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Supplementary Information

Early Warning Signals in Chemical Reaction Networks

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S1 General Procedures

S1.1 Materials

All chemicals and reagents were used as received from commercial suppliers (*e.g.* Acros, Sigma Aldrich, Ellsworth, Life Technologies) without any further treatment unless stated otherwise. We used Milli-Q (MQ) water (i.e., ultrapure deionized water) from Millipore Corporation. Trypsinogen, trypsin, (from bovine pancreas), and amino ethyl benzene sulfonyl fluoride (AEBSF) inhibitor were purchased from Sigma Aldrich, while aminopeptidase M (EC: 3.4.11.2) were received from Novabiochem. Aminopeptidase activity was measured and 1 U was defined as 1 µmol of L-leucine-*p*-nitroanilide being converted within 1 minute at pH 7.7 at 24.0 °C. Bz-Arg-7-amino-4-methylcoumarin dye was purchased from Bachem. The synthetic and characterization procedures for the compound Pro-I [Ac-Lys(Me)-Gln-4-(2-aminoethyl)benzenesulfonyl fluoride trifluoroacetate] is as reported in previous work.[1-3]

S1.2 Instrumentation

Cetoni® neMESYS, 14.5 gear high-precision pumps were used for CSTR experiments. A 2mag MIXdrive 1 XS magnetic stirrer plate is used to stir the CSTR. The outflow of CSTRs is either collected with a *Bio-Rad 2110* fraction collector or monitored by fluorescence spectrometry using a Dolomite T-junction chip with 10 channels with a four way Linear Connector mounted on an *Olympus IX81* inverted microscope with an INDO X filter cube, an *Andor iXon 888* camera, a *Olympus IX2-UCB* microscope controller, a *Lumen Dynamics X-Cite® Series 120 Q* light source with a *Lumen Dynamics 120 Watt High Pressure Metal Halide Arc Lamp* and a *Prior OptiScan II* motorised stage. The microscope was controlled with Micromanager 1.4[4] and ImageJ software was used to process fluorescence images. For CSTR experiments, temperature is controlled by a *Lauda E100* thermostat water bath and monitored using a *Warner Instrument CL-100* temperature monitor and a *Warner Instrument* TA-29 thermistor.

S1.3 Experimental Methods

S1.3.1 Flow Experiment Setup

The details of how the flow experiments are setup and performed has been previously described in the Supporting Information of Maguire *et al. Chem. Eur. J.* **2020**, *26*, 1676 –1682[5] but are included here in full to give the reader clarity on how the experiments were performed.

S1.3.2 Preparation of Continuous Stirred Flow Tank Reactor (CSTR)

The preparation of the CSTRs is as previously described.[5]

S1.3.3 Overview of Experimental setup

The setup of a continuous stirred tank reactor (CSTR) experiment is depicted in Fig.S1. The CSTR is placed upon a magnetic stirrer plate and reactant solutions (trypsinogen, trypsin, aminopeptidase, and pro-inhibitor solutions, Table S1) are flowed in from glass syringes attached to a *NEMESYS* pump system. For the detection of trypsin concentration two additional syringes containing a dilution buffer and a Trypsin cleavable fluorescent dye (Bz-Arg-7-amino-4-methylcoumarin) (Table S1) are attached to the *NEMESYS* pump system. The outflow of the CSTR is connected to a microfluidics T-junction where the solution is diluted with the buffer and then flowed into the Dolomite T-junction chip. The Bz-Arg-7-amino-4-methylcoumarin solution is flowed into the Dolomite T-junction chip and both solutions are mixed inside an internal T-junction within the chip. The chip is mounted upon an *Olympus IX81* microscope with *Andor iXon 888* camera. The microscope was centred on the 9th microfluidic channel in the 10-channel chip (Fig.S2) and fluorescence images are automatically taken every 6 minutes throughout the duration of the experiment using an INDO X filtercube and a 100 ms exposure time. Analysis of the fluorescence images was performed using *ImageJ* by calculating the mean intensity of a region on the microfluidic channel for every acquired image.

Figure S1: Setup of a typical CSTR experiment. Syringes are mounted on pumps with four of the six syringes depicted connected to the CSTR via inlet tubing and the remaining two used for detection in the microfluidics chip (not shown here). The outlet tubing is either connected to a fraction collector or a microfluidics chip. The CSTR made of PDMS, with the outlet tubing on the right and the four inlets on the left. A copper tube, which is connected to a thermostatic water bath, encircles the CSTR (without making contact with the contents of the CSTR), enables control over the temperature of the CSTR. A

magnetic stirring bar is present in the CSTR to ensure rapid mixing of the inflowing compounds. Figure reproduced from the Supporting Information of Maguire *et al. Chem. Eur. J.* **2020**, *26*, 1676 –1682.

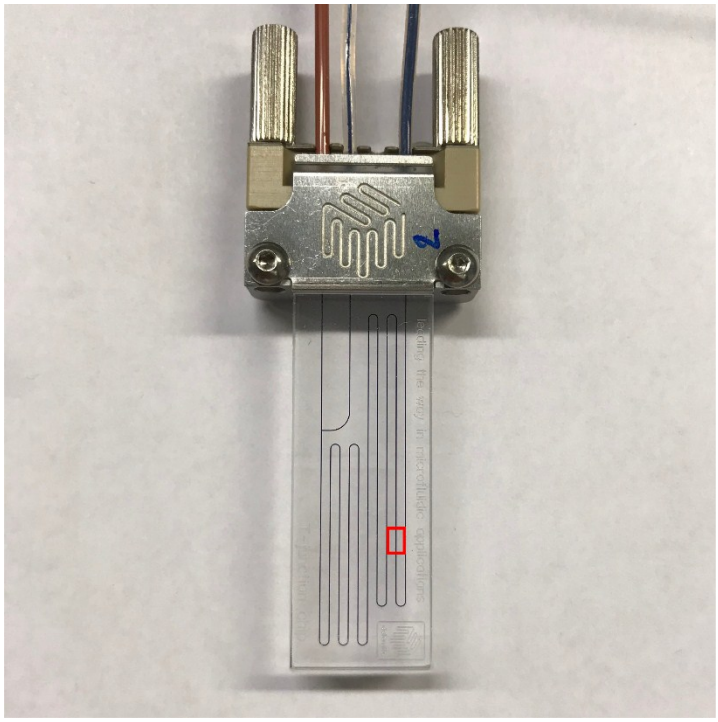

Figure S2: The Dolomite T-junction chip used for measuring the fluorescent signal. The fluorescence images in the flow experiments were taken on the 9th channel at the point marked by the red box. The internal T-junction can be seen in the top left of the chip. In this photo methylene blue dye was added into the channels of the chip for clarity. Figure reproduced from the Supporting Information of Maguire *et al. Chem. Eur. J.* **2020**, *26*, 1676 –1682.

S1.3.4 Preparation of Solutions

All experiments involved the preparation of 6 solutions. Trypsinogen (Tg), Trypsin (Tr), the Pro-inhibitor (ProI), and the aminopeptidase (Ap) were all dissolved in specific buffers whose composition and concentration is in Table S1. The buffer concentrations were used for all experiments unless other specified in the next sections and the volumes per experiment are also given in the next sections. All solutions were made with MilliQ water. For stability purposes we add calcium chloride to Trypsin and Trypsinogen solutions.[6]

Table S1: Buffer solutions used in all of the experiments unless otherwise stated.

Solutions	Buffer Solution
Tg buffer	4 mM HCl, 36 mM $CaCl_2$, pH = 2.4
Tr buffer	0.5 M Tris and 20 mM $CaCl_2$ pH = 7.7
ProI buffer	2 mM HCl, pH = 2.7
Ap buffer	10 mM Tris and 10 mM $MgCl_2$ pH = 7.7
Dilution buffer	50 mM Tris and 20 mM $CaCl_2$ pH = 7.7
CM solution	0.66 mM Bz-Arg-7-amino-4-methylcoumarin. Prepared from 132 μL of 50 mM stock solution Bz-Arg-7-amino-4-methylcoumarin in DMF, in 10 mL MilliQ water

S1.3.5 Initiating the Flow Experiment

Prior to connection of the CSTR to the chip, the chip is flushed to remove air with Bz-Arg-7-amino-4-methylcoumarin solution and dilution buffer in a 1 : 1 ratio at $1000\ \mu L\ h^{-1}$. In order to set up the CSTR the inflow tubing for each solution is filled until the solution is just about to enter the reactor cavity. To fill the reactor cavity a total flow rate of $5000\ \mu L\ h^{-1}$ is used, with individual flow rates in the same ratio as used in the experiment itself. A flow rate of $5000\ \mu L\ h^{-1}$ is maintained until all air is removed from the CSTR and the outflow tubing is filled and then the flow rates are lowered to that required for the experiment. The outflow tubing is connected to the chip via a microfluidics T-junction into which the 50 mM Tris + 20 mM $CaCl_2$ dilution buffer also flows. The internal T-junction in the chip is where the diluted outflow of the CSTR meets the Bz-Arg-7-amino-4-methylcoumarin solution and the concentration of trypsin is determined by fluorescence.

S1.3.6 Conversion of Fluorescence Intensity to Trypsin Concentration

The conversion of Fluorescence Intensity to Trypsin Concentration was performed as previously described.[5] A calibration line for the conversion of fluorescence intensity (F.I.) from 7-amino-4-methylcoumarin to trypsin concentration was established (Fig.S3). A flow experiment was set up with the Dolomite T-junction chip and four syringes containing 10 μM trypsin in 0.5 M Tris + 20 mM $CaCl_2$, MQ water, 0.66 mM Bz-Arg-7-amino-4-methylcoumarin solution and the dilution buffer 50 mM Tris + 20 mM $CaCl_2$. Tryspin and MQ water were mixed in a Y-junction at a total flow rate of 28.8 $\mu L\ h^{-1}$ prior to being flowed into a T-junction where the solution was mixed with 0.66 mM Bz-Arg-7-amino-4-methylcoumarin solution (70.4 $\mu L\ h^{-1}$). The solution was then passed into the chip and was diluted by the dilution buffer (42.0 $\mu L\ h^{-1}$) inside the internal T-junction in the

(Eq.1)

chip. The total flow rate of 141 µL h^{-1} through the chip was identical to that used in the flow experiments with the CSTR. Different concentrations of trypsin solution were generated by altering the ratio between the flow rates of trypsin solution and MQ water. The conversion for Figure S3 is:

$$[Tr] = \frac{F.I. - 96.05}{9.73}$$

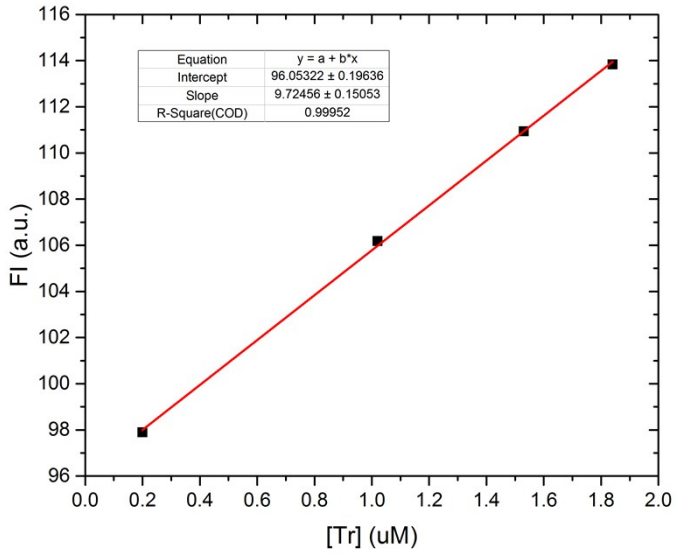

Fig.S3: Calibration lines for the conversion of fluorescence intensity to trypsin concentration at a total flow rate of = 28.8 µL h^{-1}. The red lines are the linear for through the data. The same calibration line was used in this study as that in Maguire *et al. Chem. Eur. J.* **2020**, *26*, 1676 –1682. This calibration line is also shown in Figure S3a in the Supporting Information of the aforementioned paper.

S1.3.7 Temperature control

Temperature control was performed as previously described.[5] The temperature within the CSTR was controlled by adjusting the temperature of the thermostatic water bath connected to the copper tubing encircling the CSTR (see Fig.S4a). An additional inlet was made to measure the temperature inside of the reactor using temperature monitor. The temperature in the reactor was calibrated following Fig.S4b).

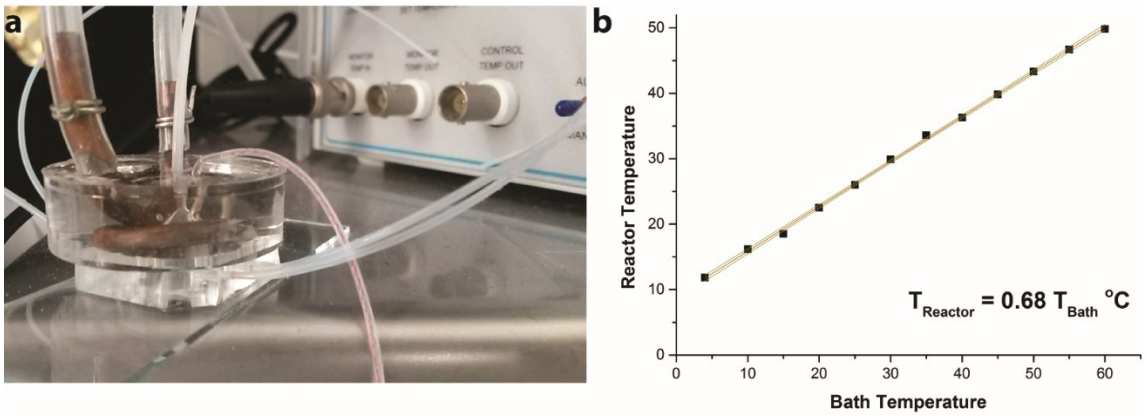

Fig.S4: Temperature control in CSTR experiments. (a) The copper tube, which is connected to a thermostatic water bath encircles the CSTR. A hole at the top of the reactor allows insertion of a micro thermistor (red wire) which is connected to the temperature monitor. (b) Calibration curve for the temperature of water measured in the reactor. The water flow in the thermostat bath was kept at a flow setting of P4, and the temperature reading was done under stirring conditions in the reactor. Figure reproduced from the Supporting Information of Maguire *et al. Chem. Eur. J.* **2020**, *26*, 1676 –1682.

S1.3.8 Data Processing

All data was processed in Origin 2018b. The data for Main Text Fig.2 and Fig.S5 in the main text was smoothed were smoothed using the Savitzky-Golay method with a window of 5 points and a polynomial order of 2.

S1.4 Simulations

Simulations were performed either with Matlab using scripts adapted from previously published scripts.[1-3, 5] Please consult the "Simulation Code.zip" which contains the code used in this paper for simulations.

S2 Early Warning Signal: Perturbation-Recovery experiments for measuring recovery time (Main Text Fig.2)

S2.1 Procedure for Perturbation-Recovery Experiment

The initial setup of the flow experiments is described in S1.3.2. Concentrations of reactants in syringes are given in Table S1. The initial temperature in the CSTR was set at one of four values a) 20.9 °C, b) 24.3 °C, c) 29.0 °C and d) 37.0 °C (Fig.S5). The Perturbation-Recovery experiments for 20.9 °C, 24.3 °C and 29.0 °C were all performed in one long continuous experiment (247 h in total) with the volume of feeder solutions in the syringes periodically replaced. The Perturbation-Recovery experiment for 37.0 °C was performed separately using identical conditions.

Before the perturbation was applied the experiment was left undisturbed until it had established sustained oscillations of identical amplitude and periodicity. The perturbation itself was performed by heating the CSTR to a temperature of 49.0 °C for a period of 2 h. The time required to go from the initial temperature to 49.0 °C during the perturbation was approximately 12 min and the 2 h was measured once the CSTR had reached 49.0 °C. To remove the perturbation, ice was added to the waterbath to rapidly cool the CSTR back to its initial temperature and the cooling process took approximately 10 min. The perturbation was applied at a 1.5π phase position in the oscillation, where the length of time between two successive peaks in trypsin concentration is defined as 2π.

After the initial temperature was restored, the time required for the system to go back into sustained oscillations was measured as the recovery time i.e. time required for the re-appearance of two peaks with an identical periodicity as before the perturbation. Observing two peaks was chosen due to limitations of reagents and the need to maximise the number of different temperatures.

Trypsin concentration was monitored using a 0.66 mM Bz-Arg-7-amino-4-methylcoumarin solution and the fluorescence was monitored using an excitation wavelength of λ_{ex} = 365 nm and monitored at emission wavelength of λ_{ex} = 440 nm.

Table S2: Experimental Conditions for the flow experiment in Figure S5.

	Concentration in Feed Syringe	Flowrate ($\mu L\ h^{-1}$)	Concentration in CSTR
Tg	236 μM	18.115	148.4 μM
Tr	1.35 μM	4.985	0.234 μM
ProI	10.0 mM	2.849	1.0 mM
Ap	1.5 U mL^{-1}	2.885	0.15 U mL^{-1}
Buffer	50 mM Tris + 20 mM $CaCl_2$	41.970	
CM	0.66 mM Bz-Arg-Coumarin	70.426	

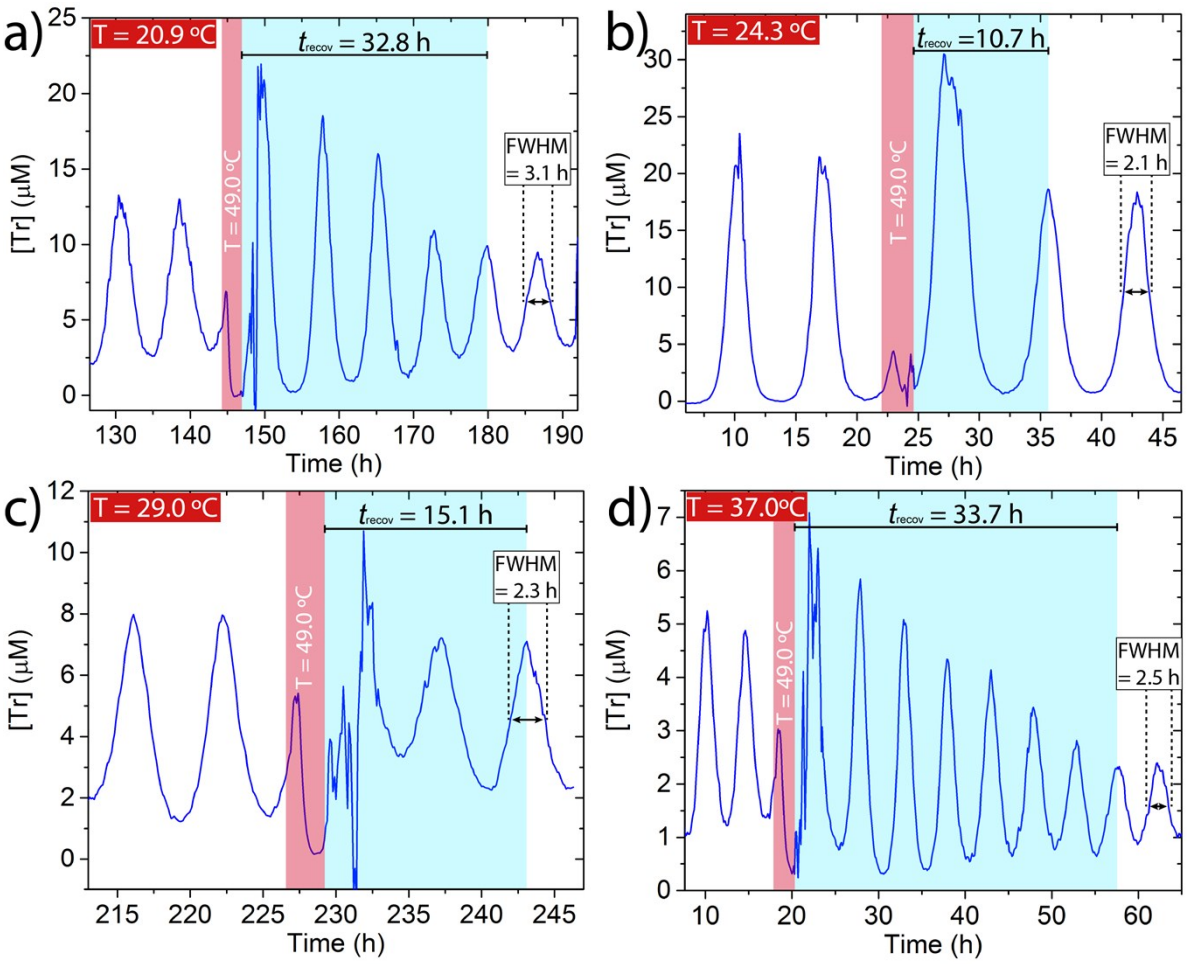

Fig.S5: Perturbation and Relaxation experiments for the trypsin oscillator at a base temperature of a) 20.9 °C, b) 24.3 °C, c) 29.0 °C and d) 37.0 °C. The noise in the fluorescent intensity after the perturbation was removed is due to air bubbles formed in the CSTR during the high temperature passing from the CSTR through the detector chip. The total flow rate in the CSTR was 28.8 μL h^{-1} and concentrations of all components are given in Table S2. The FWHM for each temperature are also shown (see Section S3 + Table S3). Note that the FWHM are measured when the system has fully recovered and whose measurement is independent of the perturbation recovery experiment.

Note that for Main Text Figure 2a (at 20.9 °C) the x-axis was normalised to start at 0 h, the actual experimental time for 20.9 °C is shown here in Figure S5a.

S3 Early Warning Signal: Experimental determination of the Full Width Half Maximum (FWHM) (Main Text Fig.3)

S3.1 Procedure for determining the experimental FWHM

The FWHM value was determined only once the system had developed sustained oscillations. In theory, our system should always produce identical oscillation peaks for a given temperature and relative flow rate and therefore the FWHM ought to be consistent for all oscillations. In practice, during experiments the FWHM does show some fluctuation due to noise from small fluctuations in e.g. temperature. Thus, not all peaks will be perfectly identical and therefore we determined the mean value of the FWHM for two peaks when it had developed sustained oscillations. For the temperatures a) 20.9 °C, b) 24.3 °C, c) 29.0 °C and d) 37.0 °C in Fig.S5 the FWHM was calculated for the last two oscillation peaks (after the oscillations had recovered, except for 29 °C where only the last peak was taken) by first determining the amplitude of the peaks through subtracting the maximum trypsin concentration from the minimum concentration for each peak. This value was halved to get the Half Maximum and the time points which corresponded to the half amplitude on either side of the oscillation peak were determined. The earlier time point was subtracted from the later time point to give the Full Width Half Maximum. The average of the FWHM value for the final two peaks was then taken as the FWHM for the temperature.

Table S3: The determination of the FWHM for the flow experiment in Figure S5.

Temperature (°C)	Peak	Amplitude (μM)	Half Amplitude (μM)	FW Min (h)	FW Max (h)	FWHM (h)	Average FWHM (h)
20.9	1	6.9	3.5	178.3	181.1	2.8	3.1
	2	6.3	3.2	185.1	188.4	3.3	
24.3	1	18.0	9.0	34.6	36.7	2.1	2.1
	2	17.4	8.7	41.8	43.9	2.1	
29.0	1	4.8	2.4	242.1	244.3	2.3	2.3 [a]
37.0	1	1.7	0.9	56.5	58.8	2.4	2.5
	2	1.7	0.9	61.1	63.7	2.6	

[a] For 29 °C, the first peak that had entered sustained oscillations (at 237 h) still displayed the tail-end of the recovery time behaviour during the initial rise in trypsin concentration and was therefore not incorporated into the FWHM calculation.

S3.2 The effect of Relative Flow Rate upon the FWHM

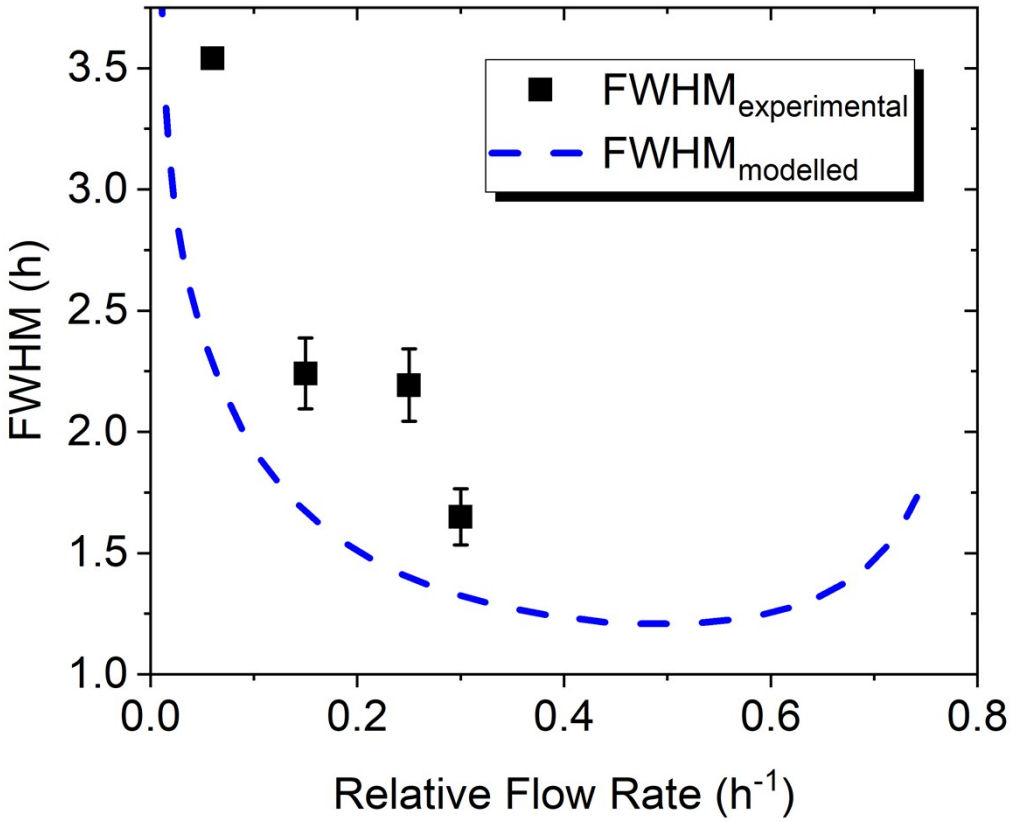

Fig.S6: A comparison of experimental and modelled results for the effect of changes in relative flow rate upon the FWHM of the Tr oscillations. Experimental and modelled conditions: $[Tg]_0$ = 167 µM, $[Tr]_0$ = 0.200 µM $[Pro\text{-}I]_0$ = 1.50 mM and $[Ap]_0$ = 0.32 U ml^{-1}, Temperature = 25.3 °C. Data for calculating the FWHM was taken from the oscillations shown in Figure S5 in the Supporting Information of Maguire *et al. Chem. Eur. J.* **2020**, *26*, 1676 –1682.

S4 Early Warning Signal: Simulations of Perturbation-Recovery experiments for measuring recovery time (Main Text Fig.2c+d)

S4.1 Matlab code for Perturbation-Recovery simulation

The Matlab code which incorporates previously reported temperature dependent rate equations[5] was adapted to perform simulated perturbation experiments. This script works by:

1) The trypsin oscillator is simulated at a given stable temperature. The script then identifies when oscillations have become sustained through the establishment of an identical amplitude and periodicity.
2) Once sustained oscillations are obtained the script will then apply a perturbation at a chosen phase of the oscillations. The length of time between two successive peaks in trypsin concentration is defined as 2π. Typically, a value of 1.5π was chosen for the time point at which the perturbation was applied.
3) Once the choice in time for when the perturbation is applied is selected the script identifies the concentrations of all components at the perturbation time for the system when it is in sustained oscillations. This set of concentrations is then used as the initial concentrations vector for the perturbation.
4) The effect of the perturbation on the system is then simulated. Perturbations are regarded as being instantaneous and the oscillations are simulated at the perturbed temperature for a set period of time, typically 2 h.

Once the perturbation time is completed, the concentrations of all components at the final time point is then transferred as the initial concentration vector for a simulation of the behaviour of the trypsin oscillator at the original temperature. The removal of the perturbation is also regarded as being instantaneous. The length of time required for the system to return to sustained oscillations at the original temperature is determined by a script which compares the oscillation pattern of trypsin concentration when it is in sustained oscillations with the oscillations in the trypsin concentration as the system is recovering back to sustained oscillations. When the oscillation patterns match the system is regarded as having returned to sustained oscillations and the time point at which this occurs is regarded as the recovery time of the system.

The code to calculate the recovery time after a perturbation was also inserted into a previously reported Matlab script[1-3, 5] for calculating a phase plot for the behaviour of the system at all

combinations of temperature between 10 – 40 °C and relative flow rates between 0 – 1.1 h^{-1}. This script was used to calculate the recovery time of the trypsin oscillator after a perturbation of 49 °C for 2 h across this entire phase space (Main Text Figure 2d).

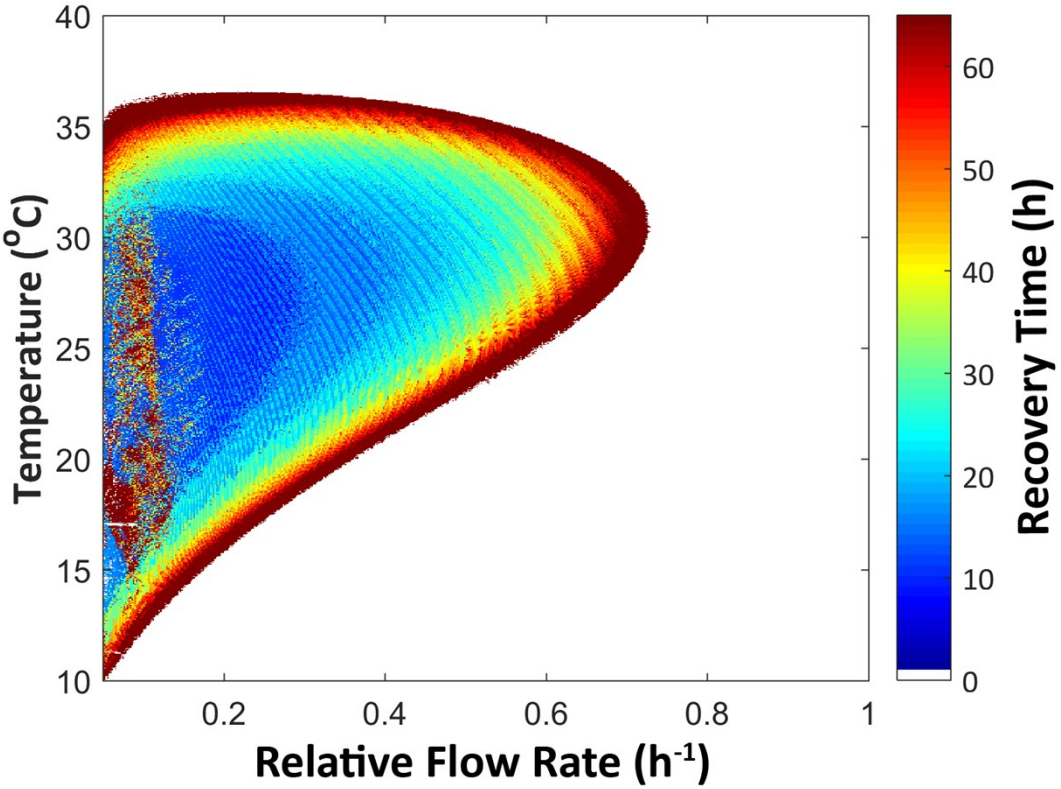

Fig. S7: The effect of a perturbation to 5 °C for 2 h is examined here. The phase plot shows the modelled dependency of recovery time on temperature and relative flow rate. Modelled conditions: $[Tg]_0$ = 148 µM, $[Tr]_0$ = 0.234 µM, $[Pro\text{-}I]_0$ = 1.00 mM, $[Ap]_0$ = 0.20 U ml^{-1}.

S5 Early Warning Signal: Full Width Half Maximum (Main Text Fig.3)

S5.1 Matlab code for Calculating the Full Width Half Maximum (FWHM)

The script used to calculate the FWHM works as follows:

1) The trypsin oscillator is simulated at a given stable temperature and flow rate. The script then identifies when oscillations have become sustained through the establishment of an identical amplitude and periodicity.
2) For all sustained oscillations the script identifies the minimum and maximum concentrations of trypsin for each oscillation peak and records them along with their corresponding time points in a matrix.
3) The amplitude of each peak is then calculated by subtracting the minimum value from the maximum value. This value is then halved to determine the Half Maximum.
4) The script then searches either side of the maximum of the oscillation peak to identify the two trypsin concentrations that have the closest value to the Half Maximum. The time points at which these half amplitudes occurs are then obtained and the earlier time point subtracted from the later time point in order to determine the Full Width Half Maximum.
5) The mean of the Full Width Half Maximum values for all sustained oscillation peaks was determined and output as the FWHM value.

The code to calculate the FWHM was also inserted into the previously reported Matlab script[1-3, 5] for calculating a phase plot for the behaviour of the system at all combinations of temperature between 10 – 40 °C and relative flow rates between 0 – 1.1 h^{-1}. This script was used to calculate the FWHM across this entire phase space (Main Text Figure 3b).

S6 References

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