Phosphorylation Analysis Platform

“Phospho-Totum”

SOCIUM Inc.

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2. Basic Concept (Differentiation)
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5. A proposal for use

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We developed the original phosphorylation array for reproducing the phosphorylation state in the cell onto the glass, named "***Phospho-Totum***".

Patent Application No. JP6550571
Patent Application No. JP6270221 (US11238959)

Input Data

Many sample types are available

Cell line

biopsy

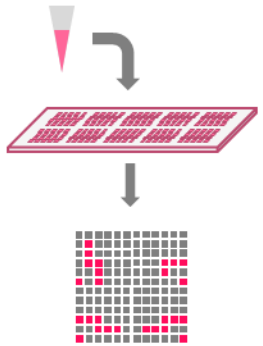
xenograft

Lysis

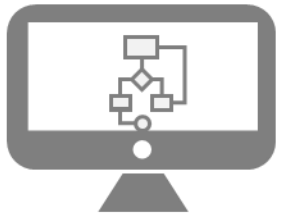
Phosphorylation Analysis

Only 1 month

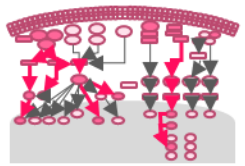
1 — 2 — 3



Kinase in lysis phosphorylate proteins on array



Our software enable to estimate phosphorylation with array data precisely



Whole picture of all phosphorylation pathway

Target of kinase inhibitors

Measure → Analyze → Ineterpret

Key Applications

Discover Research

- Pathway evaluation
- Compound MoA
- Target discovery
- Biochemical characterization
- Substrate identification

Biomarker and Clinical Research

- Classification Biomarker
- Prognostic Biomarker
- Therapy-predictive Biomarker
- Pharmacodynamic Biomarker

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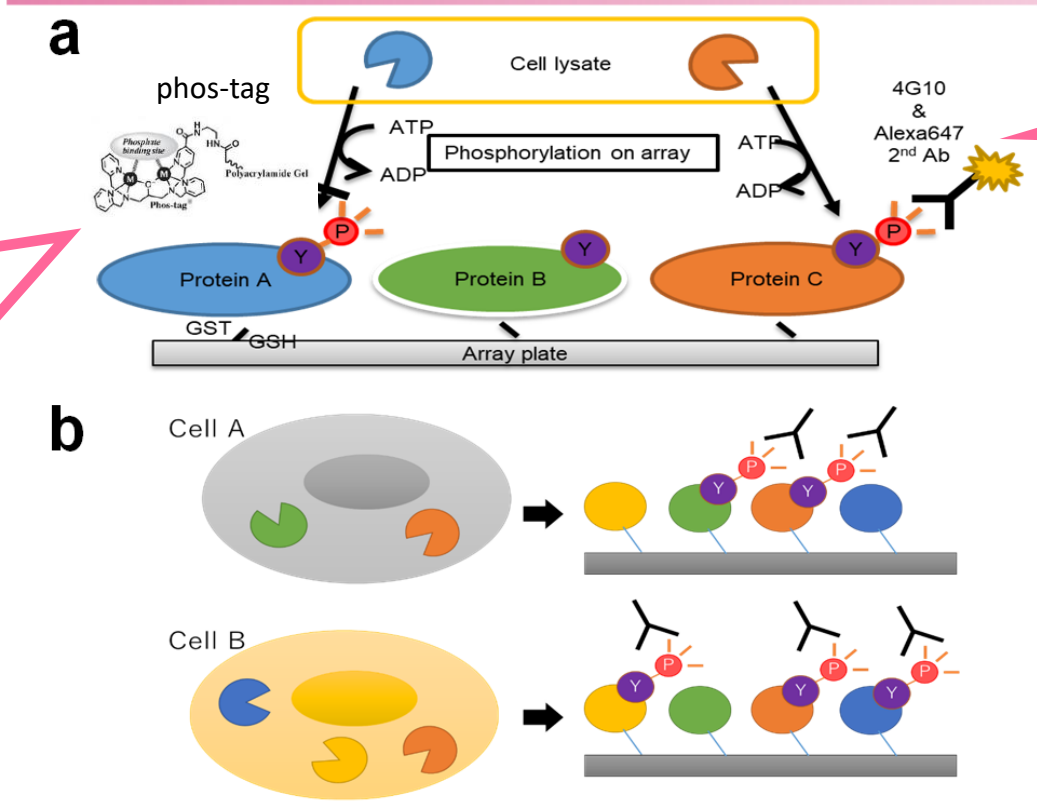
Phospho-Totum measures "activity" of kinases in cell lysates, not the amount of phosphorylated proteins.

For detecting the phosphorylation by Ser/Thr kinases and Tyrosine kinases as well (Updated in Aug. 2024)

Experimental procedures



HTP assay of phosphorylation upon **1492 proteins located on protein array**
a Kinase reaction and detection on protein active array
b Kinase activity profiling of different source or state of cell groups

For detecting the phosphorylation by Tyrosine kinases

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Differentiation of "***Phospho-Totum***" from Other modalities

	"Phospho-Totum."	other company's modalities
Composition	**Whole proteins** (kinases and their substrate proteins)	**Portion** (peptide or designated antibody molecule)
Object of measurement	**Protein**	**Residues** (part of protein)
Measurement Environment	**Cellular** environment (**many-to-many** kinases and target molecules)	**Artificial** environment (**1:1** kinase and target molecule)
Number of measurements	**One** time	**Multiple** times depending on the purpose
Estimated Target	**Activity**	**Amount**

◆ Measurement in a state closer to the intracellular environment than

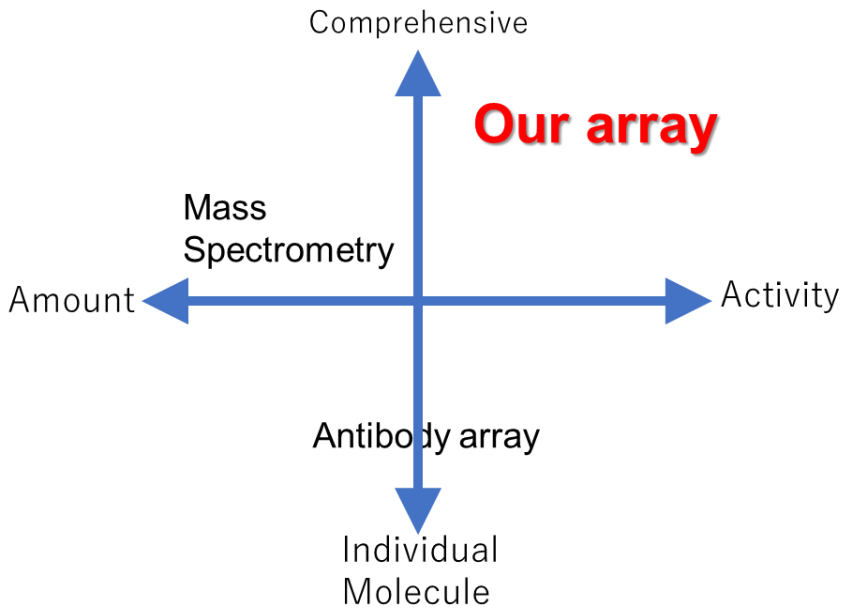
→

measurement of the phosphorylation of all proteins in cell lysate

◆ Extraction of a wealth of information from a single measurement

→

From the measurement data, it is possible to estimate the experimental condition-specific phosphorylated molecules, activation levels (kinase, pathway), etc.



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One-stop service for phosphorylation analysis

We can

① **simultaneously measure phosphorylation levels of 1492 proteins** that belong to 273 pathways and cover substrates of all 190 kinases coded on human genome.

② **estimate differentially phosphorylated proteins and their pathways**, from multiple perspectives.

③ **statistically estimate active (and inactive) pathways in 273 pathways**, based on the consistency between pathway connectivity and phosphorylation levels of constituent proteins

④ **quantitatively estimate activities of 190 kinases**, based on the knowledge of the relationship between kinases and their substrates

Catch the dynamical activity changes on kinases and pathways

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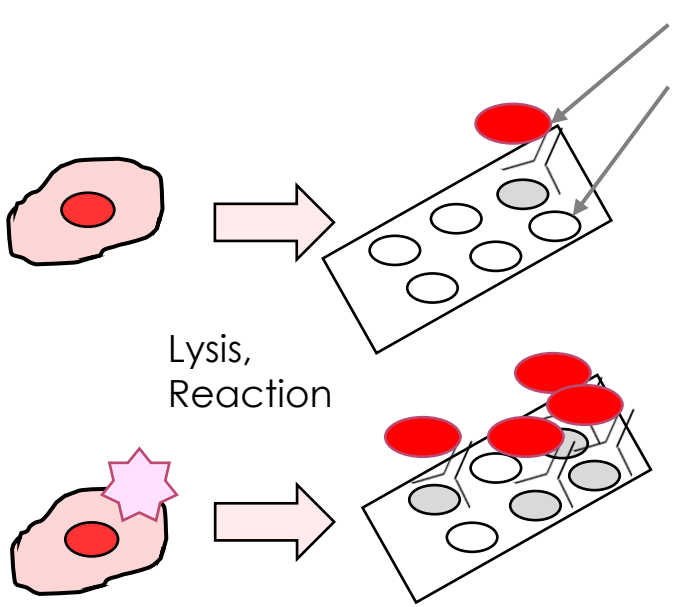
① Phosphorylation array focuses on a comprehensive phosphorylation of whole proteins, NOT of peptides or of residues in a protein.

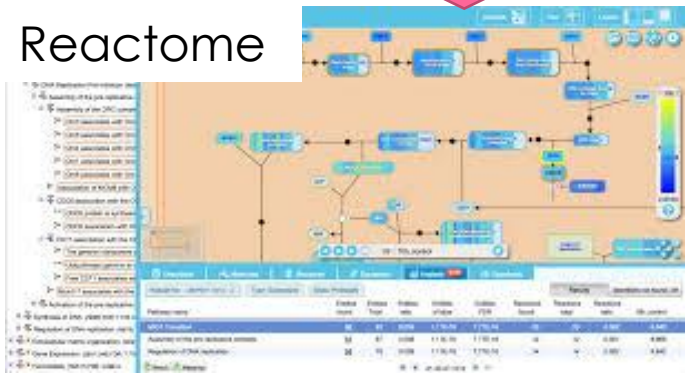
Design of Our Phosphorylation Array



Fluorescence conjugated Anti-pY antibody or phos-tag
GST-tagged recombinant protein

Kinds of Proteins ①
Tyrosine and Serine/Threonine Kinase: 190
Substrate (Y) : 816

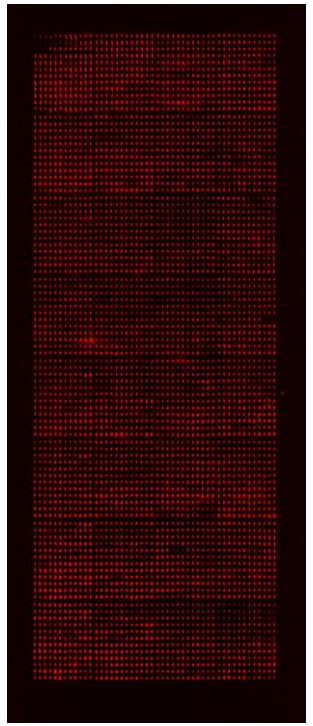
Reactome



Extraction of signaling pathway regulated by tyrosine phosphorylation. (273 pathway)

Kinds of Proteins ②
Proteins belonging to 273 pathways: 845

Not peptides but whole proteins on the glass



Anti-GST

Anti-pTyr or phos-tag

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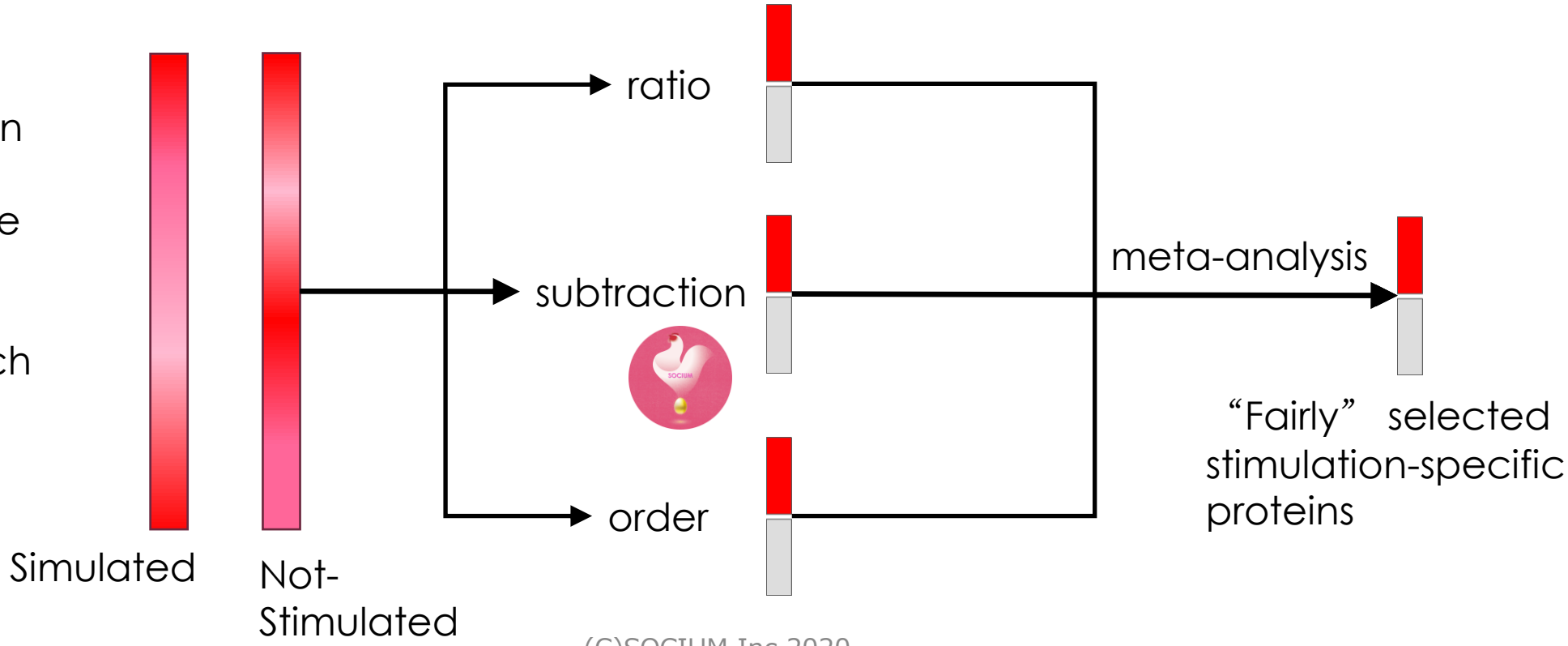
② We can estimate the difference between two groups in terms of ratio, subtraction, and rank of phosphorylation degrees between two compared groups, and unify the differences into one index by meta-analysis technique

[Analysis Policy]
The "difference" in the amount of phosphorylation is defined from **three perspectives**, and the results are **integrated**.
To detect differences "impartially," **analyst preferences for "differences" are eliminated** as much as possible.

Phosphorylation levels of 1492 proteins can be measured by our arrays for two groups such as simulated and not-simulated.



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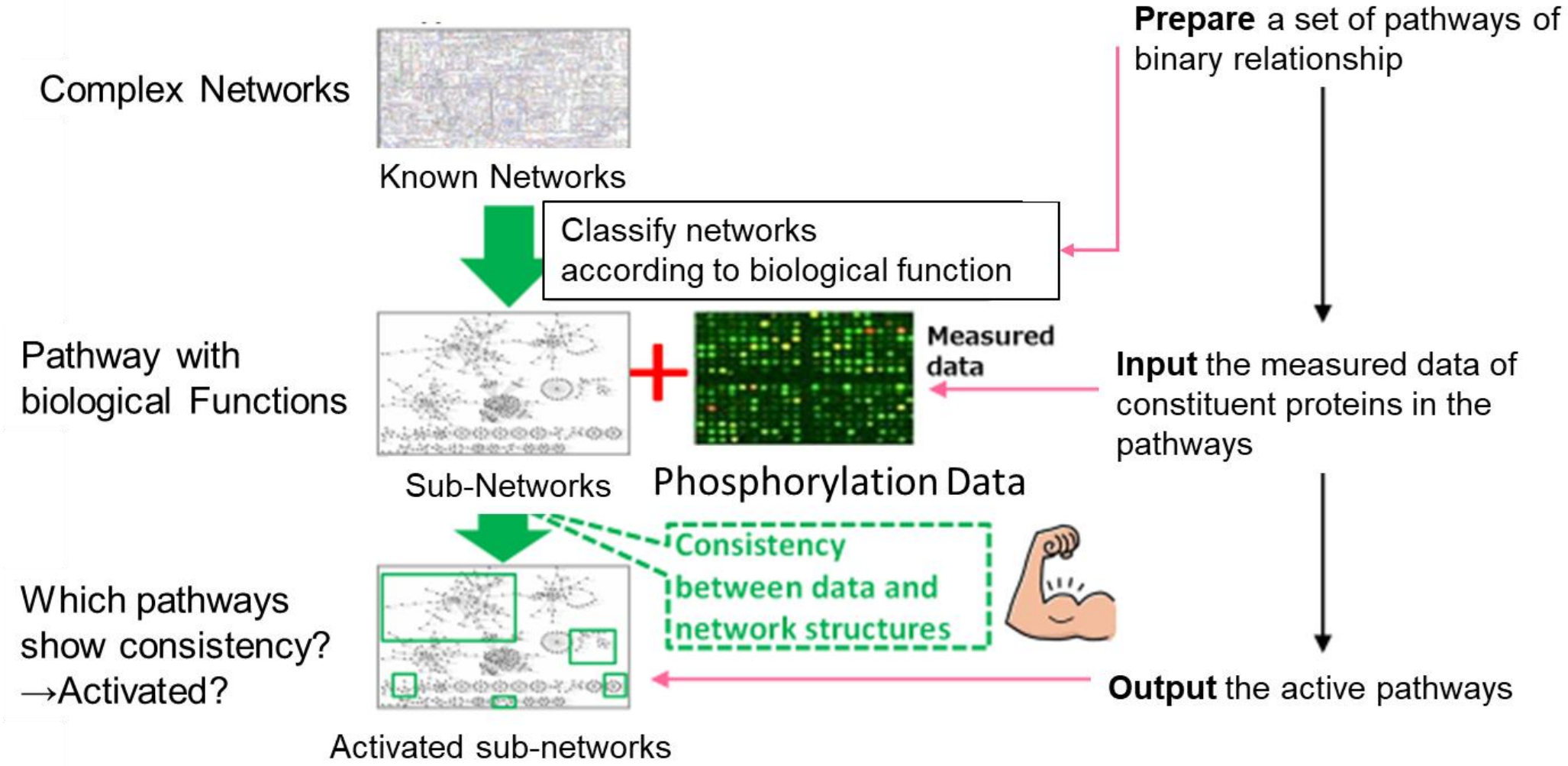
③ Identifying Activated/Inactivated Pathway

Saito, S., Aburatani, S. and Horimoto, K, BMC Sys. Biol. 2, 84, 2008.

What's "*Pathway Screening*" ?



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④ We can estimate the activity of kinases in sample based on phosphorylation levels of corresponding substrates on the array

Phosphorylation levels
of 816 substrates of
190 kinases

Activity of 185 kinases

Mathematical Ways

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In general, one kinase phosphorylates different substrates , and the information on the pairs of kinase and its substrate have accumulated in the database. Here, we assume that as a first approximation, the phosphorylation degrees of substrates are expressed by a linear combination of kinase activity as follows:

$$\begin{bmatrix} p_1 \\ p_2 \\ \vdots \\ p_s \end{bmatrix} = a_1 \begin{bmatrix} \delta_{11} \\ \delta_{12} \\ \vdots \\ \delta_{1s} \end{bmatrix} + a_2 \begin{bmatrix} \delta_{21} \\ \delta_{22} \\ \vdots \\ \delta_{2s} \end{bmatrix} + \cdots + a_k \begin{bmatrix} \delta_{k1} \\ \delta_{k2} \\ \vdots \\ \delta_{ks} \end{bmatrix} \quad (1)$$

Where p_i (i=1,2, ⋯, s) and a_j (j=1, 2, ⋯, k) are phosphorylation degree of i th substrate and phosphorylation activity of k th kinase, respectively, and

$$\delta_{ks} = \begin{cases} \frac{1}{n_s} & if\ protein\ s\ is\ a\ substrate\ of\ k\ th\ kinase, \\ & and\ n_s\ is\ a\ total\ number\ of\ subsurates \\ & of\ k\ th\ kinase \\ 0 & otherwise \end{cases}$$

In Eqn (1), the problem is attributed to solve a system of linear simultaneous equations for phosphorylation activity of kinases { a_j} from the measured phosphorylation degrees of substrates {p_i} and the information on kinase-substrate pairs {δ_{ks}}.

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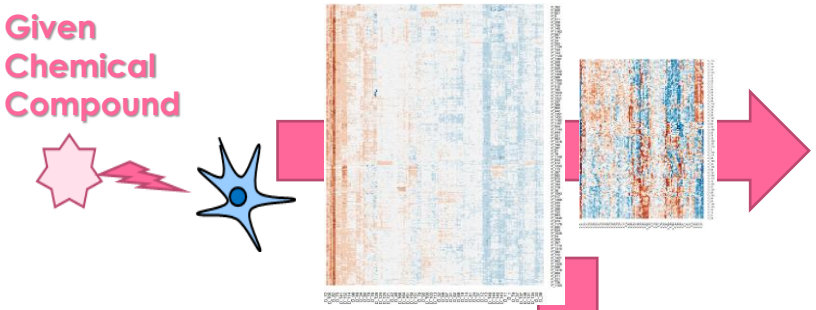
- Formulate the following relationship: [Substrate phosphorylation level]~[Kinase-substrate relationship] ⊗[Kinase activity]
- Numerical analysis of the above equation system
- Define the activity score based on the numerical values obtained from the above analysis

High-content analysis

Characterization of Chemical Compound

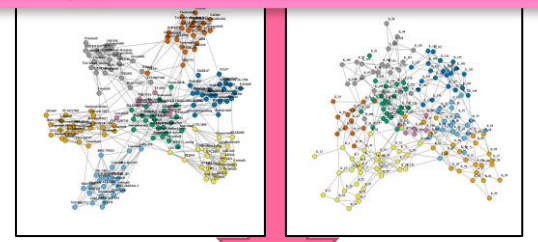
Data set of phosphorylation patterns of 167 commercialized TKI
(Phosphorylation pattern of 1471 proteins (left) and Activity patterns of 106 kinases (right)

Given Chemical Compound



Similar patterns of TKIs with given chemical compound

Phenotypic Netwrok Screening



High correlated TKI with given chemical compound

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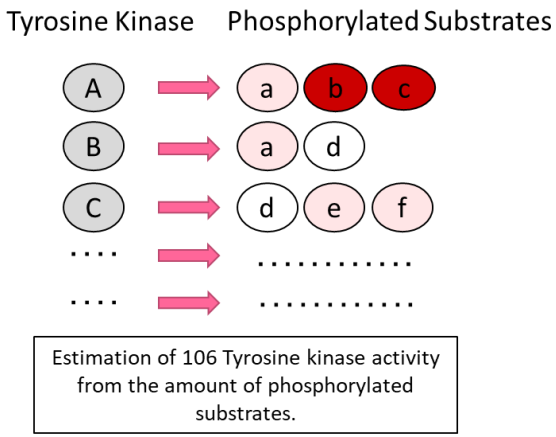
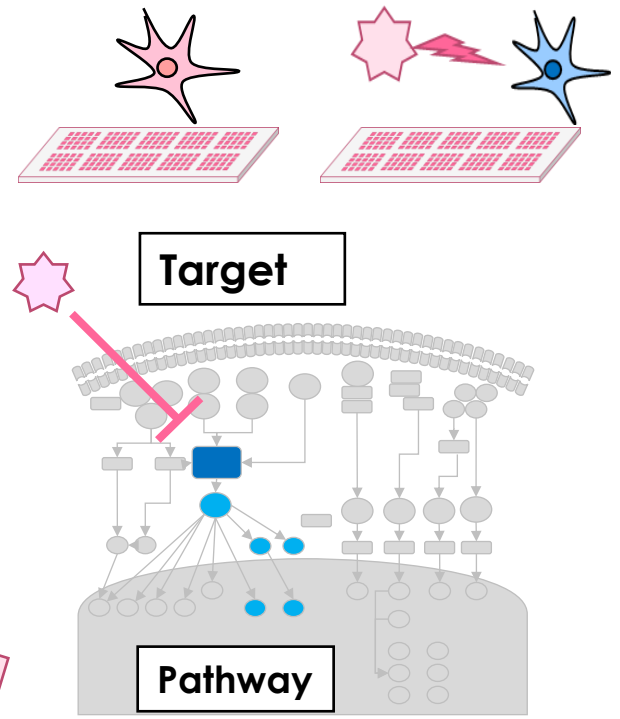
③&④ SOCIUM can identify the target and pathway of kinase inhibitor from two analytical methods.

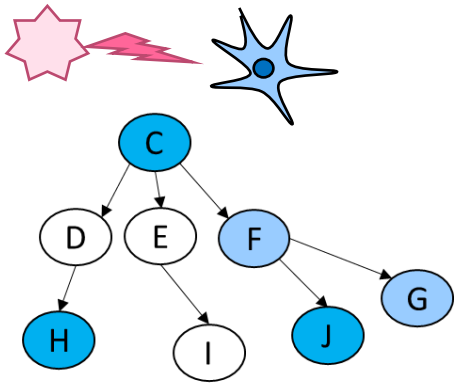
④ Analysis of Kinase Activities



Just 1 experiment ・・・

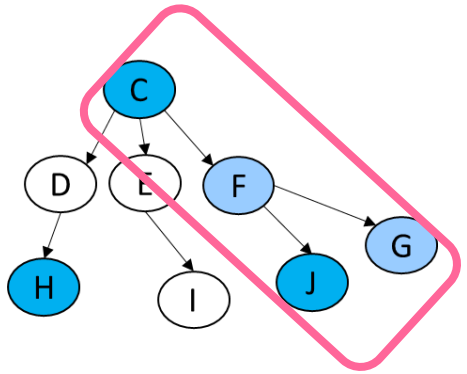


③ Analysis of Active Pathway



“Pathway Screening”

Inhibitory Pathway



Two analytical methods can be used in one experiment to infer targets and mechanism of actions.

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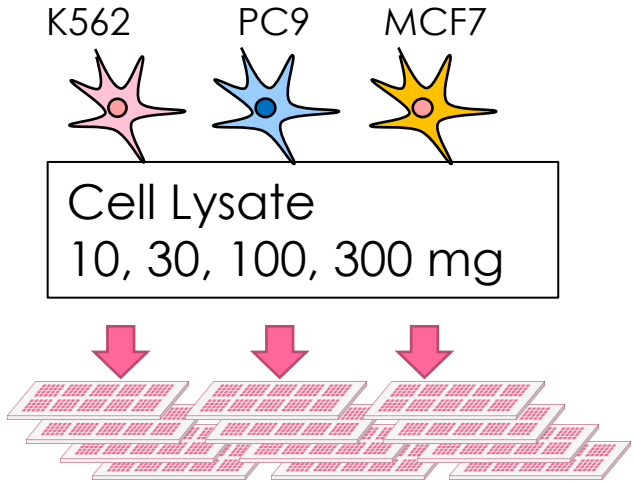
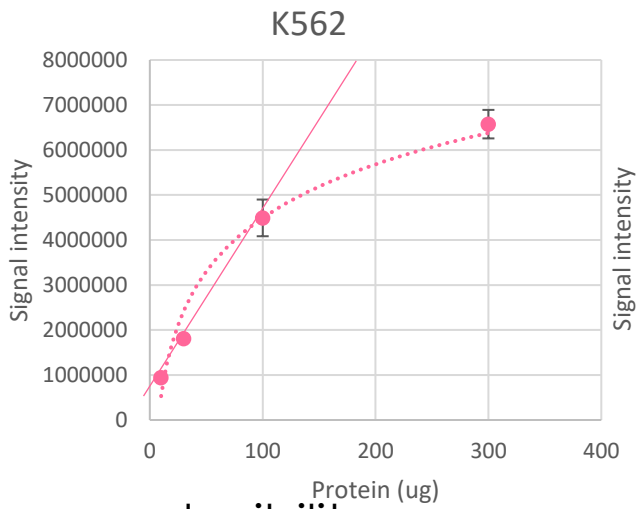
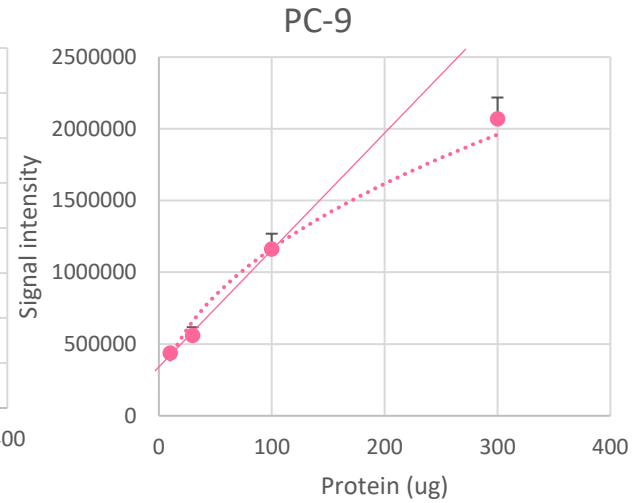
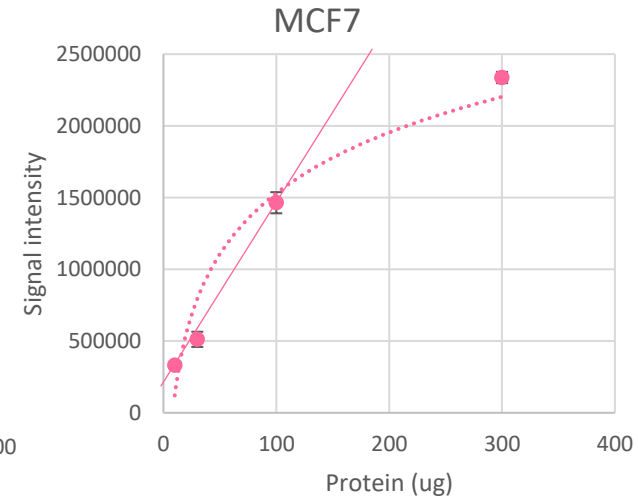
Phospho-Totum show high accuracy with small sample volumes.

Examination of required amount of protein from cell.



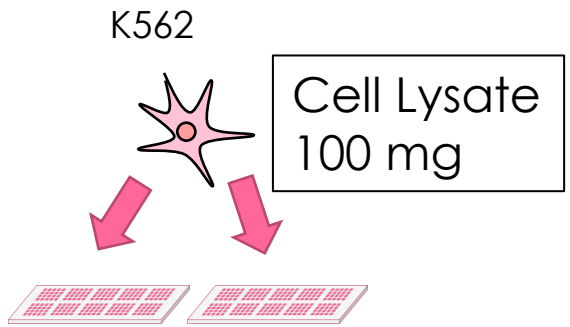




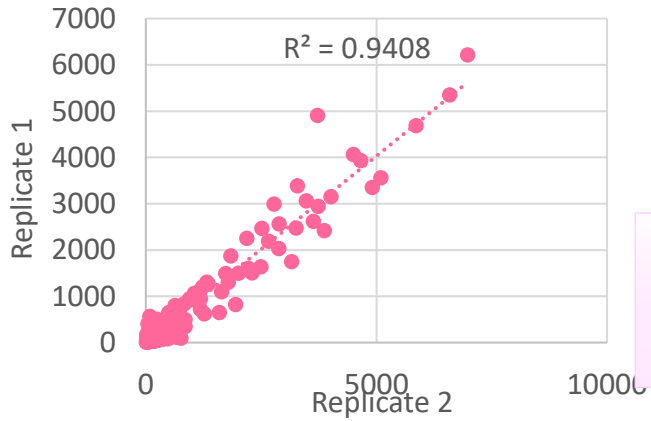


The total signal intensity increased linearly when reacting cell lysates up to 100 mg from 10 mg.

Examination of simultaneous reproducibility.



Use the same cell extract.



Phospho-Totum represented high simultaneous reproducibility (R^2=0.94).

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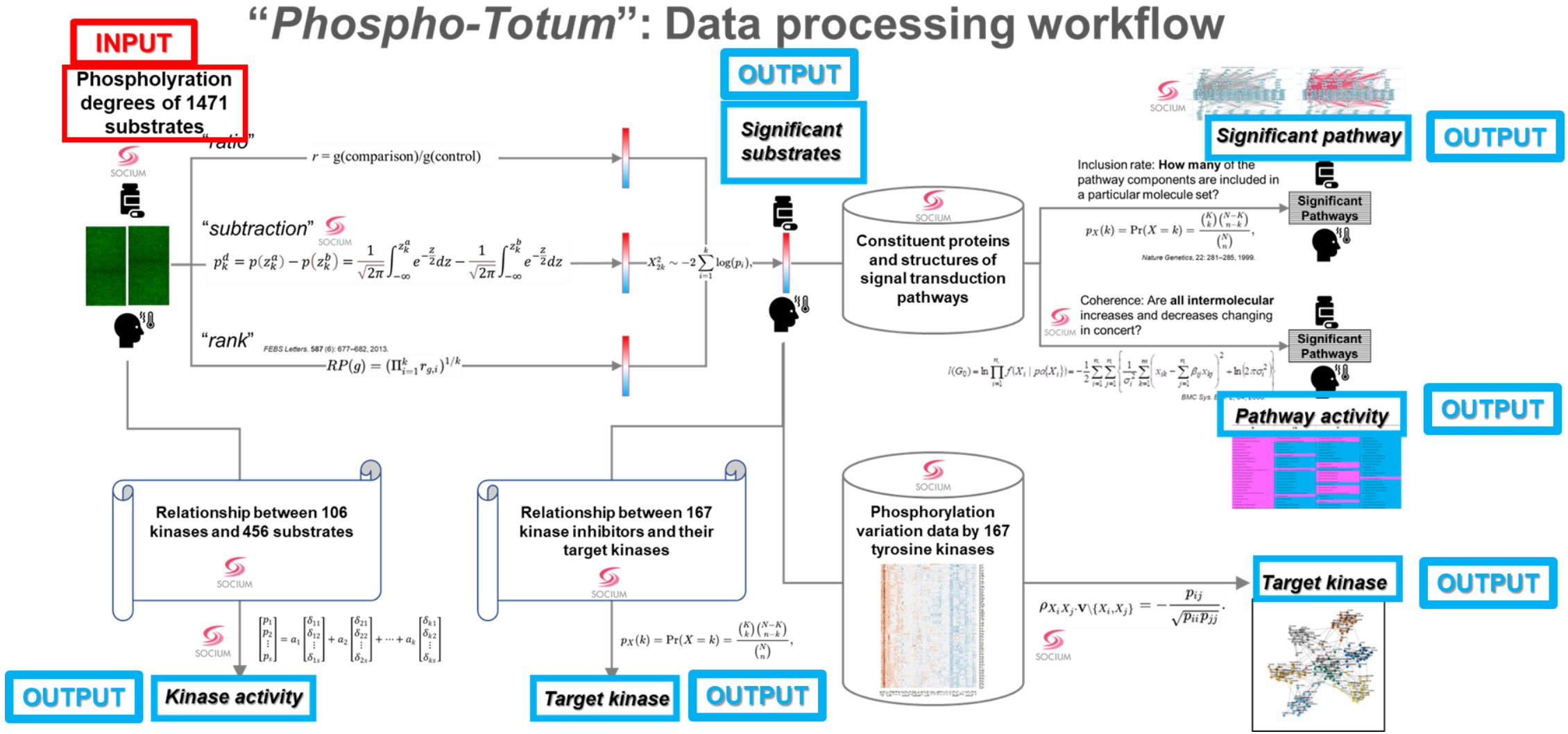
Overview of Computational System

Horimoto, K., Suyama, Y., Sasaki, T., Fukui, K., Sun, M., Feng, L., Tang, Y., Zhang, Y., Chen, D., Han F., The Journal of Biomedical Research. 38(3): 195-205, 2024



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Various application fields in "Phospho-Totum"

1. Examination of the time course of activated pathway after EGF treatment.
2. Identification of tyrosine kinases with changed activity by treatment with EGFR inhibitor.
3. Examination of the difference in activated pathway by treatment with 5 Bcr-Abl inhibitors.
4. Examination of TKI-resistance acquisition mechanism in lung cells
5. Target kinase identification of chemical compound
6. Elucidation of the mechanism of the synergistic effect of using a combination of two drugs.

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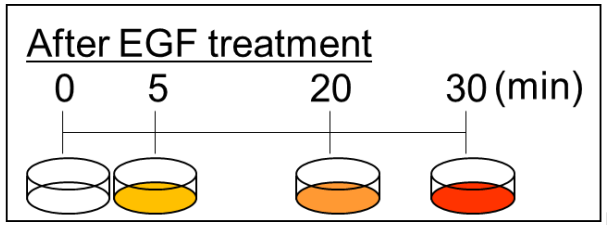
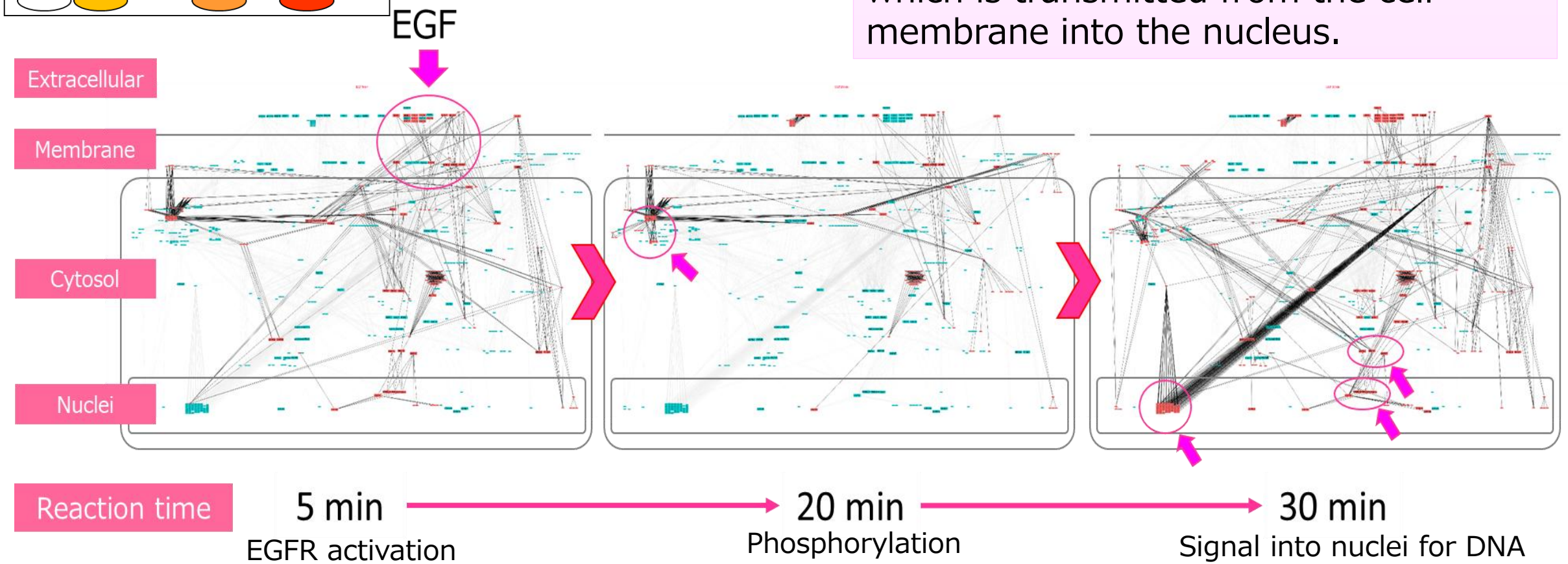
1 **Trace time course of activation pathway** after EGF treatment.

H. Kagiwada, T. Kiboku, H. Matsuo, M. Kitazawa, K. Fukui, K. Horimoto: Proteomics, 21(16):e2000251, 2021.



We were able to visualize the signal transduction flow after EGF treatment, which is transmitted from the cell membrane into the nucleus.



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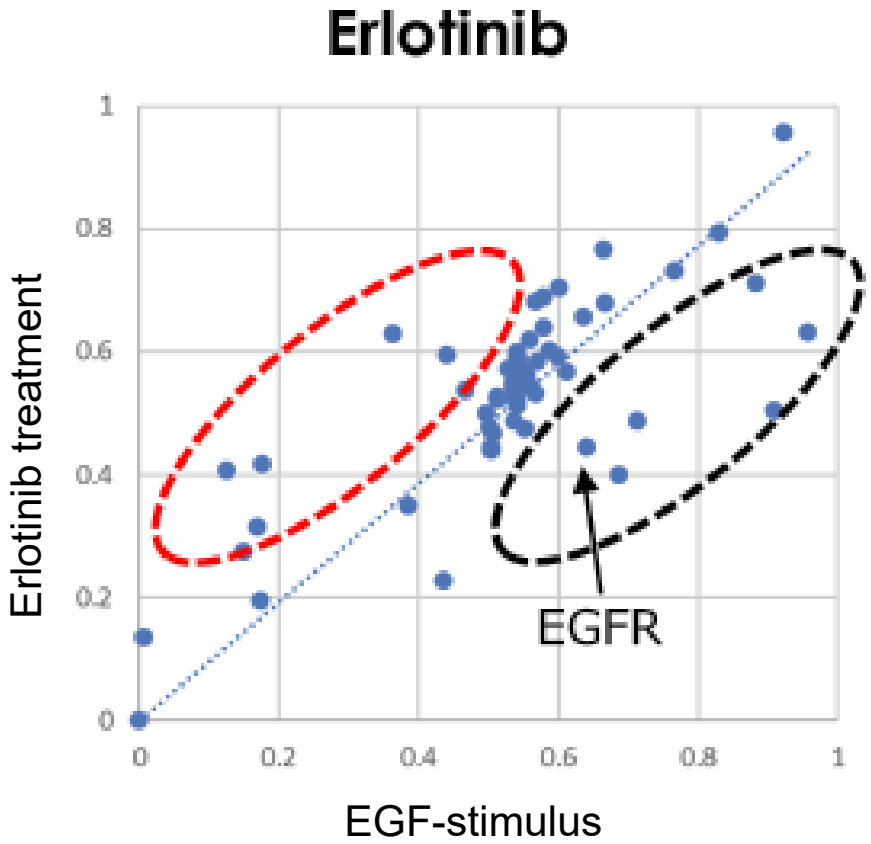
2 **Target/Off-target tyrosine kinase** by treatment with EGFR **inhibitor.**

Feng, L., Chen, X., Sheng, G., Li, Y., Li, Y., Zhang, Y., Yao, K., Wu, Z., Zhang, R., Kiboku, T. Kawasaki, A., Horimoto, K., Tang, Y., Sun, M., Han, F., Chen, D. Journal of Medicinal Chemistry (in press, Publication Date (Web):October 20, 2023)



EGF-stimulus: up
Erlotinib treatment : down ➡Erlotinib target kinase

EGF-stimulus: down ➡Bypass by Erlotinib treatment
Erlotinib treatment : up ➡Next drug target?

We were able to investigate the target and detour route of Erlotinib by estimating the degree of each tyrosine kinases activity.

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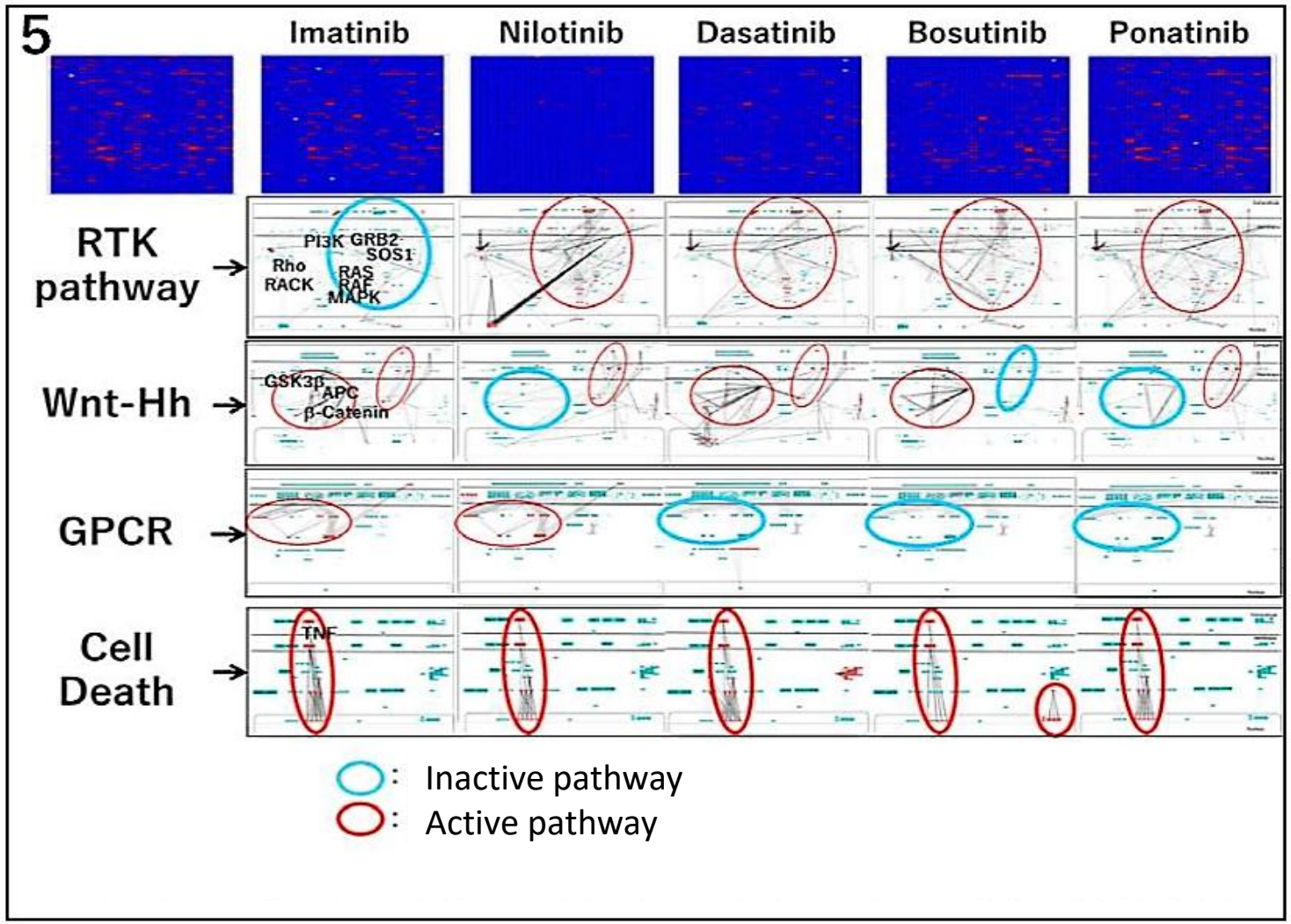
3 **A discrepancy in mechanism of action** among 5 Bcr-Abl **inhibitors.**

Purpose :
Identify difference in MoA among Bcr-Abl inhibitors

Sample :
Cell lysate after drug treated K562 cells

Difference of active pathways among Bcr-Abl inhibitors were detected.



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4 **Acquisition mechanism** of EGFR **inhibitor resistance.**

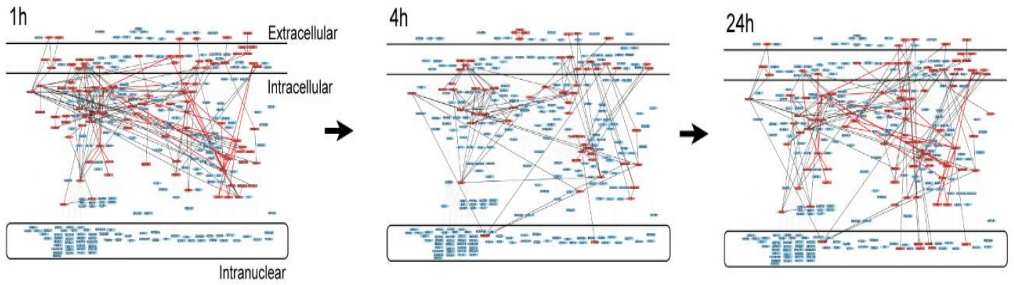
Keio University

MOLECULAR CANCER RESEARCH

IGF2 Autocrine–Mediated IGF1R Activation Is a Clinically Relevant Mechanism of Osimertinib Resistance in Lung Cancer

Tadashi Manabe et al. (2020) *Mol Cancer Res*. 18:549-559.

Investigation of active pathway by treatment with EGFR inhibitor using Phospho-Totum.



Identification of activated pathway in EGFR resistant cell using Phospho-Totum.

Pathway	PC9ER	PC9GR	PC9AZDR
VEGFR2-mediated vascular permeability B	0.47	0.77	0.43
GAB1 signalosome A	0.56	0.35	0.38
Phosphorylation and activation of VAV1	0.74	0.92	0.99
p-Y-IRS1 p-Y-IRS2 bind PI3K	0.32	0.55	0.06

Note: Pathway activity was estimated by "network screening." The thresholds of the probabilities were set to 0.3 in "network screening."

IGF1R bypass contributes the acquisition of EGFR inhibitor resistance

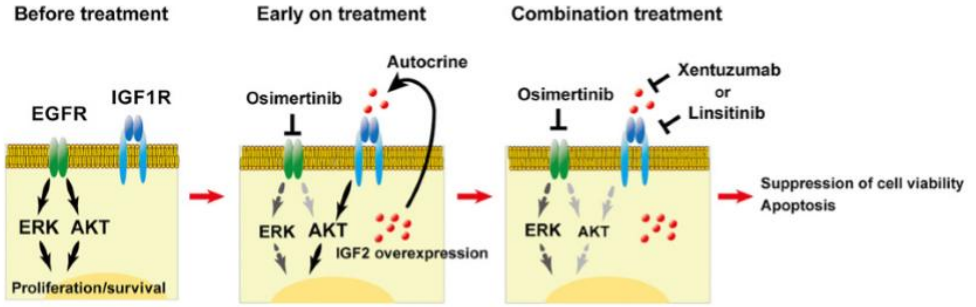


Figure 6.
Schematic representation of the resistant mechanism induced by IGF2 overexpression. Proposed model of IGF2 overexpression inducing an IGF1R bypass and contributing to the acquired resistance to osimertinib.

Using Phospho-Totum, we could provide the data to help elucidate the mechanism of acquisition of EGFR inhibitor resistance.

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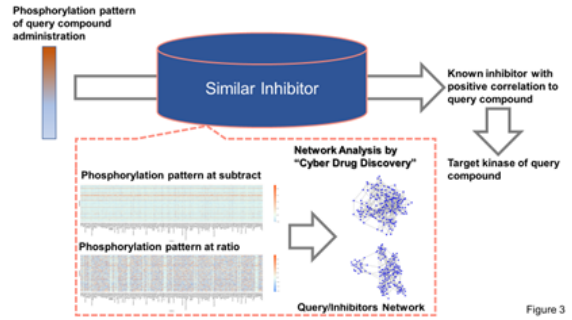
5 **Target kinase identification** of chemical compound

Journal of Medicinal Chemistry 66(21): 14609-14622, 2023
Journal of Biomedical Research. 38(3): 195-205, 2024

Application of Network Analysis Approach for Drug Discovery to Target Identification

Known (Dasatinib) case

✓ **Learning by network inference**



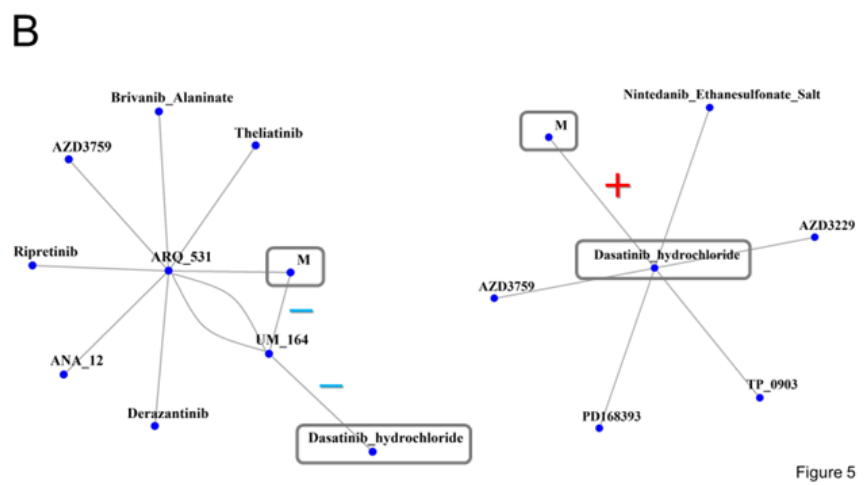
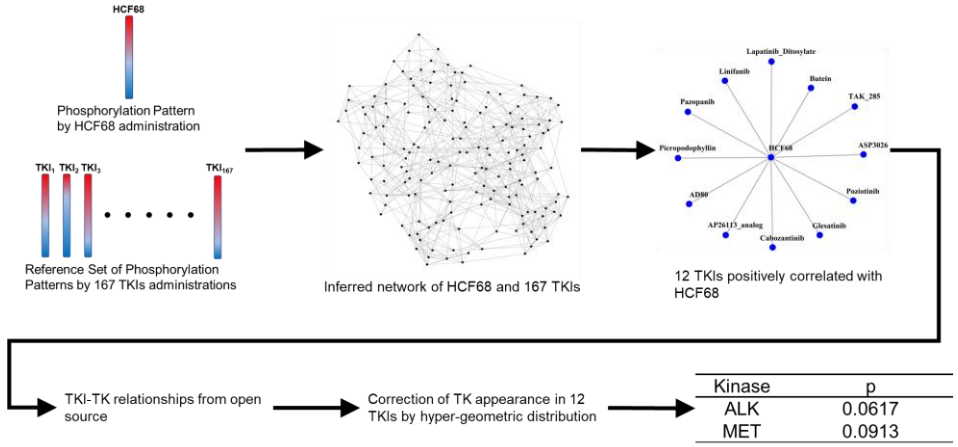


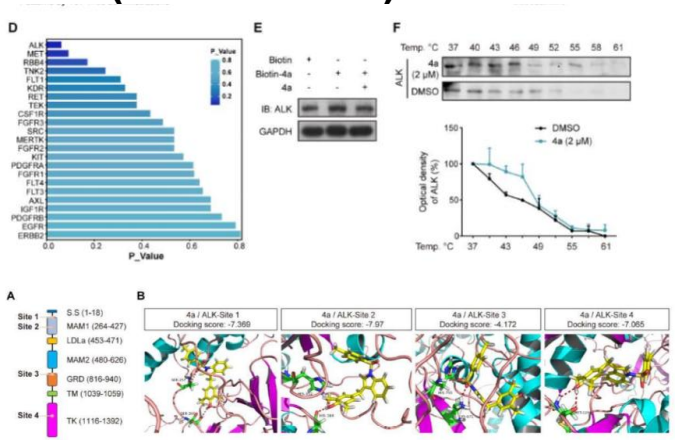
Figure 5

Target-Unknown case

(Prediction)



(Verification)

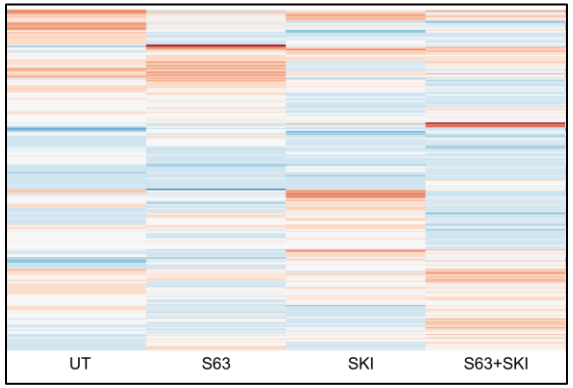


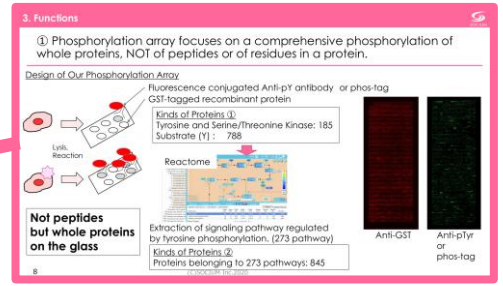
6. Elucidation of the mechanism of the synergistic effect of using a combination of two drugs.

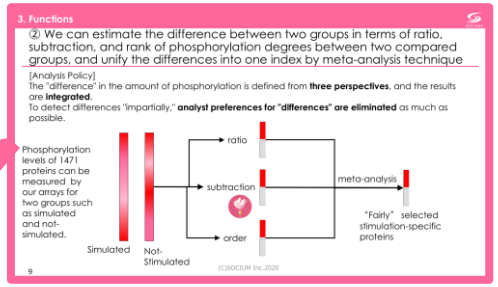
Signal transduction and Target Therapy, 10, 50 (2025).

Detecting differences in mechanisms between a single drug and a combination of two drug administrations.

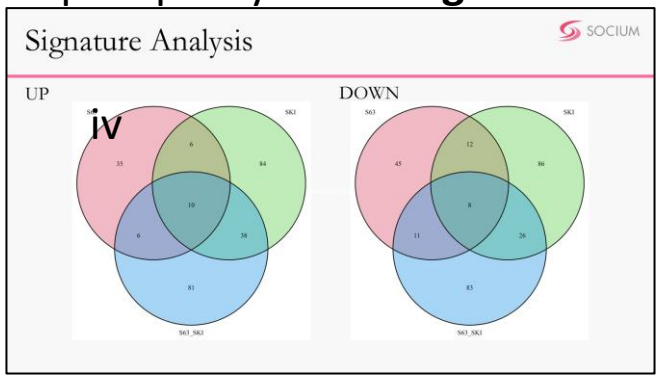
Comprehensive measurement of **phosphorylation degrees**



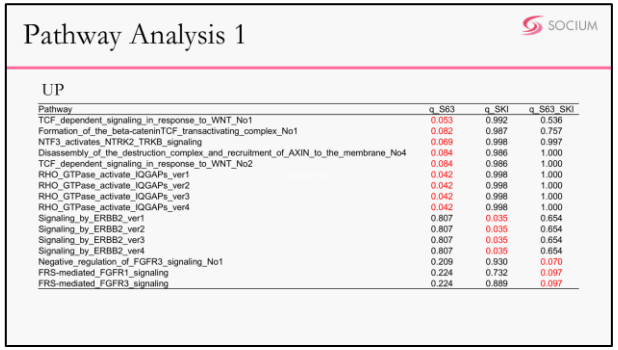




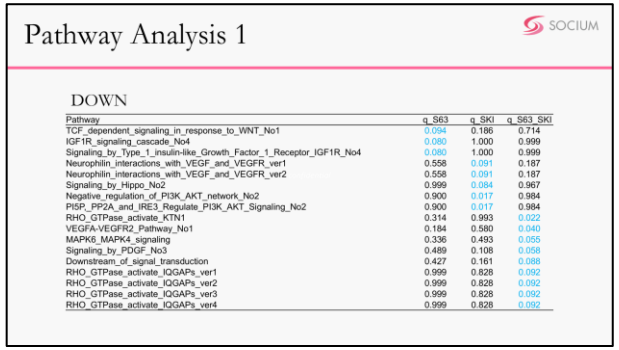
Changes of phosphorylation **degrees of substrates**



Changes of phosphorylation **degrees of pathways**



Pathway	q_S63	q_SKI	q_S63_SKI
TCF_dependent_signaling_in_response_to_WNT_No1	0.053	0.992	0.536
Formation_of_the_beta-cateninTCF_transactivating_complex_No1	0.082	0.987	0.757
NTF3_activates_NTRK2_TRKB_signaling	0.069	0.998	0.997
Disassembly_of_the_destruction_complex_and_recruitment_of_AXIN_to_the_membrane_No4	0.084	0.986	1.000
TCF_dependent_signaling_in_response_to_WNT_No2	0.084	0.986	1.000
RHO_GTPase_activate_IQGAPs_ver1	0.042	0.998	1.000
RHO_GTPase_activate_IQGAPs_ver2	0.042	0.998	1.000
RHO_GTPase_activate_IQGAPs_ver3	0.042	0.998	1.000
RHO_GTPase_activate_IQGAPs_ver4	0.042	0.998	1.000
Signaling_by_ERBB2_ver1	0.807	0.035	0.654
Signaling_by_ERBB2_ver2	0.807	0.035	0.654
Signaling_by_ERBB2_ver3	0.807	0.035	0.654
Signaling_by_ERBB2_ver4	0.807	0.035	0.654
Negative_regulation_of_FGFR3_signaling_No1	0.209	0.930	0.070
FRS-mediated_FGFR1_signaling	0.224	0.732	0.097
FRS-mediated_FGFR3_signaling	0.224	0.889	0.097



Pathway	q_S63	q_SKI	q_S63_SKI
TCF_dependent_signaling_in_response_to_WNT_No1	0.094	0.186	0.714
IGF1R_signaling_cascade_No4	0.080	1.000	0.999
Signaling_by_Type_1_insulin-like_Growth_Factor_1_Receptor_IGF1R_No4	0.080	1.000	0.999
Neurophilin_interactions_with_VEGF_and_VEGFR_ver1	0.558	0.091	0.187
Neurophilin_interactions_with_VEGF_and_VEGFR_ver2	0.558	0.091	0.187
Signaling_by_Hippo_No2	0.999	0.084	0.967
Negative_regulation_of_PI3K_AKT_network_No2	0.900	0.017	0.984
PI5P_PP2A_and_IRE3_Regulate_PI3K_AKT_Signaling_No2	0.900	0.017	0.984
RHO_GTPase_activate_KTN1	0.314	0.993	0.022
VEGFA-VEGFR2_Pathway_No1	0.184	0.580	0.040
MAPK6_MAPK4_signaling	0.336	0.493	0.055
Signaling_by_PDGF_No3	0.489	0.108	0.058
Downstream_of_signal_transduction	0.427	0.161	0.088
RHO_GTPase_activate_IQGAPs_ver1	0.999	0.828	0.092
RHO_GTPase_activate_IQGAPs_ver2	0.999	0.828	0.092
RHO_GTPase_activate_IQGAPs_ver3	0.999	0.828	0.092
RHO_GTPase_activate_IQGAPs_ver4	0.999	0.828	0.092

6. Elucidation of the mechanism of the synergistic effect of using a combination of two drugs.

Signal transduction and Target Therapy, 10, 50 (2025).

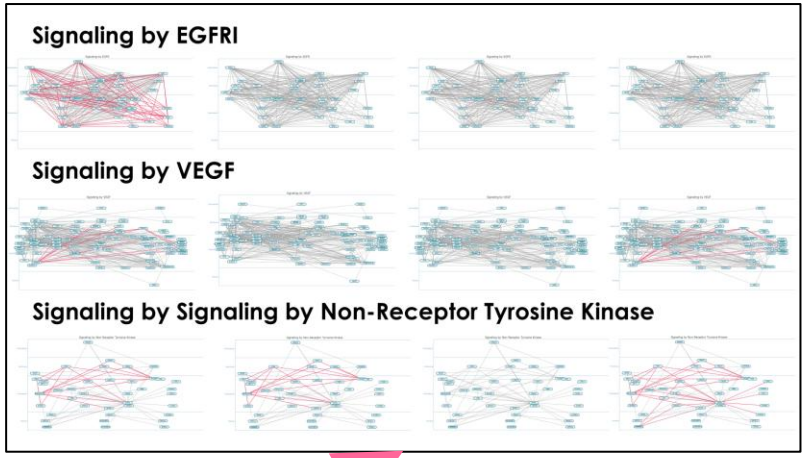
Detecting differences in mechanisms between a single drug and a combination of two drug administrations.

Changes in **pathway activity** (numerical)

Pathway Analysis 2

Pathway	Category	p_Control	p_S63	p_SKI	p_S63_SKI
NRAGE_signals_death_through_JNK_No1	Deth Receptor Signaling	0.47	0.68	0.1	0.1
GRB2_SOS_provides_linkage_to_MAPK_signaling_for_Integrins_ver1	Integrin signaling	0.5	0.98	0.01	0.03
CaMK_IV-mediated_phosphorylation_of_CREB_No2	Intracellular signaling by second messengers	0.44	0.25	0.29	0.13
Cam-PDE1_activation	Intracellular signaling by second messengers	0.2	0.25	0.36	0.01
Negative_regulation_of_MAPK_pathway_No3	MAPK Family signaling Cascades	0.07	0.92	0.79	0.01
IRS_Activation	Signaling by Insulin receptor	0.59	0.88	0.02	0.13
PTK6_Actives_STAT3	Signaling by Non-Receptor Tyrosine Kinase	0.58	0.52	0.81	0.12
PTK6_Regulates_Cell_Cycle	Signaling by Non-Receptor Tyrosine Kinase	1	0.8	0.64	0.17
PTK6_Regulates_RHO_GTPases,_RAS_GTPase_and_MAP_kinases	Signaling by Non-Receptor Tyrosine Kinase	0.07	0.01	0.5	0.01
Downstream_of_signal_transduction	Signaling by PDGF	0.01	0.05	0.01	0.01
Signaling_by_PDGF_No3	Signaling by PDGF	0.43	0.09	0.09	0.02
VEGFA-VEGFR2_Pathway_No5	Signaling by VEGF	0.13	0.67	0.43	0.01
VEGFR2_mediated_cell_proliferation_No2	Signaling by VEGF	0.09	0.65	0.38	0.03
Binding_of_TCF_LEF_CTNNB1_to_target_gene_promoters_No1	Signaling by WNT	0.95	0.15	0.82	0.16
Negative_regulation_of_TCF-dependent_signaling_by_DVL-interacting_proteins_ver1	Signaling by WNT	0.48	0.79	0.5	0.12
FasL_CD95L_signaling	Deth Receptor Signaling	0.1	0.68	0.01	0.84
Signaing_by_Leptin	Signaling by Leptin	0.29	0.37	0.08	0.28
GAB1_signalosome	Signaling by EGFR	0.02	1	0.62	0.78
MET_activates_PTPN11_ver1	Signaling by MET	0.01	0.96	0.99	1
ERK_MAPK_targets_No2	Signaling by NTRKs	0.11	0.01	0.52	1
Disassembly_of_the_destruction_complex_and_recruitment_of_AXIN_to_the_membrane_No2	Signaling by WNT	0.29	1	0.1	0.99
Disassembly_of_the_destruction_complex_and_recruitment_of_AXIN_to_the_membrane_No5	Signaling by WNT	0.17	0.98	0.01	0.38
TCF_dependent_signaling_in_response_to_WNT_No2	Signaling by WNT	0.17	1	0.17	0.97
TCF_dependent_signaling_in_response_to_WNT_No5	Signaling by WNT	0.17	0.97	0.02	0.3
RHO_GTPase_activate_CFTRtrafficking	Signalingby Rho GTPases	0.68	0.01	0.01	0.63
RHO_GTPase_activate_IQGAPs_ver2	Signalingby Rho GTPases	0.92	1	0.19	0.93
RHO_GTPase_activate_IQGAPs_ver3	Signalingby Rho GTPases	0.92	1	0.17	0.95

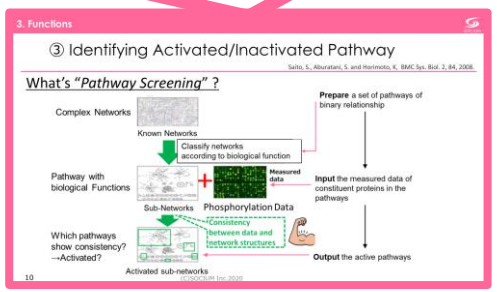
Changes in **pathway activity** (visualization)

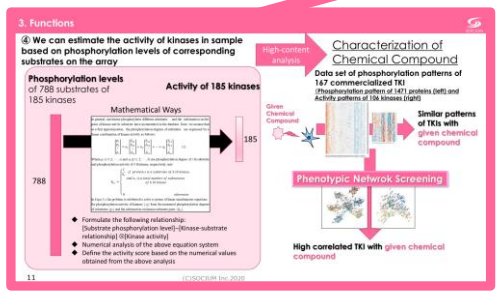


Changes in **kinase activity**

Kinase Activity

kinase	DOWN S63 vs. S63&SKI	DOWN SKI vs. S63&SKI	kinase	UP S63 vs. S63&SKI	UP SKI vs. S63&SKI
FER	① 0.018	② 0.003	LIMK1	③ 0.045	④ 0.026
MAPKAPK5	0.020	0.031	MAPK9	0.023	0.008
PIK3CA	0.283	0.002	CHEK1	0.015	0.007
AURKB	0.264	0.015	ABL2	0.143	0.041
CSNK1A1	0.464	0.024	RPS6KA3	0.127	0.039
CSNK1G2	0.736	0.036	KDR	0.675	0.037
PRKCQ	0.070	0.039	MTOR	0.668	0.034
PBK	0.023	0.115	ERBB2	0.439	0.033
CDK1	0.040	0.140	HIPK2	0.085	0.015
PAK2	0.043	0.150	IKBKE	0.089	0.011
MATK	0.018	0.182	MAP2K2	0.189	0.005
AKT2	0.042	0.202	AXL	0.073	0.001
RET	0.026	0.278	PRKCE	0.046	0.800
MAP2K1	0.049	0.281	RPS6KA5	0.019	0.310
IRAK1	0.021	0.363	RPS6KB2	0.045	0.252
MAP3K14	0.022	0.379	LIMK2	0.013	0.231
BMX	0.037	0.749	CAMK2D	0.002	0.218
			PLK1	0.013	0.151





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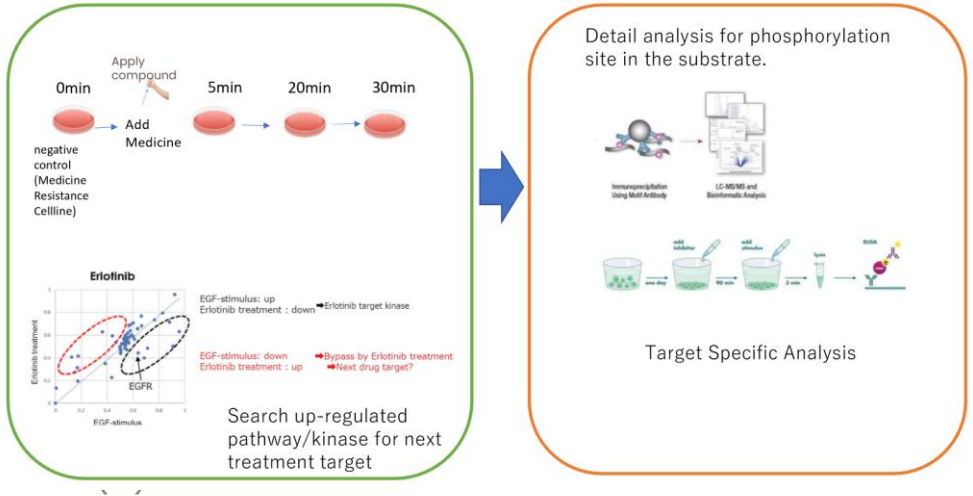
First, predict the whole from the overall picture, and then check the details later.

Example 1) Developing Kinase Drug & Elucidating Resistance Mechanisms

	SOCIUM	other companies
Evaluate **Kinase Activity (or inhibitory effects)**	kinase activity estimation	experiment
Decipher the **Compound's Mechanism of Action**	pathway activity estimation	experiment
Kinase Selectivity Profiling (or off-target effects)	substrate measurement & kinase and pathway activity estimation	experiment
Target Engagement and Functionality **inside Living Cells**	computational prediction including the network analysis	combination of experiments
Further work such as **preclinical testing**	X	brute force (?)

"First, predict the whole"

✓ **One experiment and computational predictions**



"check the details later."

✓ **Various experiments**

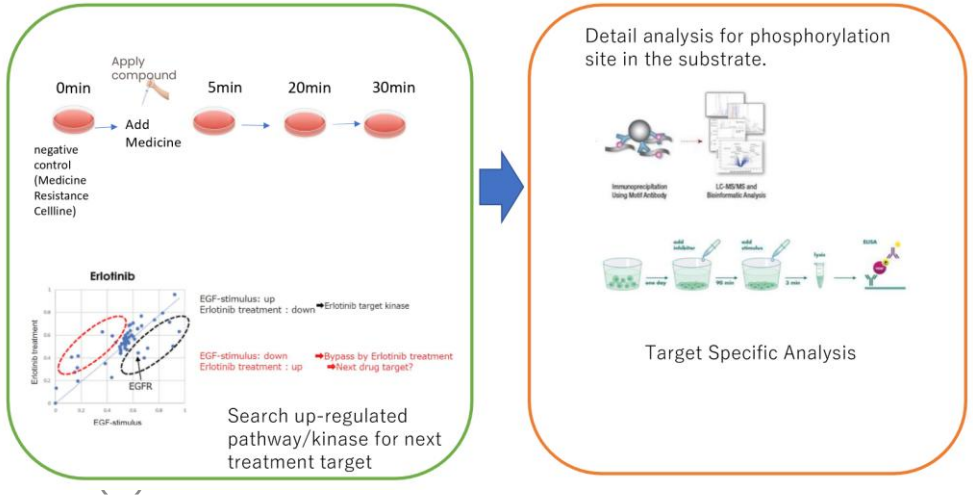
First, predict the whole from the overall picture, and then check the details later.

Example 2) Quantitively estimating the phosphatase effects on the phosphorylation state

	Cell Lysate *phosphatase inhibitors*	Phosphorylation Reaction *phosphatase inhibitors*	
DMSO	○	○	catch the **phosphorylation activity**
Condition 1	○	○	
DMSO	×	×	catch the **phosphatase effects**
Condition 1	×	×	

"First, predict the whole"

✓ **One experiment and computational predictions**



"check the details later."

✓ **Various experiments**

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