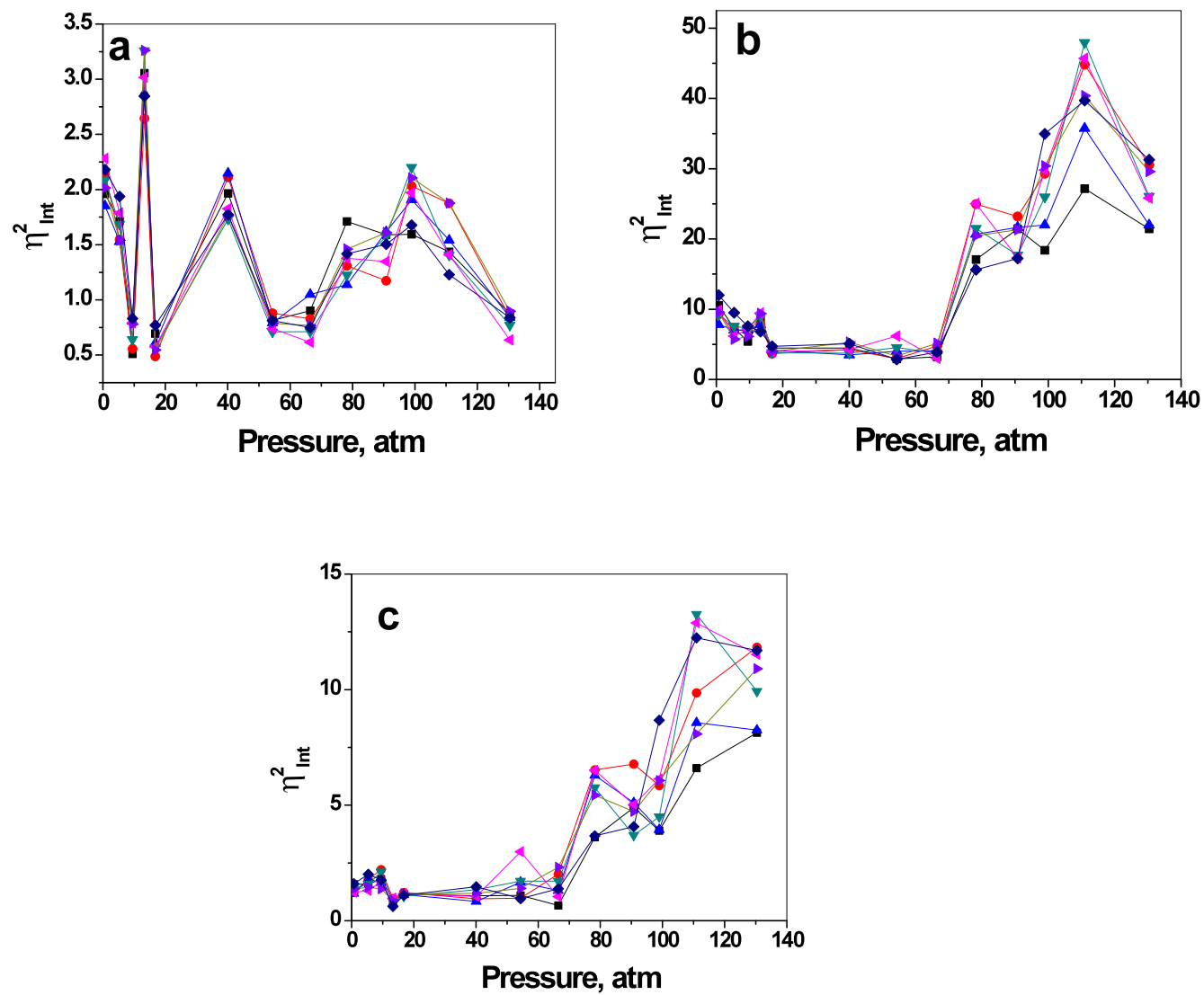


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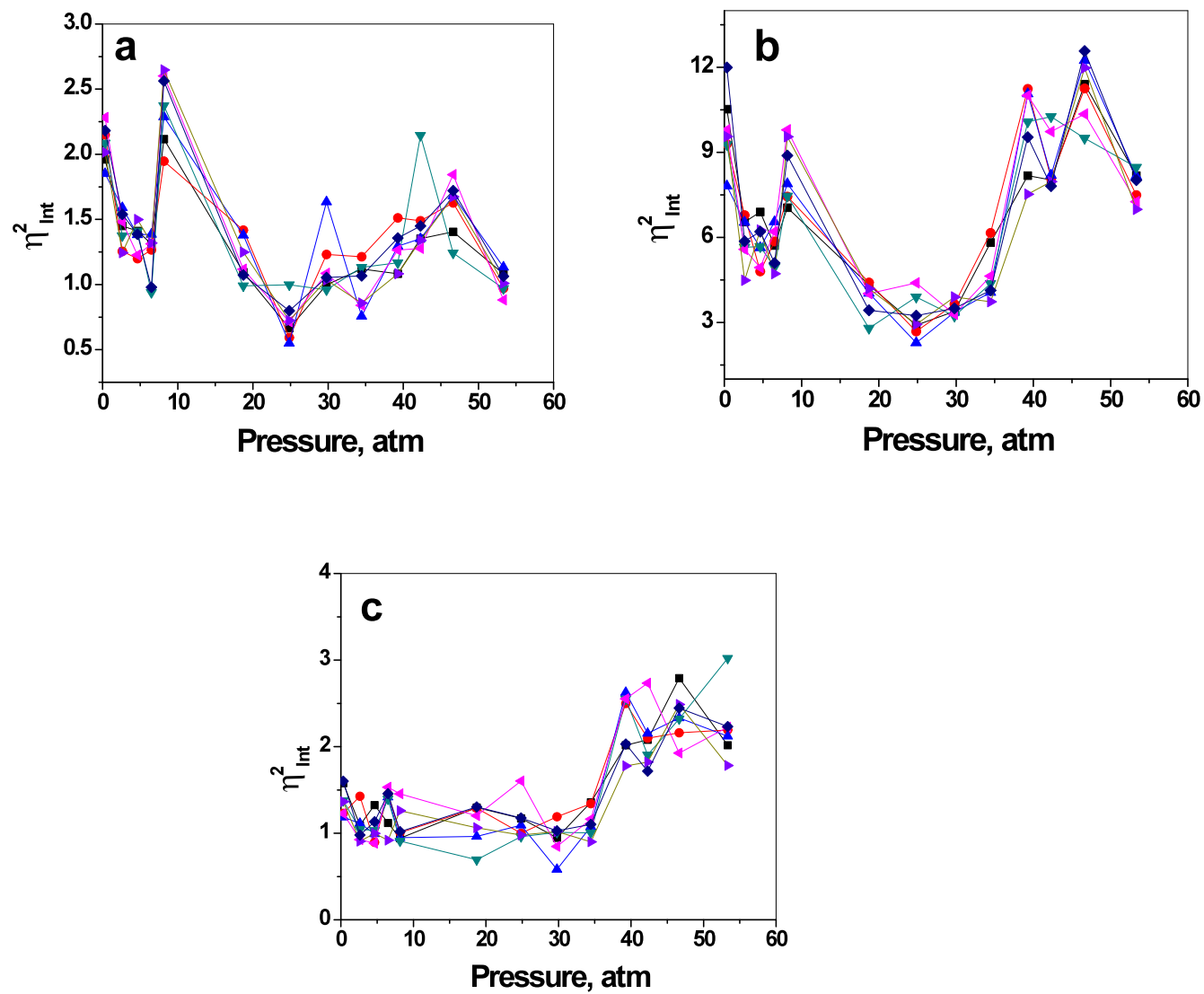


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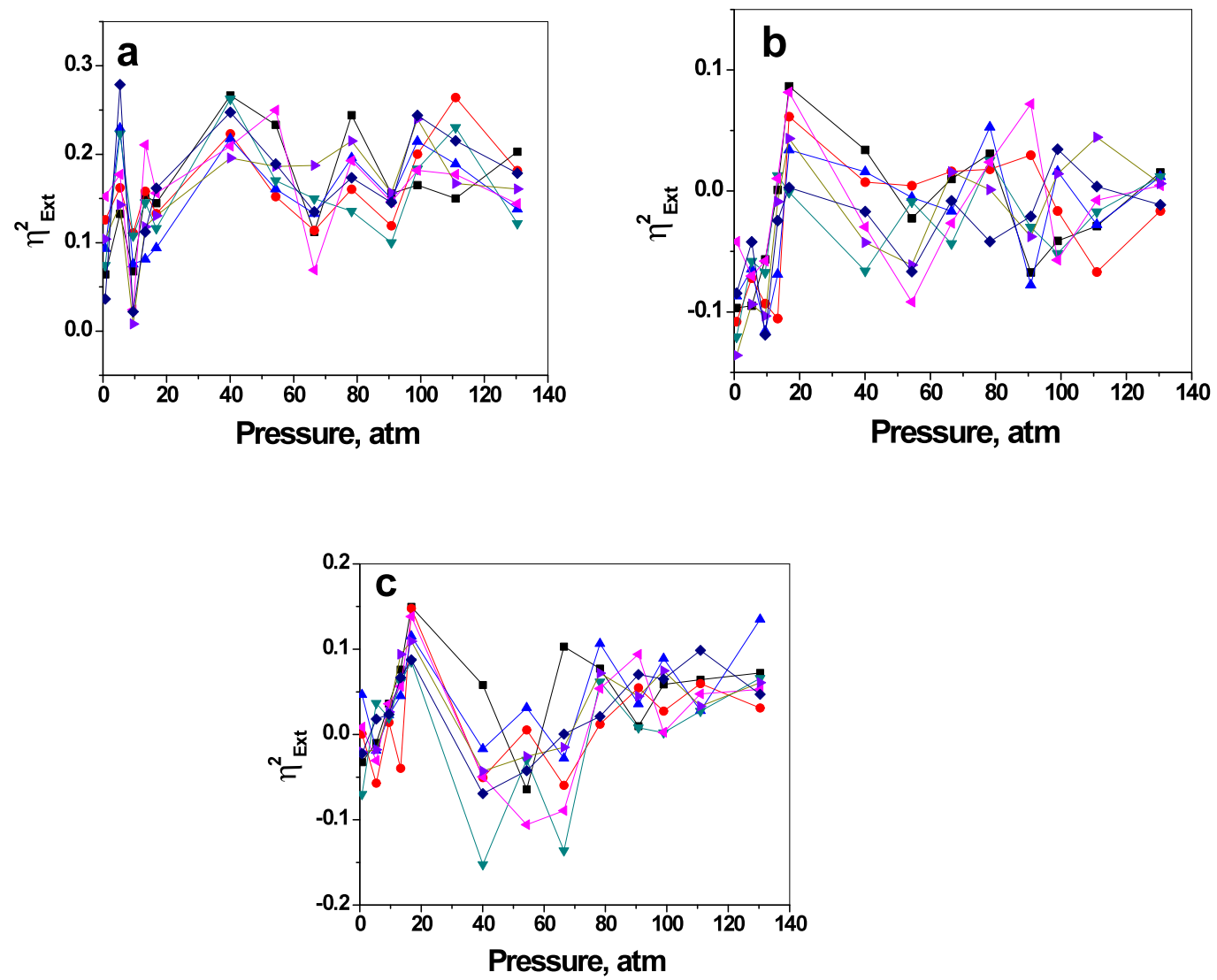


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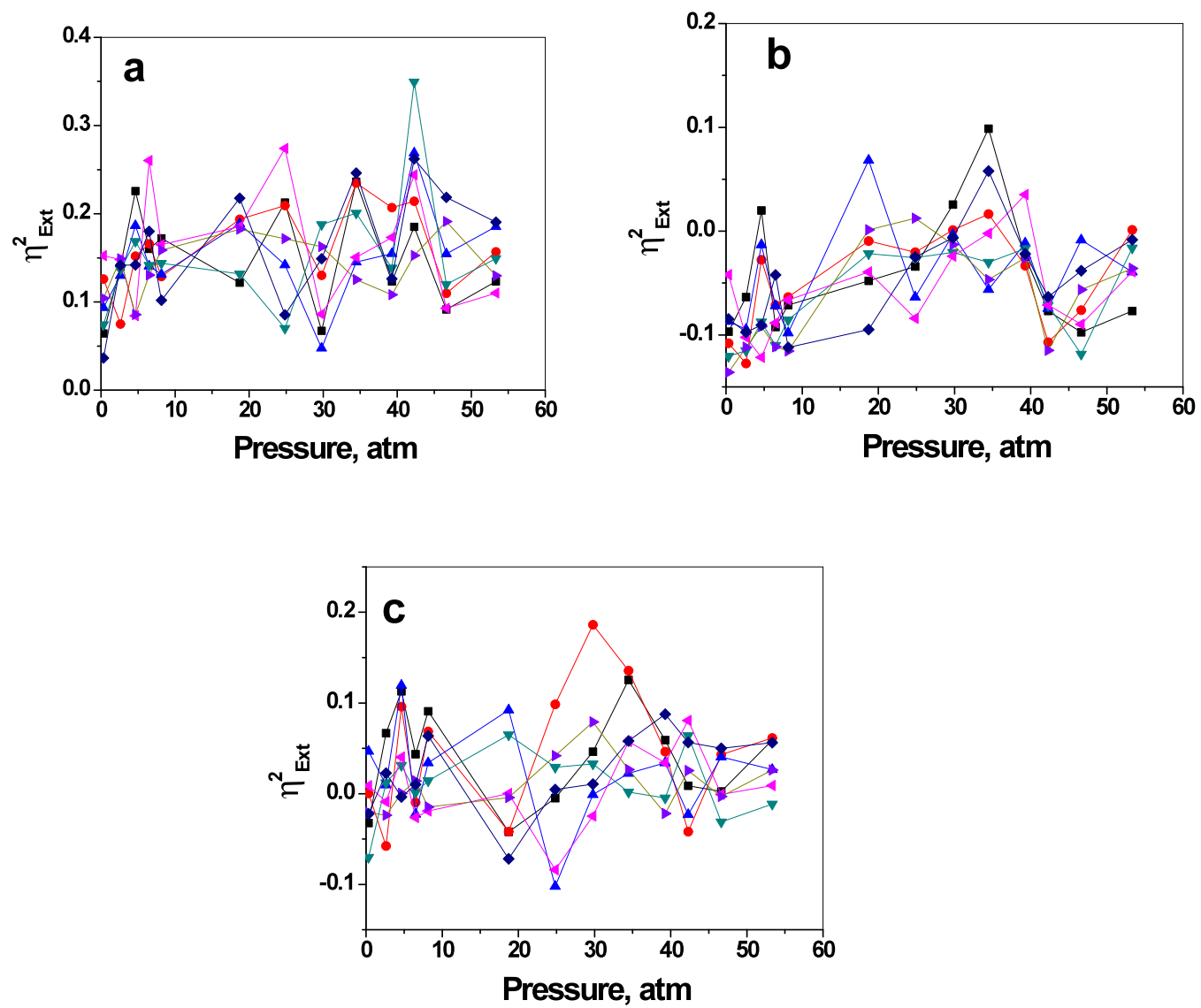


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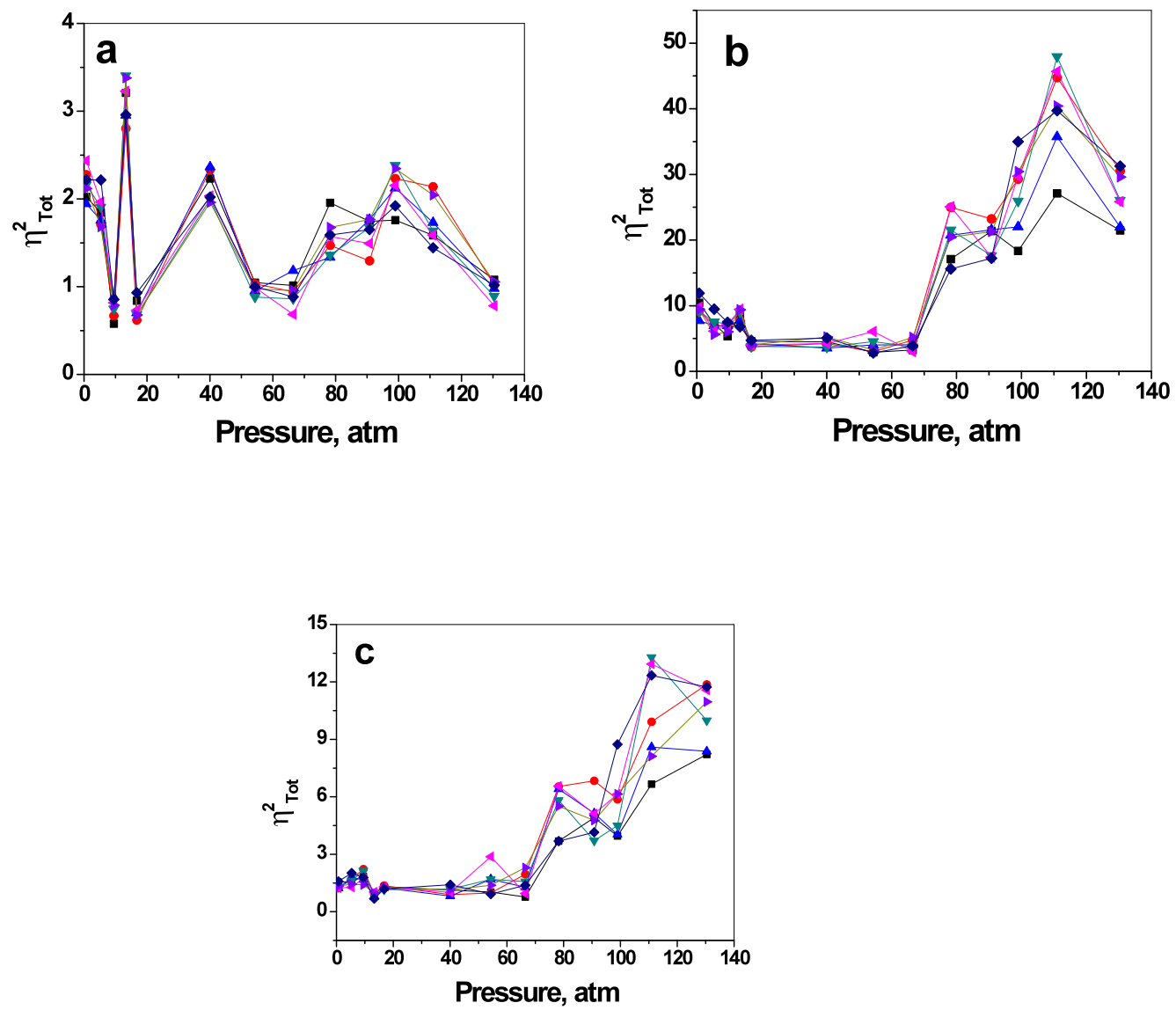


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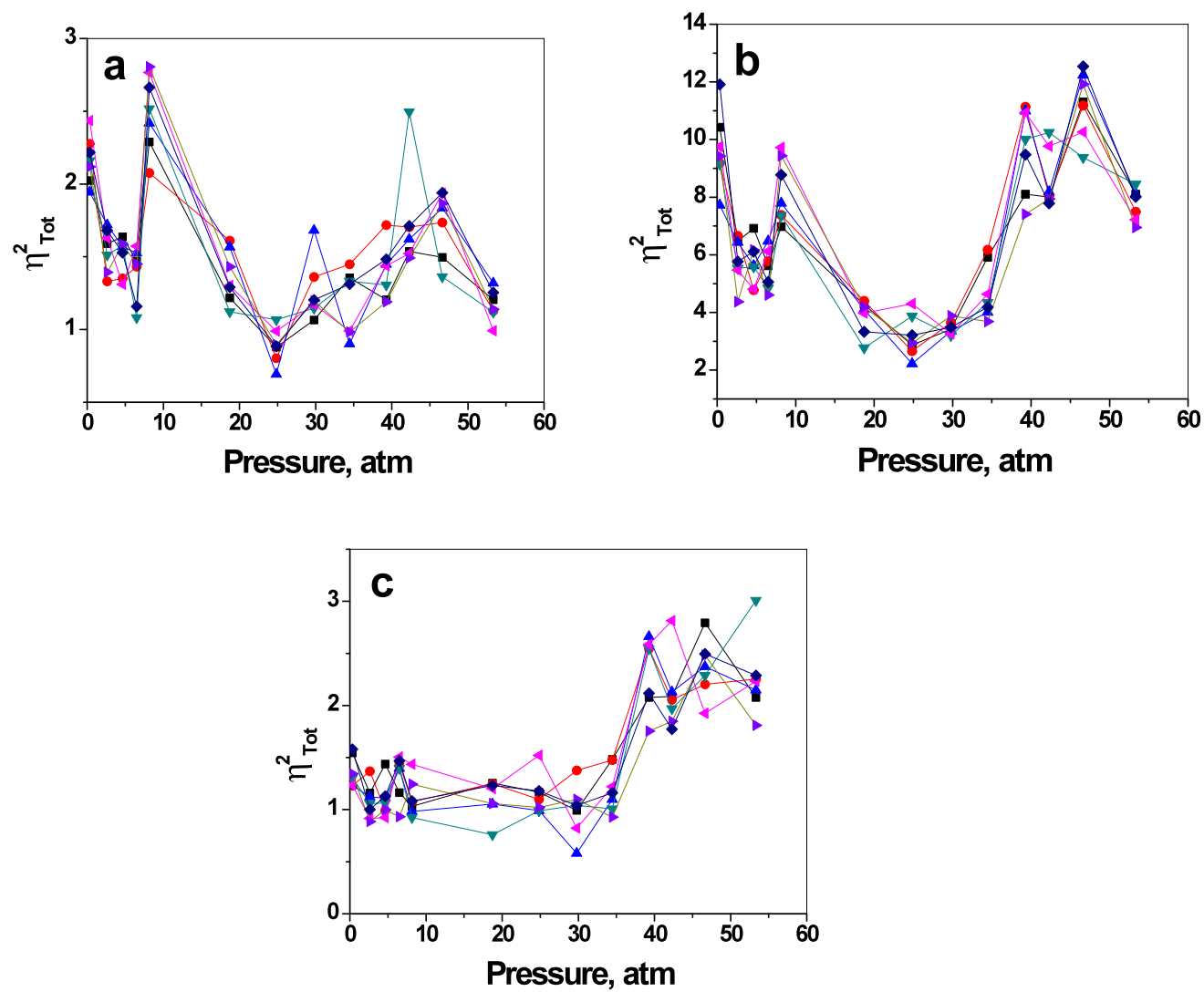


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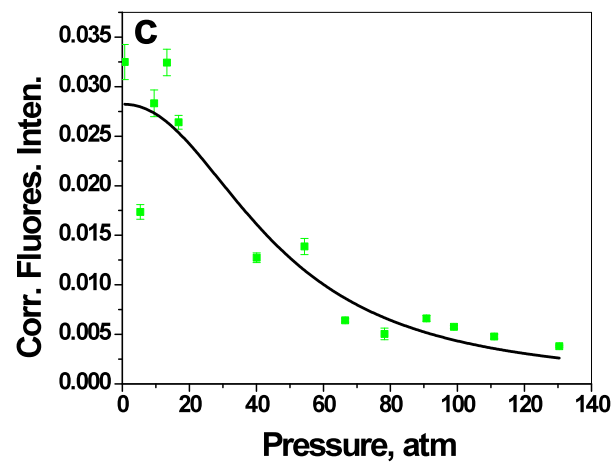
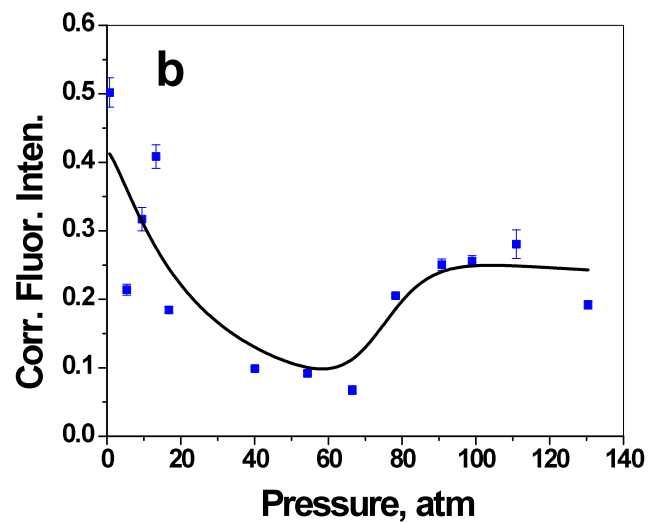
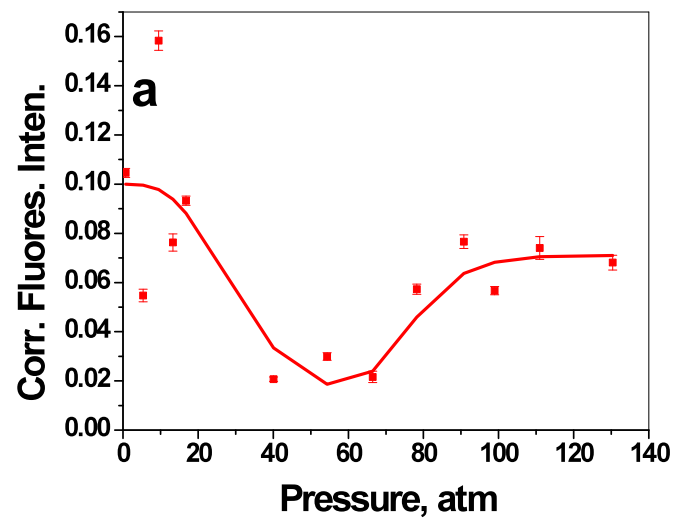


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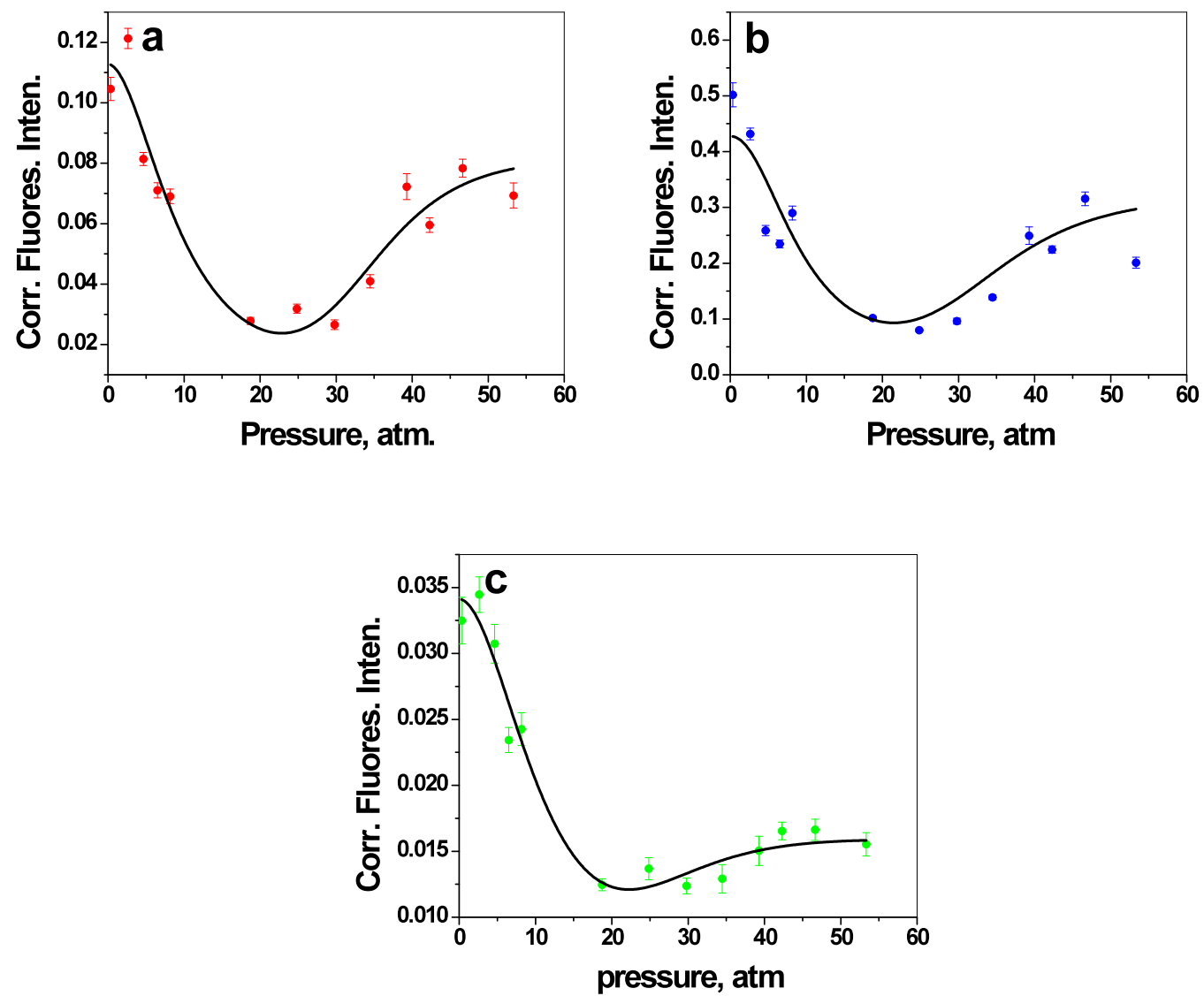


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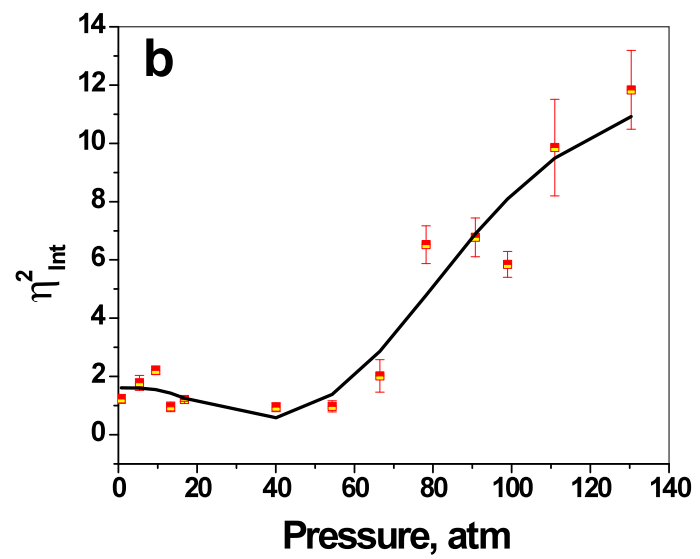
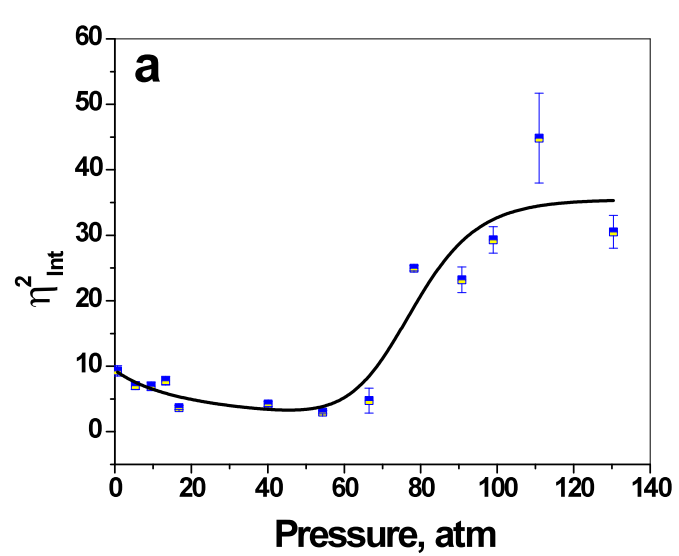


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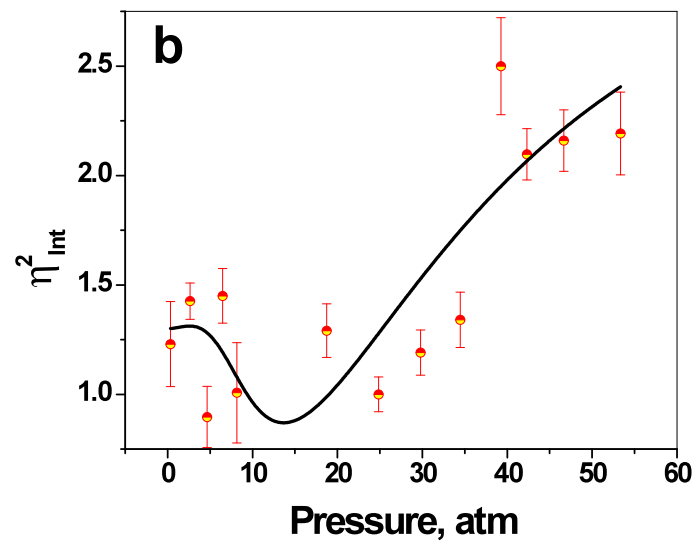
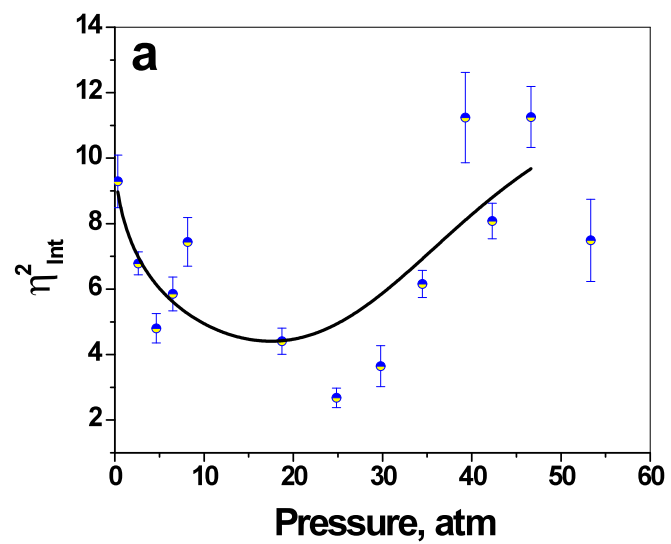


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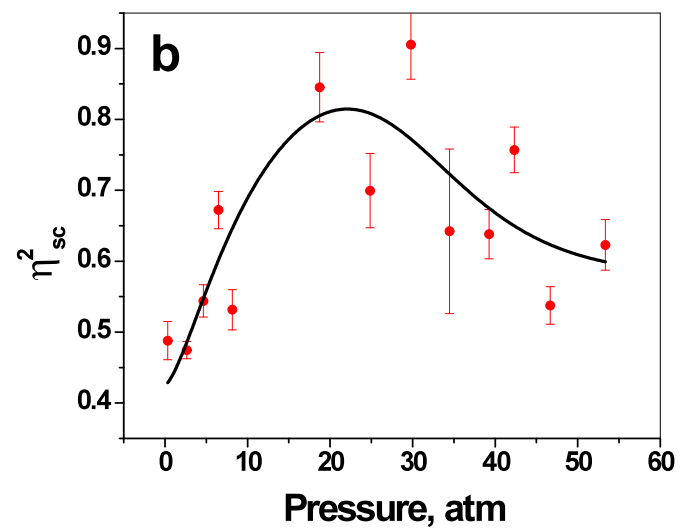
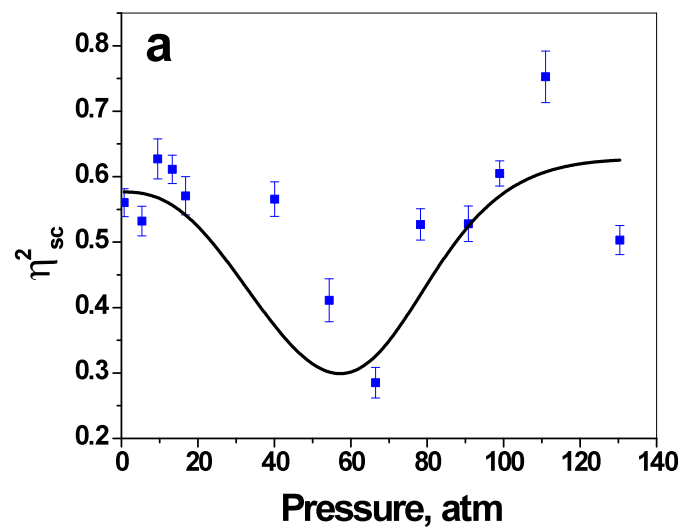


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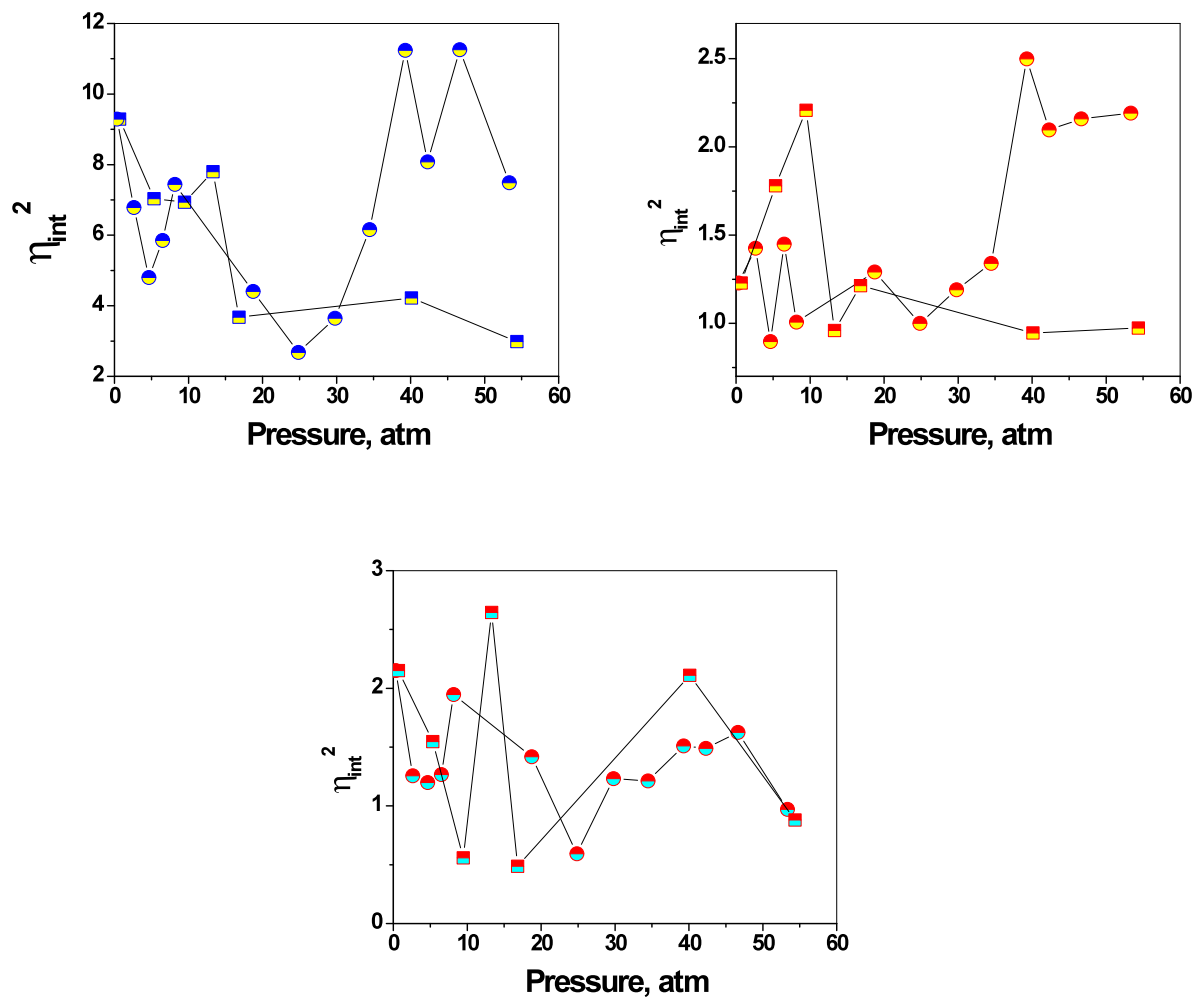


Table S1. Intrinsic noise vs pressure fitted to the bimodal equation

Gene pair	NaCl						Sucrose					
	Repression			Activation			Repression			Activation		
	a ₁	k ₁	n ₁	a ₂	k ₂	n ₂	a ₁	k ₁	n ₁	a ₂	k ₂	n ₂
<i>cfp-yfp</i>	9.4±2.9	22.2	1	34.3±4.5	78.4±4.9	9.2±4.5	9.9±1	9.5	0.6±0.2	11	30	3.9
<i>yfp-rfp</i>	1.6±0.5	24	3.5	12.7±0.8	87.4	4.6±0.9	1.3	10	3.2±1	3.5	35	1.8±0.4
<i>rfp-cfp</i>	2.1±0.4	20	0.97	1.9±0.5	90	1.47	2.2	8	1.8±0.7	2.6	33.1	1.4±0.6

Table S2. Intensity vs. pressure fitted to the bimodal equation

Protein	NaCl						Sucrose					
	Repression			Activation			Repression			Activation		
	a ₁	k ₁	n ₁	a ₂	k ₂	n ₂	a ₁	k ₁	n ₁	a ₂	k ₂	n ₂
RFP	0.1	32.1±9.2	3.1± 1.7	0.07	76	10	0.11±0.01	9.7±1.7	1.9±0.5	0.08	35.4±3.1	6.1±1.9
CFP	0.4±0.05	22	1.3±0.6	0.2±0.06	76±10.9	12	0.43±0.03	9.6	2.1±0.6	0.32	35	5±1.9
YFP	0.03±0.003	45.7±10.2	2.2±0.8	-	-	-	0.03±0.001	12.2±1.6	1.9±0.4	0.015	28.7±6.1	4.1±1.1

Table S3. η^2_{sc} vs. pressure fitted to the bimodal equation

Protein	NaCl						Sucrose					
	Repression			Activation			Repression			Activation		
	a ₁	k ₁	n ₁	a ₂	k ₂	n ₂	a ₁	k ₁	n ₁	a ₂	k ₂	n ₂
RFP	0.8	30	0.4	0.8	90	0.89	0.4±0.03	35	4.7±2	0.6	12	1.5±0.3
CFP	0.6	50	2.5±0.8	0.6	80	6.6±2	0.32±0.006	12	3.83	1.04±0.09	35	0.49
YFP	0.33	92.7	8.4±4	0.85	30	0.12	0.72±0.03	35	1.6±0.3	0.46±0.03	20	8.2±3