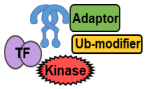
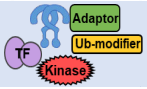
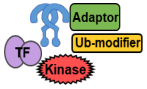
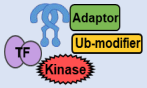
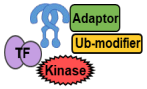
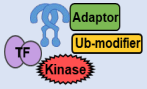
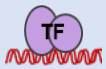
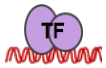
Common Experimental Approaches for Systems Analysis of Immune Response Signaling Networks (1/2)

Method	Information gained	Pathway components interrogated	Resolution	Degree of multiplexing	Quantitation, reliability, sensitivity	Data generation challenges	Data interpretation challenges	Example protocol or study reference
Flow Cytometry of cell surface proteins	Protein abundance, internalization kinetics		Single cell snapshot	Multi-analyte (typically <10, more possible with Cy-TOF)	Relative quantitation (protein abundance/cell), absolute quantitation (fraction of cells expressing specific protein); sensitivity depends on antibody quality and probe.	Flow cytometer, specific antibodies against proteins of interest required; need to optimize fluorescence panel.	Fluorescence compensation if using multi-color panel; gating strategies and multi-dimensional analysis.	Yu et al., 2016, PMID: 26938654. Rigo and Vinante, 2017, PMID: 27342211.
Co-immuno-precipitation (Co-IP), + Western Blotting or + Mass Spec	Protein-protein interactions		Bulk	Typically single analyte, but can be linked to mass spec	Relative quantitation; not reliable for weak or transient interactions.	High specificity, high affinity antibodies required; need to optimize precise experimental conditions; time-intensive.	Potential for artefact requires good negative and positive controls. Not clear whether association is direct or indirect.	Tang and Takahashi, 2018, PMID: 29855966.
***In situ* Proximity Ligation Assay (PLA) with microscopy**	Protein-protein interactions		Single cell snapshot	Single analyte	Absolute quantitation of complexes possible; high sensitivity.	Fluorescence microscope, antibodies against both proteins required; need to optimize experimental conditions.	Automated cell segmentation and spot count.	Hegazy et al., 2020, PMID: 33044803.
(Phospho-) proteomics by Mass Spectrometry using labeling (e.g. SILAC)	Protein abundance, Protein activation		Bulk	'omic	Relative quantitation; high accuracy.	Mass Spec required; chromatography for fractionation and phosphopeptide enrichment; very time-intensive; only applicable to metabolically active model systems.	Peptide identification using databases and commerical software; correct analysis of pooled samples; time-intensive.	Rathore and Nita-Lazar, 2020, PMID: 32936995. Ankney et al., 2018, PMID: 29894226.
(Phospho-) proteomics by Mass Spectrometry using peptide spike-in (e.g. AQUA)	Protein abundance, Protein activation		Bulk	Single analyte, multi-analyte	Absolute quantitation; high accuracy.	Mass Spec required; stable isotope labled synthetic peptides for proteins of interest required; time-intensive.	Peptide identification using databases and commerical software.	Ankney et al., 2018, PMID: 29894226. Kirkpatrick et al., 2005, PMID: 15722223.
Immuno-blotting (for phospho-proteins)	Protein abundance, Protein activation		Bulk	Single analyte, (multi-analyte possible)	Relative quantitation; dependent on skilled protocol implementation; sensitivity varies with antibody affinity and detection chemistry.	High specificity antibodies required; need to optimize experimental conditions: knowledge about phosphorylation sites; time-intensive.	Need to determine dynamic range of antibody; appropriate loading; correct band identification; careful normalization, etc.	Pillai-Kastoori et al., 2020, PMID: 32007473. Esser-Nobis et al., 2020, PMID: 32571931.
Protein turnover assays (e.g. pulse-chase (P-C), cycloheximide (CHX) inhibition, Mass Spec)	Protein half-life/ degradation		Bulk	'omic (mass spec), targeted (pulse-chase, CHX, etc.)	Rate constants can be determined from relative abundance measurements; mass spec: high sensitivity, CHX: low sensitiivty.	Metabolically active system required; classical P-C requires IP, radioactivity; CHX induces cells stress; mass spec: sample complexity, much input material required.	Accurate calculation of turnover rate; mass spec: peptide identification using databases across time points; Validation of measured rates often required.	Hinkson and Elias, 2011, PMID: 21474317.
Kinase activity assays	Protein activity		Bulk	Single-analyte	Absolute quantitation possible (nmole of phosphate incorporated/min); good sensitivity and accuracy.	Radioactivity required; high quality protein preparation/IP required; time-intensive; need to determine linear range of kinase.	Calculating the kinase activity for true quantitation is not easy.	Hastie et al., 2006, PMID: 17406331.
Live-cell Microscopy	Subcellular localization of protein		Single cell time-lapse	Single analyte, multi-analyte	Relative quantitation; dynamic range strongly dependent on reporter protein design.	Cells with fluorescently-tagged reporters required; advanced microscope with live cell incubator and multi-position automated time series image acquisition required.	Accurate image segmentation and reporter quantification pipeline required; need to relate localization to activity.	Kudo et al., 2018, PMID: 29266096; Regot et al., 2014, PMID: 24949979 ; Adelaja et al., 2021, DOI: 10.1101/ 2020.05.23.11286.
Electrophoretic Mobility Shift Assay (EMSA)	DNA-protein interactions, protein activation		Bulk	Single analyte	Good but relative quantitation of DNA binding activity, and composition of the DNA binding complex.	Radioactivity required; phosphor-imager required; time-intensive.	Quantitation procedure and appropriate controls are well established.	Juvekar et al., 2012, PMID: 22113267.
TransAM® assay	DNA protein interactions, protein activation		Bulk	Single analyte	Relative quantitation, absolute quantitation possible; high sensitivity.	High costs for kits; commercial kit for TF of interest needs to be available.	Data analysis is standardized, but determining the complex composition is a challenge.	Challis et al., 2006, DOI: 10.1038/nmeth907.

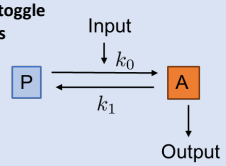
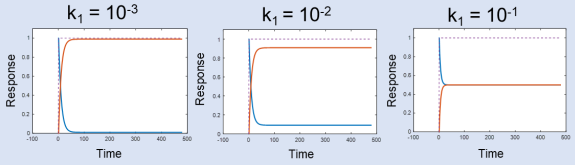
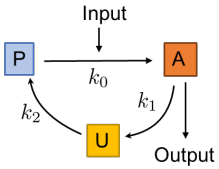
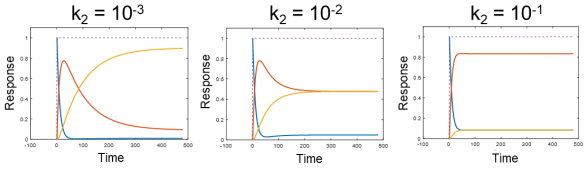
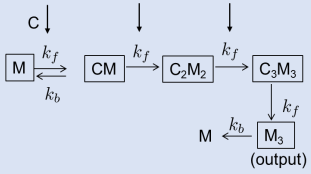
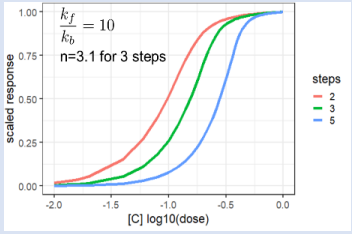
Common Experimental Approaches for Systems Analysis of Immune Response Signaling Networks (2/2)

Method	Information gained	Pathway components interrogated	Resolution	Degree of multiplexing	Quantitation, reliability, sensitivity.	Data generation challenges	Data interpretation challenges	Example protocol or study reference
Bulk epigenetic assays, e.g. ATACseq, ChIP-Seq, CUT&RUN, CUT&Tag	Chromatin state		Bulk	'omic	Relative quantitation; ChIP-Seq, C&R, C&T: normalized to background or spike-in control.	High cost; ChIP-Seq, C&R, C&T require high specificity, high affinity antibodies; optimization of protocol to cell types; ChIP-Seq: large number of cells required.	Next Gen Sequencing data processing/ interpretation; may need to develop new algorithms for analysis.	Buenrostro et al., 2013, PMID: 24097267. Corces et al., 2017, PMID: 28846090. Skene et al., 2017, PMID: 28079019.
Single cell epigenetic assays, e.g. ATACseq, CUT&Tag	Chromatin state		Single cell snapshot	'omic	Relative quantitation; C&T: normalized to background or spike-in control; some dropout events common.	High cost; separating cells; C&T require high specificity, high affinity antibodies; need to optimize protocol for each condition.	High dimensional data; batch effects; dropouts and sparsity; technical noise vs. biological effects.	Buenrostro et al., 2015, PMID: 26083756. Kaya-Okur et al., 2019, PMID: 31036827.
Sequencing of Intron-Containing RNA (e.g. caRNA-Seq, total RNA Seq)	Abundance of nascent RNA		Bulk	'omic	Relative quantitation; low sensitivity; accurate quantitation difficult due to variable intron excision rates across time course and genes.	High costs for library kits and Next Gen Sequencing.	'omic data processing/ interpretation; caRNA-Seq: non-linear quantitation, some non-nascent RNA species also sequenced.	Wissink et al., 2019, PMID: PMID: 31399713.
qRT-PCR	RNA abundance		Bulk	Single analyte	Relative quantitation; absolute quantitation possible using standard.	qRT-PCR instrument required; entry level skill sets.	Appropriate normalization; standard methods.	Forlenza et al., 2012, PMID: 22131023.
Bulk mRNA Sequencing	mRNA abundance		Bulk	'omic	Relative quantitation; absolute quantitation with spike-ins.	High costs for library kits and Next Gen Sequencing.	'omic data processing/ interpretation.	Su et al., 2014, PMID: 25150838. Kukurba et al., 2015, PMID: 25870306.
Single cell mRNA Sequencing	mRNA abundance		Single cell snapshot	'omic or targeted multi-analyte	Absolute quantitation possible with UMI-based approaches; some dropout genes common.	Very high costs for kits or service; separating cells; maintaining cell health during assay; access to cell capture device and sequencing core.	High dimensionality data; batch effects; dropouts and sparsity; technical noise vs biological effects.	Shalek et al., 2014, PMID: 24919153. Luecken et al., 2019, PMID: 31217225.
Fluorescence In Situ Hybridization (FISH), e.g. MERFISH	RNA abundance		Single cell snapshot	Multi-analyte	Absolute quantitation of mRNA spots; high sensitivity.	Good resolution fluorescence microscope required; If multiplexed: microfluidics required, time-intensive, complex protocol, correct hybridization probe design, feasable sample size is limited.	Cell segmentation, automated quantitation of RNA spots; If multiplexed: optical crowding of RNA spots esp. for highly expressed genes, analysis and interpretation is challenging	Chen et al., 2015, PMID: 25858977. Xia et al., 2019, PMID: 31118500.
Metabolite Analysis by Mass Spectrometry	Metabolic state		Bulk	Omics, (single analyte for absolute quantitation)	Relative quantitation; absolute quantitation possible using spike-ins; high sensitivity.	Mass spec required.	Optimizing parameters for peak identification in software.	Abuawad et al., 2020. PMID: 32114632.
Intracellular Cytokine Staining (ICS) by flow cytometry	Protein abundance		Single cell snapshot	Multi-analyte	Relative quantitation (protein abundance/cell), absolute quantitation (portion of cells expressing specific protein)	Flow cytometer, specific antibodies against proteins of interest required; need to identify time point, to optimize permeabilization procedure.	Fluorescence compensation if using multi-color panel, gating strategies and multi-dimensional analysis.	Lovelace and Maecker, 2018, PMID: 29071680.
Enzyme-linked Immunosorbent Assay (ELISA)	Cytokine secretion		Bulk	Single analyte	Relative quantitation, absolute quantitation of cytokine concentration possible using standard.	Relatively expensive; plate reader required; antibodies or ready-made ELISA kit for cytokine of interest required.	Analysis is generally not very challenging.	Chiswick et al., 2012, PMID: 22262432.
Enzyme-linked Immunospot (ELISPOT) assay	Cytokine secretion		Single cell snapshot	Single analyte, (multianalyte possible)	Absolute quantitation of number of cells secreting cytokine of interest.	Relatively expensive; antibodies against cytokine of interest required; cell health during incubation time.	Analysis is generally not very challenging.	Kouwenhoven et al., 2001, PMID: 11687471.
LUMINEX®	Cytokine secretion		Bulk	Multi-analyte	Relative quantitation, absolute quantitation possible using standard; large dynamic range.	LUMINEX® instrument required; expensive kits; kit for desired analyte needs to be available.	Analysis is generally not very challenging.	Surenaud et al., 2016, PMID: 27835944.

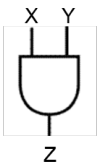
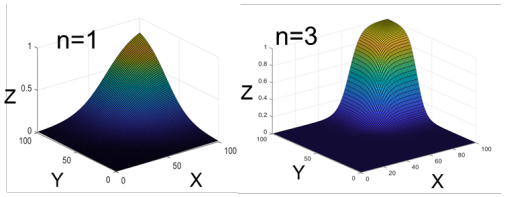
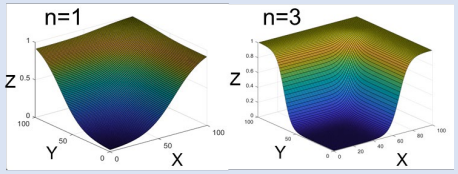
Common Perturbation Approaches for the Study of Immune Response Signaling Networks (1/1)

Perturbation	Purpose	Features	Challenges	Example protocol or study reference
Pharmacological inhibitors	Reduce enzymatic activity, or block protein-protein interactions	- Easy to administer - Usually transient effect, with defined t = 0 - Usually high efficiency if using established inhibitor	- Cytotoxicity depending on concentration, target, and solvent - Significant off-target effects a common problem, depends on available inhibitors, requires careful controls	Van den Blink et al., 2001, PMID: 11123340. Greten et al., 2007, PMID: 17803913.
RNA interference by siRNA, often delivered by transfection or electroporation	Reduce protein production	- Easy to implement - Strength of knockdown can be titrated - Transient effect, but timecourse not well defined	- Off-target effects require controls, such as suppressing the phenotype by complementary expression of an siRNA-resistant variant - May activate type I IFN - Effect may be cell-to-cell heterogeneous	Birmingham et al., 2007, PMID: 17853862. Troegeler et al., 2014, PMID: 24890643.
RNA interference by shRNA, often vector-based delivery by viral transduction	Reduce protein production	- Permanent knockdown through genomic integration possible - Inducible knockdown systems available - Viral transduction often well tolerated	- Off-target effects require controls, such as suppressing the phenotype by complementary expression of an shRNA-resistant variant - May activate type I IFN - May require selection of cell clone to avoid heterogeneity	Moore et al., 2013, PMID: 20387148.
Traditional knockout in mice (or DT40 cell line)	Eliminate expression	- Permanent and complete - Perfectly specific for gene of interest - Conditional/ organ-specific knockouts possible - Inducible knockout possible	- Genetic compensation by family members may mask true function - Embryonic lethality may be a problem - Long and expensive process, low efficiency	Hall et al., 2010, PMID: 19731224.
CRISPR knockout	Eliminate expression	- Easy to design and implement compared to traditional knockout - Many design and delivery options available - Avoids embryonic lethality if cell type of interest tolerates loss of protein - Permanent knockout - Inducible knockout possible	- Genetic compensation by family members may mask true function - Off target effects possible, can be reduced by careful design, but when clonal analysis is needed, multiple clones are required - Often relatively low efficiency - May result in cytotoxicity	Ran et al., 2013, PMID: 24157548. Giuliano, et al., 2019, PMID: 31503414.
Modulation of gene expression via catalytically inactive CRISPR variants	Transcriptionally activate or repress target gene without altering protein coding sequence	- Applicable also to non-coding RNA expression - CRISPRa: allows overexpression of endogenous genes - Inducible designs available	- Off targets effects possible, depend on design of guide RNA - Designing guide RNA targeting transcriptional start site can be difficult (accessiblity, promoter use, lack of annotation)	Qi et al., 2013, PMID: 23452860. Konerman et al., 2015, PMID: 25494202. Joung et al., 2017, PMID: 28333914.
Transient Transfection	Overexpress signal transducer or transdominant inhibitory mutant, introduce tagged or mutated genes, complement knockout.	- Easy to implement in some cell lines - Expression starts within 12-24 h after transfection, reverses over time in most cells - Expression levels can be titrated	- Works well for some cell lines, but not for all, and rarely for primary cells - Cell-to-cell heterogeneity of expression - May activate type I IFN	Kim et al., 2010, PMID: 20549496. Longo et al., 2014, PMID: 24011049. Zhang et al., 2010, PMID: 19347315.
Stable Transfection	As above	- Permanent genomic integration - Cloning and/or selection used to achieve pure cell population - Inducible expression systems available	- Random genomic integration may disrupt gene functions - Epigenetic silencing may occur - Low efficiency, and long time required to grow pure population - Cell health depends on nature of protein expressed	Zhang et al., 2010, PMID: 19347315.
Viral Transduction (e.g. lenti- or retroviruses)	As above	- Ensures long-lasting expression - Cloning and/or selection used to achieve pure cell population - Inducible expression systems available	- Epigenetic silencing may occur - Cell health depends on nature of protein expressed - Random genomic integration may disrupt unrelated gene functions, so when a clonal analysis is needed, multiple clones are required	Leyva et al., 2011, PMID: 21281514. Zhang et al., 2010, PMID: 19347315.
CRISPR Knockin	Mutate or tag endogenous genes	- Targeted alterations of genes in endogenous locus possible - Permanent alteration of gene - Selection/cloning/sorting results in pure cell population	- Low efficiency, sometimes difficult to select successful event - Careful design necessary - Delivery of protein/nucleic acid may not work for all cells	Koch et al., 2018, PMID: 29844520.

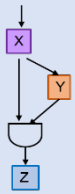
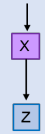
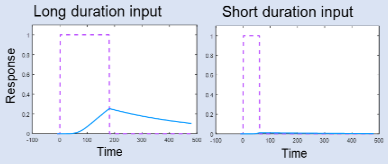
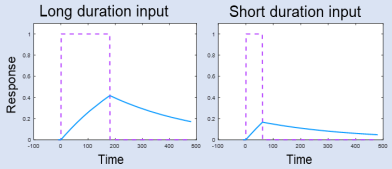
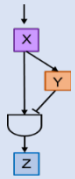
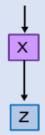
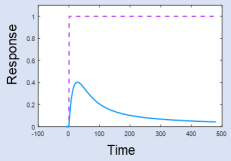
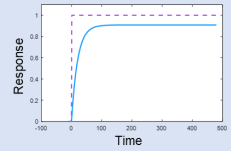
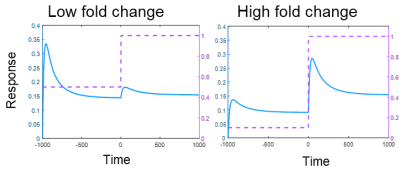
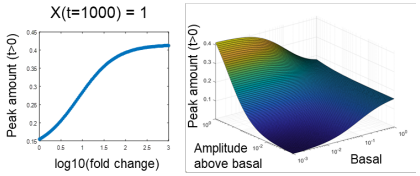
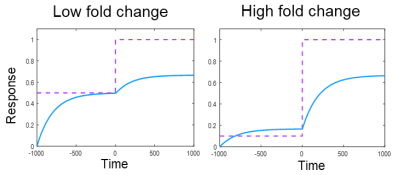
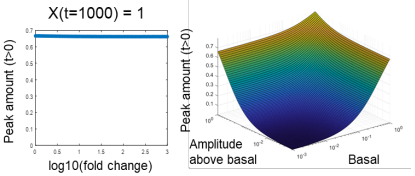
Regulatory motifs in innate immune signaling networks (1/5)

Motif	Diagram	Equation	Simulations	Description	Examples
1.1. 2-state toggle switches		$\frac{dP}{dt} = k_1 A - k_0 I(t) P$ $\frac{dA}{dt} = k_0 I(t) P - k_1 A$		**Simple two-state toggle switch** • **P (poised state)** • **A (active state)** • The incoming signal ("Input") enhances the forward reaction • Dotted line: strength of the input signal • Orange line: amplitude of the output • k_1: the reverse reaction that controls output amplitude. As k_1 increases, the sensitivity to input decreases.	**Kinase – phosphatase toggle:** Phosphatase PP2A terminates transcription factor IRF3 activity when kinase activity has diminished (Long et al., 2014).
1.2. 3-state switch cycles		$\frac{dP}{dt} = k_2 \mathrm{U} - k_0 \mathrm{I(t)P}$ $\frac{dA}{dt} = k_0 \mathrm{I(t)P} - k_1 \mathrm{A}$ $\frac{dU}{dt} = k_1 A - k_2 U$		**Three-state cycles** • **P (poised state)** • **A (active state)** • **U (unavailable)** • The forward reaction predominates over backwards reactions. • The molecule cycles from P to A to U, before returning to P. The intermediate unavailable pool U leads to a dynamically transient response for a persistent signal. The active species A becomes trapped in the U pool, thereby generating a pulse-like transient response. • As the recycling rate k_2 increases, the cycle behavior becomes more toggle-switch-like.	**Receptor recycling:** Slow reappearance of receptors at the cell surface after endocytosis or degradation leads to a refractory period. • Receptors available for ligand binding at the surface are "poised" (P). • Bound receptors are "active" (A). • Receptors "unavailable" (U) are returned to the surface later (Becker et al., 2010). **Kinase cycles (e.g. IKK):** • Kinases are "poised" (P) for activation. • Phosphorylated to an active "on" state (A) • IKK is further phosphorylated into an "off" state and "unavailable" (U) for a certain time (Behar and Hoffmann, 2013).
2. Multi-step reaction cascades		$\frac{k_f}{k_b} = \frac{C^n}{K_D^n + C^n}$ $\frac{dC}{dt} = k_b C_1 M_1 - k_f C_1 M_1 + 3k_f C_3 M_3$ $\frac{dM}{dt} = k_b C_1 M_1 - k_f C_1 M_1 + 3k_b M_3$ $\frac{dC_1 M_1}{dt} = k_f CM - 2k_f C_1 M_1 C_1 M_1 - k_b C_1 M_1 - k_f C_2 M_2 C_1 M_1$ $\frac{dC_2 M_2}{dt} = k_f C_1 M_1 C_1 M_1 - k_f C_2 M_2 C_1 M_1$ $\frac{dC_3 M_3}{dt} = k_f C_2 M_2 C_1 M_1 - k_f C_3 M_3$ $\frac{dM_3}{dt} = k_f C_3 M_3 - k_b M_3$	 • Response is the ratio of oligomers M_3 formed relative to the monomer concentration M. • When testing the model with a range of doses (i.e., a range of concentrations for the ligand-receptor complex, C), the response is thresholded. • Different approximated Hill coefficients depend on the number of oligomerization steps when $\frac{k_f}{k_b}$ is held constant.	**Multistep reaction cascades** • Hill function-like ultra-sensitivity • Increasing $\frac{k_f}{k_b}$ approximates a Hill function with increasing Hill coefficient n. • A multistep mechanistic model can suggest the molecular mechanisms behind a Hill function approximation. • To model e.g. oligomerization, monomers (M) dimerize when receptor-ligand complex (C) forms. The input dose (C) accelerates the multiple forward reactions. • The first aggregated complex C_2M_2 then aggregates again, with forward rate k_f, up to the most aggregated form, here called C_3M_3. The reverse rate k_b is the dissociation of the oligomers into monomers.	**Examples of multistep reaction cascades:** • Receptor-ligand binding • Kinase cascades (MAPK) • Signal adapter oligomerization (e.g. MyD88 and MAVS) The multistep process generates digital rather than linear dose response curves, as shown by the sequential oligomerization of the Myddosome (Cheng et al., 2015).

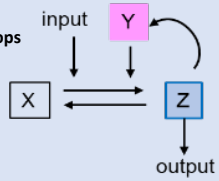
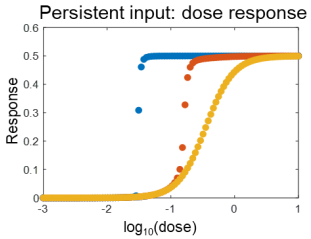
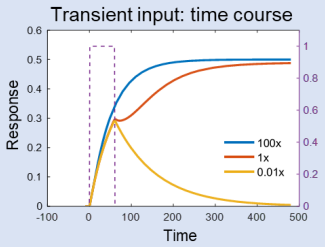
Regulatory motifs in innate immune signaling networks (2/5)

Motif	Diagram	Equation	Simulations	Description	Examples
3. Boolean logic gates	When two signals combine to produce a single output, the combinatorial relationship between output and the two inputs can be abstracted as a **Boolean logic gate**. In the diagrams of *AND* and *OR* gates shown below, • **X** represents the concentration of S_1, • **Y** represents the concentration of S_2. • $\boldsymbol{n}$ represents the Hill coefficient, which affects the steepness of the output $\mathrm{G}(t)$ (**Z**) • $\boldsymbol{n}$ is a parameter that can be fit to data.				**Logic gates in signaling:** • IKK activation by TAK1-dependent and -independent mechanisms **Logic gates in gene transcription:** • Combinatorial control of gene promoters by *AND* and *OR* gates (Cheng et al., 2017). • For any particular mRNA: $\frac{dmRNA}{dt} = k_{syn}G(t) - k_{deg}mRNA.$ Synthesis term depends on the logic gate $G(t)$.
3.1 Boolean logic gates: **AND gate**	AND gates  	$\mathrm{G}(t) = \frac{S_1^n}{K_1^n + S_1^n}\frac{S_2^n}{K_2^n + S_2^n}$	 	***AND* gates reflect synergistic (super-additive) behavior** • Modeled by the product of two or more Hill functions that represent the activity of a **species (S)** (transcription factors, kinases, etc.) • $\boldsymbol{K_1}$ **and** $\boldsymbol{K_2}$ are the dissociation constants reflecting each species' binding affinity to the target. • When both species concentrations are high, the output $G(t)$ approaches 1. • When either species concentration is low, the dissociation constant in the denominator dominates, and the output $G(t)$ becomes low.	**AND gates in gene regulation:** • TNF is transcribed by NFκB AND post-transcriptionally stabilied by MAPK-p38 activity (sequential AND gate). (Cheng et al., 2017)
3.2 Boolean logic gates: **OR gate**	OR gates  	$\mathrm{G}(t) = 1 - \frac{K_1^n}{K_1^n + S_1^n}\frac{K_2^n}{K_2^n + S_2^n}$	 	***OR* gates reflect redundant (sub-additive) behavior** • Intuition behind the *OR* gate equation is that the response to an activating species can be modeled by Hill function $\frac{S_1^n}{K_1^n+S_1^n}$, representing the probability that species S is bound. • The probability of not bound is $1 - \frac{S_1^n}{K_1^n+S_1^n}$, which can be rewritten as $\frac{K_1^n}{K_1^n+S_1^n}$. • In an *OR* gate, there is activity unless both species are not bound, or $1 - \frac{K_1^n}{K_1^n+S_1^n}\frac{K_2^n}{K_2^n+S_2^n}$.	**OR gates in gene regulation:** • NFκB and ISGF3 activity form a sequential OR gate, allowing sustained responses for a cluster of about 100 macrophage immune response genes (Cheng et al., 2017).

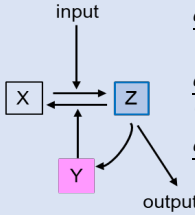
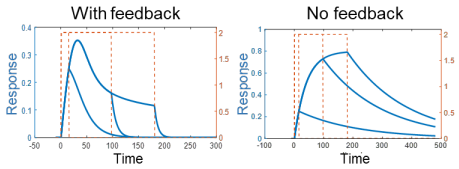
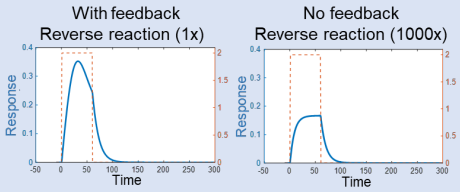
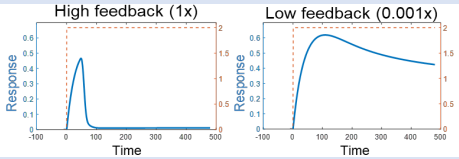
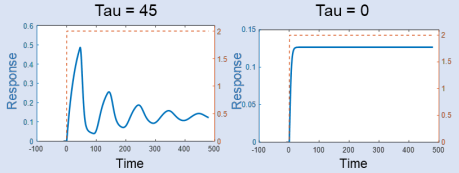
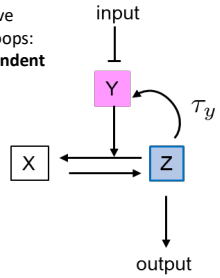
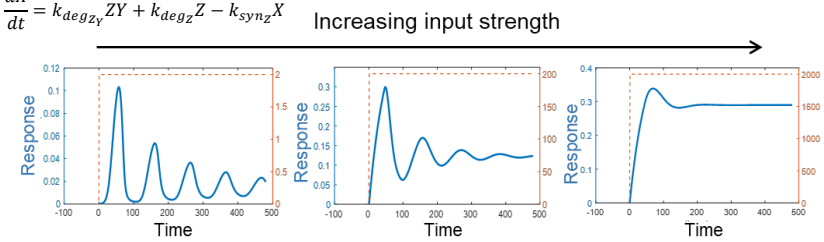
Regulatory motifs in innate immune signaling networks (3/5)

Motif	Diagram	Equation	Simulations	Description	Examples
4. Coherent feedforward loops (FFLs)	**Coherent FFL:**  **Direct activation:**  	$\frac{dY}{dt} = k_{syn_Y} f_x(t) - k_{deg_Y} Y$ $\frac{dZ}{dt} = k_{syn_Z} f_x(t) f_y(t) - k_{deg_Z} Z$ with activating Hill input functions for both $f_x(t)$ and $f_y(t)$: $f_x(t) = \frac{X(t)^n}{K^n + X(t)^n}$ $f_y(t) = \frac{Y(t)^n}{K^n + Y(t)^n}$	**Coherent feedforward loop:**  **Direct activation:**  	**Coherent feedforward loop (type 1) (duration decoder):** Occurs when a signal splits into two branches that then combine again through an *AND* gate. • X induces the production of Y, • Y then promotes the production of Z. • X also directly promotes the production of Z, but cannot activate Z alone, since it operates in an AND gate with Y. Thus, Y needs to be present in sufficient amounts for production of Z, resulting in a delay in production of Z. For direct activation, Z is produced under both long and short duration inputs. For the coherent feedforward motif, if X is transient, Z may not be produced, as the indirect path (via Y) may be delayed vis-à-vis the direct path. The motif is thus a duration decoder.	**Expression of *Il6:*** • Requires binding of both NFκB and NFκB-induced C/EPBδ (Litvak et al., 2009).
5. Incoherent FFLs	**Incoherent feedforward loop (type 1):** Occurs when a signal splits into two branches that then combine again through an *AND* gate. However, the indirect path functions to inhibit the positive effect of the direct path.				
5.1. Incoherent FFLs: **Pulse generator**	**Incoherent FFL:**  **Direct activation:**  	$\frac{dY}{dt} = k_{syn_Y} f_x(t) - k_{deg_Y} Y$ $\frac{dZ}{dt} = k_{syn_Z} \left(f_x(t) f_y(t)\right) - k_{deg_Z} Z$ $f_x(t) = \frac{X(t)^n}{K^n + X(t)^n}$ $f_y(t) = \frac{K^n}{K^n + Y(t)^n}$	**Incoherent feedforward loop:**  **Direct activation:**  	**Incoherent FFL:** • X promotes production of Y and Z, but production of Y represses the production of Z. • The action of X on Y and Z can be modeled with an activating Hill function for $f_x(t)$. • The action of Y on Z can be modeled with a repressive Hill function for $f_y(t)$ (rather than activating as in the coherent feedforward case). **Incoherent FFL (pulse generator):** • An incoherent FFL acts as a pulse generator for Z. • Initial production by X is dampened as the X-induced production of Y increases. • When X is high, increased production of Y dampens overproduction of Z.	**Controlling overproduction of inflammatory cytokines:** • NFκB-induced ATF3 represses NFκB-induced expression of IL6 and IL12B (Gilchrist et al., 2006).
5.2. Incoherent FFLs: **Fold Change Detector**			**Incoherent feedforward loop:**   **Direct activation:**   	**Incoherent FFL (fold change detector):** • If basal levels of Y are already high, then X's activation effect on Z is dampened, even when absolute abundance of X is high. • Whereas simple direct activation of Z by X is solely a function of the amplitude of X, the incoherent feedback motif is also a function of the basal level of X such that peak activity of Z is more proportional to the fold change in X.	**NFκB-induced transcription:** • NFκB induction of a transcriptionally incompetent competitor (p50:50 homodimer) inhibits NFκB-induced gene activation. • It thus provides memory of the cell's pre-stimulation state (Lee et al., 2014).

Regulatory motifs in innate immune signaling networks (4/5)

Motif	Diagram	Equation	Simulations	Description	Examples
6. Positive feedback loops		$\frac{dY}{dt} = k_{syn_Y}\frac{Z}{K+Z} - k_{deg_Y}Y$ $\frac{dZ}{dt} = k_{syn_Z}X\left(1 - \frac{K}{K+I(t)}\frac{K}{K+Y}\right) - k_{deg_Z}Z$ $\frac{dX}{dt} = k_{deg_Z}Z - k_{syn_Z}X\left(1 - \frac{K}{K+I(t)}\frac{K}{K+Y}\right)$		**Positive feedback motif:** • The Input signal produces Z, • Z then produces its positive feedback regulator Y, which continues to promote the production of Z even after the input signal has gone.	
6.1. Positive feedback loops: **Ultrasensitivity**				**Ultrasensitivity (a feature of positive feedback motifs):** • The Input signal produces Z; positive feedback loops reinforce the activity of the signal • The dose-response curve is ultrasensitive, or thresholded with a high Hill coefficient. • The strength of the positive feedback determines the threshold and steepness of the dose response curve.	**Stimulus-response:** • Positive feedback loops providing ultrasensitivity may filter out low-input noise, while triggering complete activation when a threshold is reached (Shinohara et al., 2014).
6.2. Positive feedback loops: **Bistability**				**Bistability (a feature of positive feedback motifs):** • In contrast to the toggle switch, which loses activity once the input signal is gone, positive feedback motifs allow for bistability. • The on-state remains stable, even after the incoming signal has terminated. • One can describe the system as having two different attractor steady states, as is illustrated below with the on-state being reached even if the signal is terminated after only partial activation has been achieved.	**Signaling by caspase 8/3**: • Cleaved and hence active caspase will trigger downstream effects, but also generate more active caspase. • This contributes to the irreversibility of programmed cell death decisions (Eissing et al, 2004).

Regulatory motifs in innate immune signaling networks (5/5)

Motif	Diagram	Equation	Simulation	Description	Examples
7. Negative feedback loops		$\frac{dY}{dt} = k_{syn_{Y_0}} + k_{syn_Y} Z(t-\tau) - k_{deg_{Y_2}} Y$ $\frac{dZ}{dt} = k_{syn_Z} I(t) X - k_{deg_{Z_Y}} ZY - k_{deg_Z} Z$ $\frac{dX}{dt} = k_{deg_{Z_Y}} ZY + k_{deg_Z} Z - k_{syn_Z} I(t) X$		• **Basic formulation:** an activating input signal converts X to Z. Z produces its own inhibitor Y. Y then promotes the transition of the active state Z back to an inactive form X. • This simple motif is capable of numerous functional responses that depend on the quantitative characteristics of the components and their connections (see below).	
7.1. Negative feedback loops: **Track input duration**				• Feedback may ensure that the output is only active so long as there is an input signal. • In response to transient inputs, Z will track the duration of the input. • When feedback is low or absent, it takes longer for Z to return to baseline.	SOCS proteins regulating IRF/ISGF3 responses (Liu et al., 2015).
7.2. Negative feedback loops: **Improve Responsiveness**				• In principle, a high reverse reaction rate will allow the effector Z to track the transient input, but the responsiveness of Z suffers. • Hence in scenarios where both the feedback and no-feedback systems are able to track transient input, the negative feedback allows improved responsiveness.	NFκB-IκBα (Hoffmann et al., 2002, Kearns et al., 2006).
7.3. Negative feedback loops: **Pulse generator**				• When Y's inducibility involves a delay and its half-life is long, the negative feedback loop may shut down responses to a persistent input after an initial pulse. • Hence it may function as a pulse generator.	NFκB negative feedback from delayed IκBδ expression (Shih et al., 2009).
7.4. Negative feedback loops: **Oscillation generator**				• When Y's half-life is short, the same motif may lead to oscillations in response to a persistent input as the transiently induced Y decays rapidly to allow for a second pulse of activity. • The robustness of the oscillations depends on the delay τ.	A highly active inhibitory enzyme such as MAPK phosphatase can mediate oscillatory activity of MAPKp38 (Tomida et al., 2015).
7.5. Negative feedback loops: **Input-dependent oscillation generator**		$\frac{dY}{dt} = k_{syn_{Y_0}} + k_{syn_Y} Z(t-\tau) - k_{deg_Y} I(t) Y - k_{deg_{Y_2}} Y$ $\frac{dZ}{dt} = k_{syn_Z} X - k_{deg_{Z_Y}} ZY - k_{deg_Z} Z$ $\frac{dX}{dt} = k_{deg_{Z_Y}} ZY + k_{deg_Z} Z - k_{syn_Z} X$		• **A subclass of negative feedback loops** involve the input signal-induced destruction of the negative feedback inhibitor. In this case, the inhibitor Y has a long half-life. • In the absence of the input signal, Y has a long half-life such that Z is maintained in a low basal state and is rapidly attenuated when the signal ceases. However, when the input is active, the inhibitor Y is quickly degraded repeatedly, thus creating robust oscillations. • In this motif the strength of the oscillations is a function of the strength of the input signal: When the input signal is too high, it may counteract the negative feedback inhibitor before this has built up to produce a trough of activity. Instead of oscillations, a non-oscillatory second phase may result.	**NFκB-IκBα negative feedback loop:** • The inactive state X (cytoplasmic NFκB) toggles to an active state Z (nuclear NFκB), which induces the production of Y with a delay. • When there is a persistent inhibitory input signal on Y (IκBα) by IKK, this generates oscillations under large enough values of τ. • However, when the input signal is high (in response to MyD88 pathways), then NFκB activity is largely non-oscillatory (Adelaja et al, 2021).