Flow Cytometry and Molecular Medicine

David W Hedley MD
Division of Medical Oncology and Haematology,
Princess Margaret Hospital
Senior Scientist, Ontario Cancer Institute
University of Toronto, Canada

Molecular medicine

- Understanding mechanisms of disease as alterations in molecular processes that occur inside the cell
- Many of these processes are candidates for drug treatment
- Flow cytometry has unique capabilities:
  - identify subpopulations based on light scatter and surface markers
  - large numbers of antibodies available to label key intracellular markers
- We discuss sample preparation in our lab session

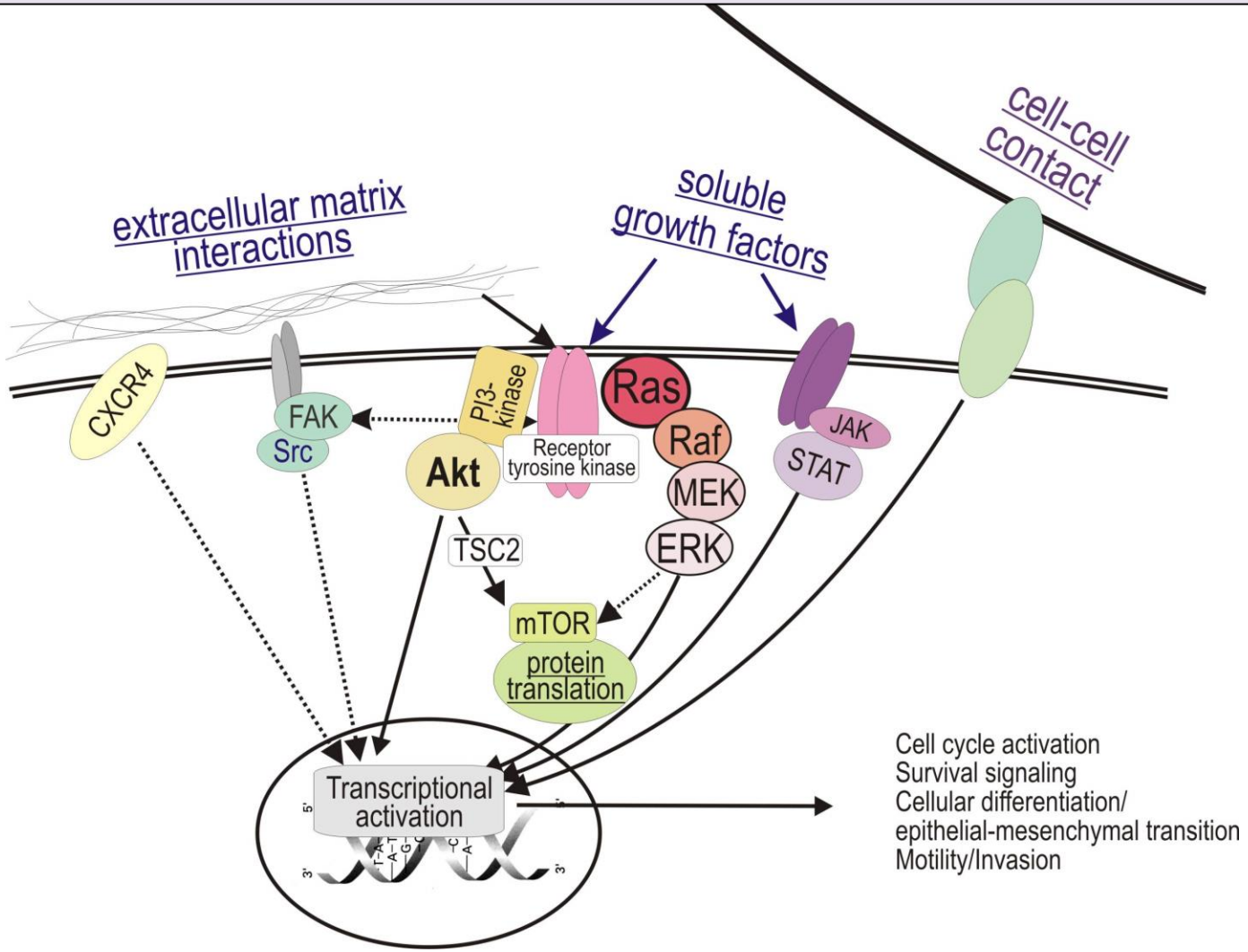
Scope of flow cytometry in molecular medicine

- Cell cycle regulation
- Signal transduction
- DNA damage responses and repair
- Cellular differentiation and stem cell biology
- Metabolic regulation
- Antioxidants and xenobiotic detoxification
- Epigenetic regulation of genes

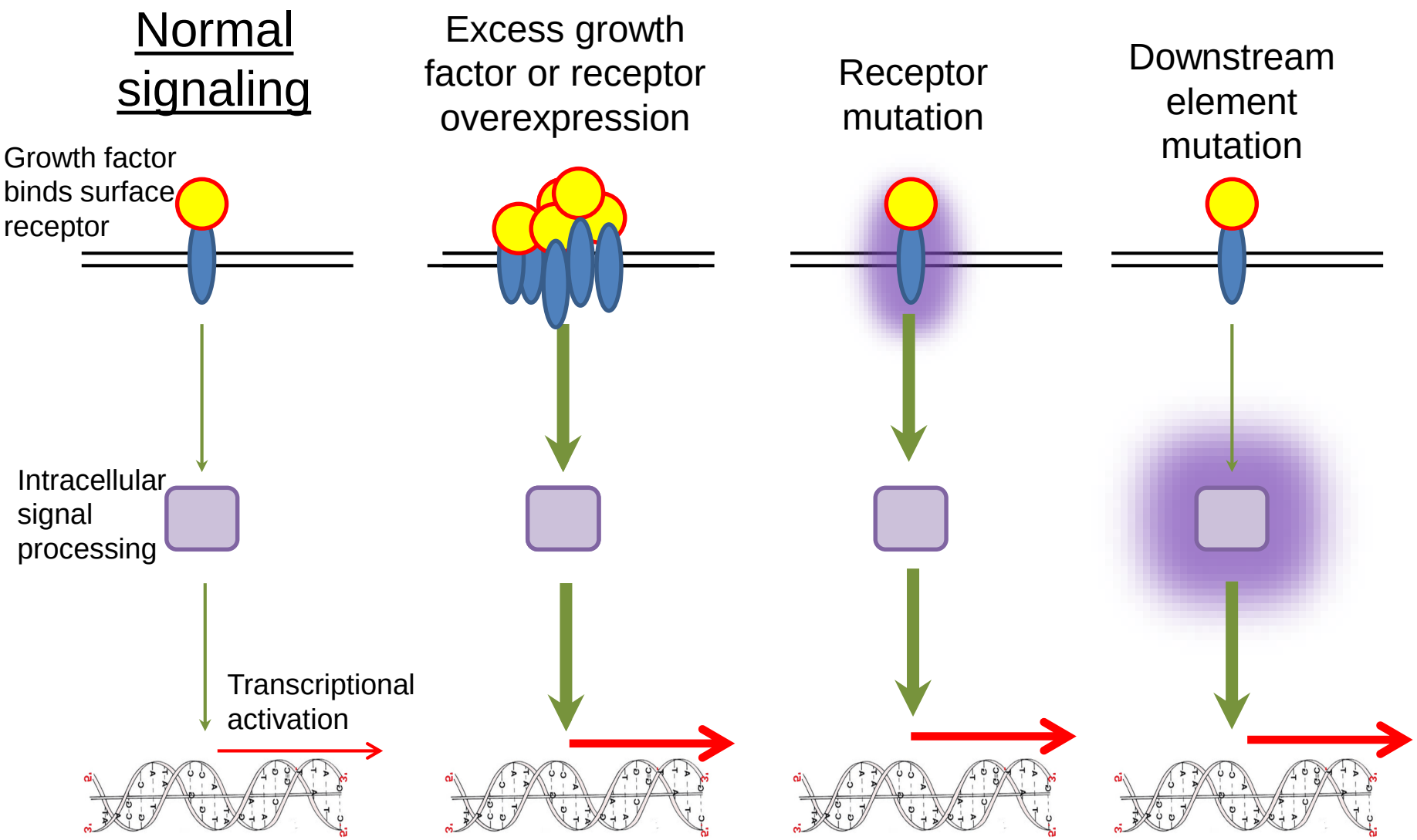
Cell signaling basics

- Information transmitted from cell surface by growth factor receptors (also direct interactions between cells, or with extracellular matrix)
- Regulate cell function (metabolism, cell growth, cell survival, cell differentiation)
- Signaling pathways are highly regulated through branch points and feedback loops
- Abnormal cell signaling occurs in cancer, and is a potential drug target

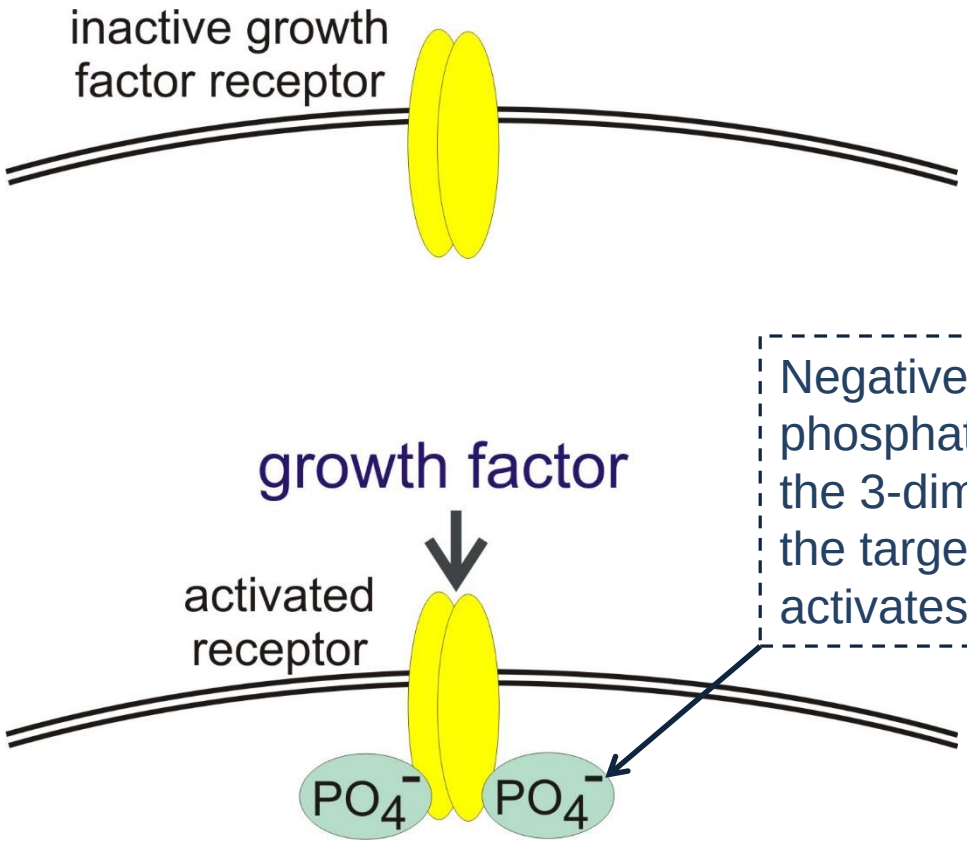
Signaling from outside the cell can be transmitted by soluble growth factors, cell-cell contact, or through contacts with the extracellular matrix. In flow cytometry we mainly study effects of soluble growth factors



Alterations that can drive oncogenic cell signaling

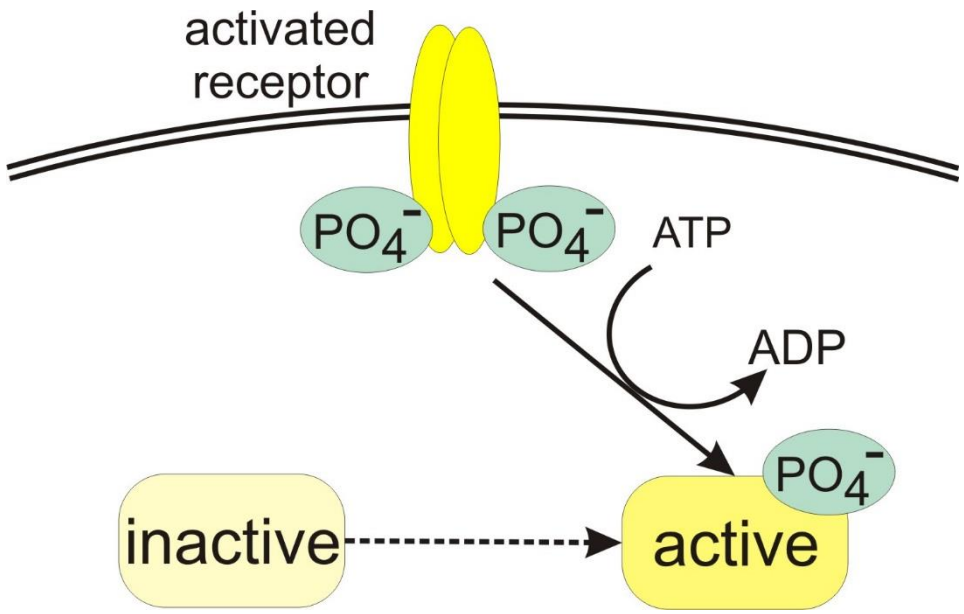


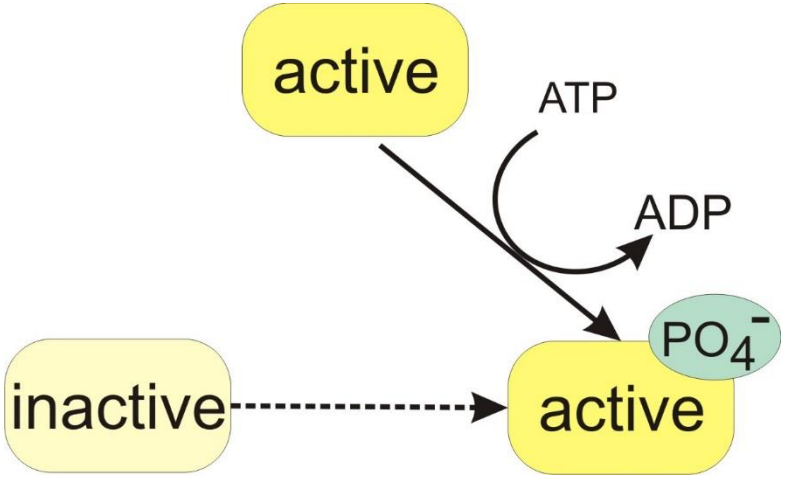
Cell surface signaling mainly through reversible phosphorylation of –OH groups of lysine, serine or threonine. Typically, growth factor binding activates kinase function of the receptor.



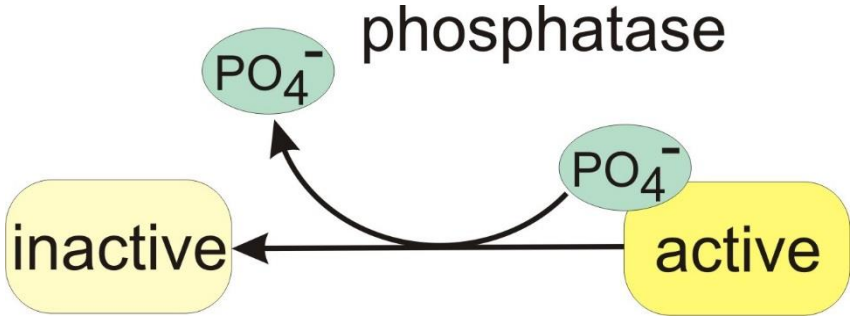
Negative electric charge on phosphate group changes the 3-dimensional shape of the target protein. This activates its kinase function

Signaling propagated into the cell through the phosphorylation of downstream signaling proteins. These become active kinases when phosphorylated, and phosphorylate the next member of the signaling cascade.

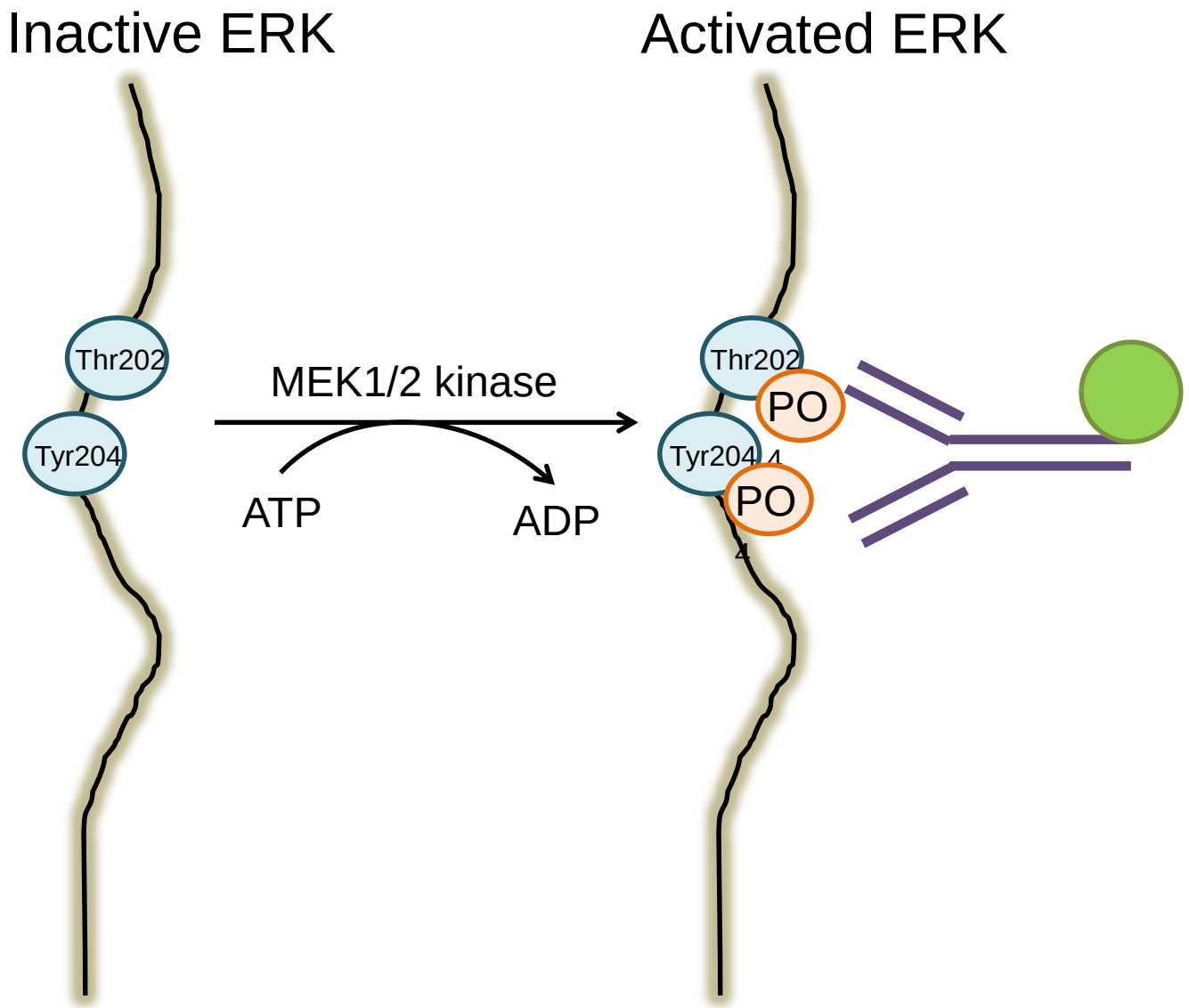




Removal of phosphate by **phosphatases** shuts down signaling pathways

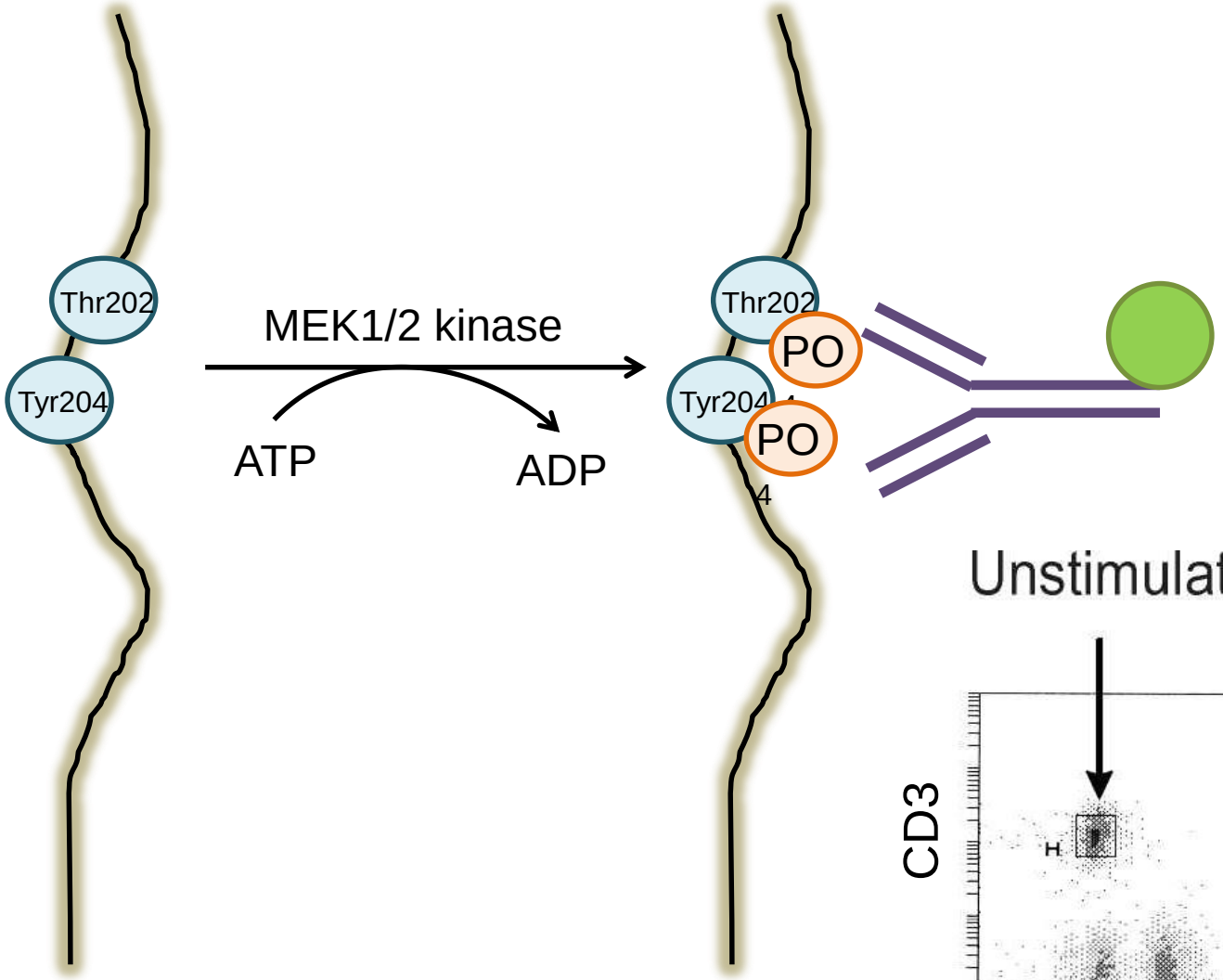


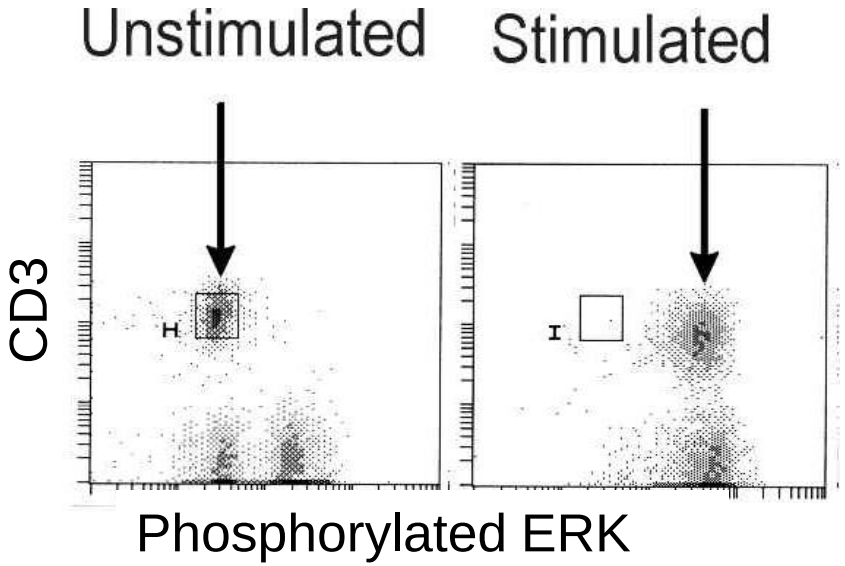
Phosphospecific antibodies recognize the protein only when specific amino acids are phosphorylated



Inactive ERK

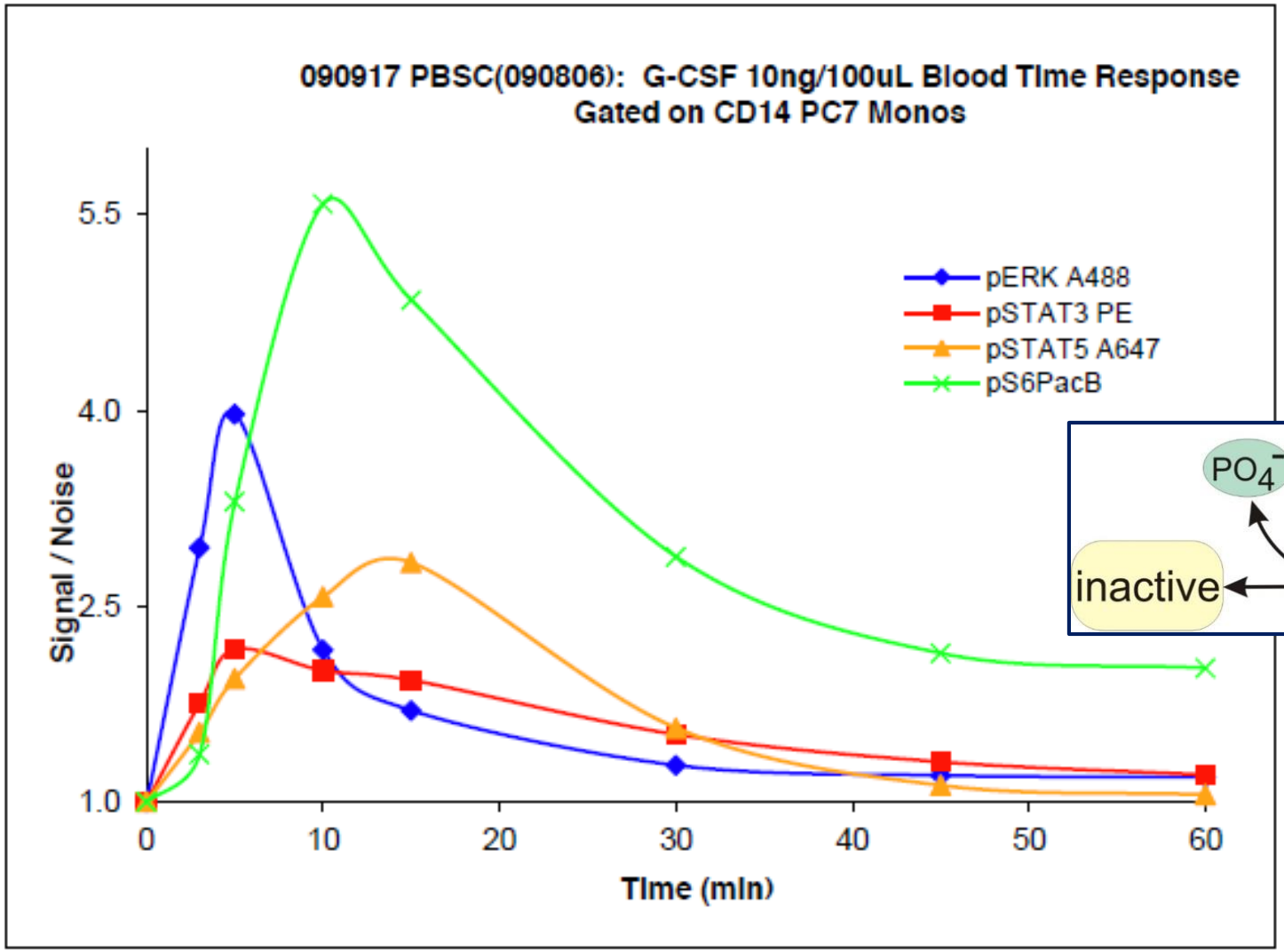
Activated ERK

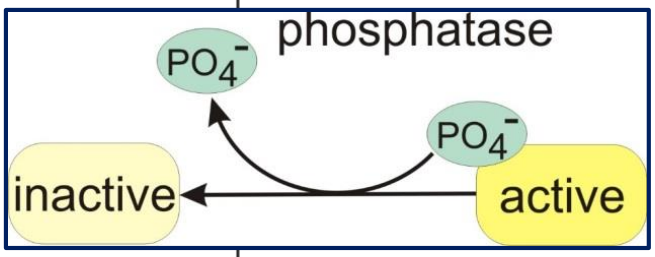




Chow, Patel and Hedley, Cytometry 2001

Pathway activation can be very fast, with rapid decay due to phosphatase activity: *Important to choose the right time to make the measurement!*





Basic protocol for whole blood samples

Chow et al. Cytometry A 2005;67:5-17

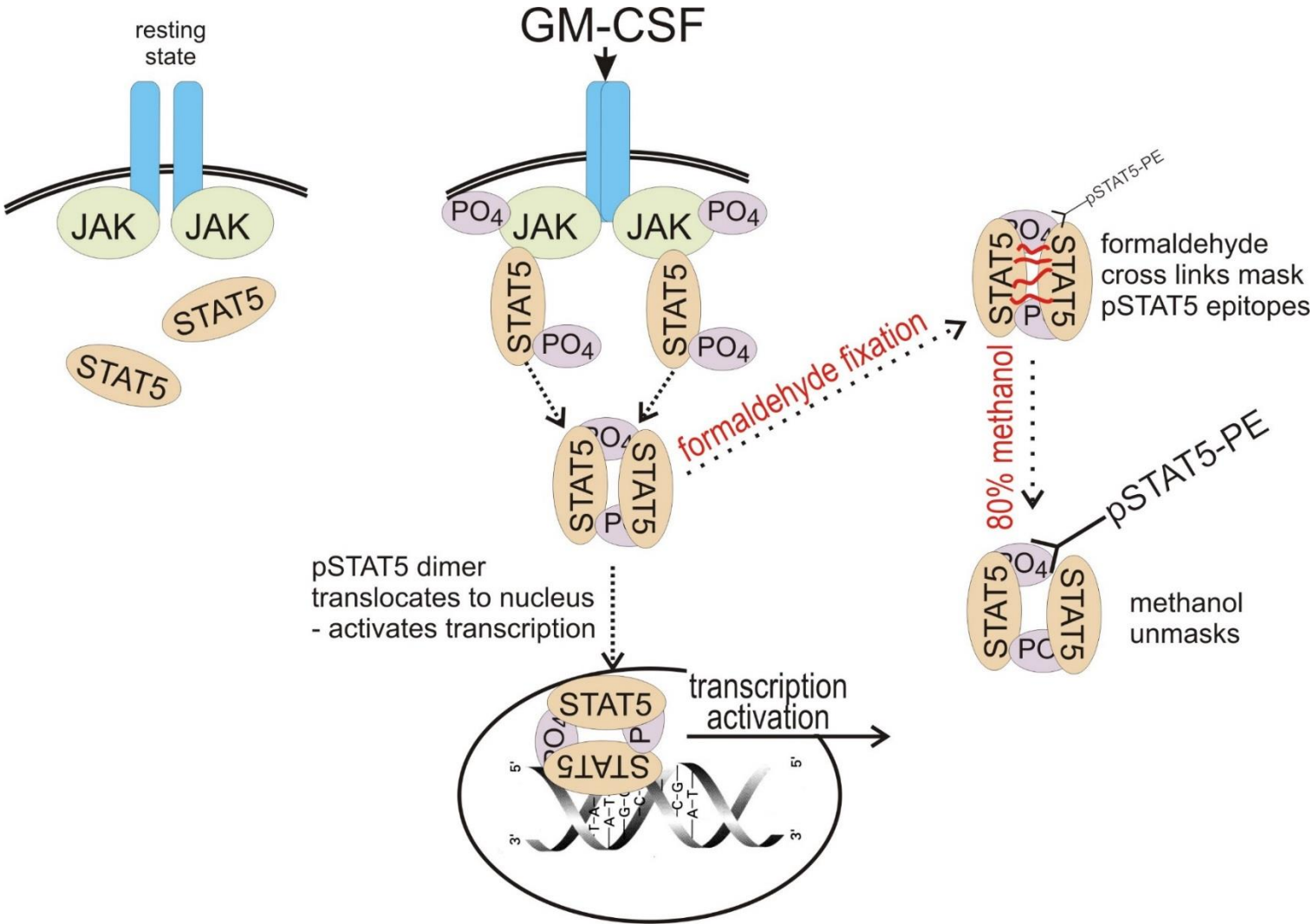
- Activators and inhibitors of pathway added to 100μl aliquots – 37°
- Fix by adding formaldehyde to whole blood (this destroys the activity of kinases and phosphatases, so stabilizes phosphorylation states, as well as fixing the cells)
- Add Triton X-100 to lyse red cells and permeabilize leukocytes
- Combined staining for intracellular phospho-epitopes and surface markers

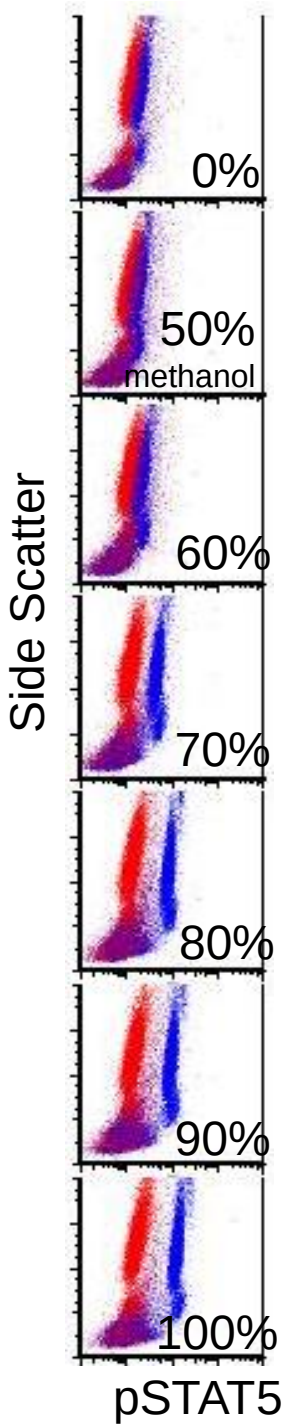
Antigen retrieval

- Antigen masking can occur following treatment with cross linking fixatives like formaldehyde
  - signaling proteins most affected by this are STAT1,3, and 5.
- Treatment with methanol causes protein unfolding; exposing masked antigens

Activation of STAT5 in normal granulocytes

- phosphorylated STAT5 proteins form dimers, with phospho groups closely attached to the binding partner. Formaldehyde fixation prevents access by anti-P-STAT5





Effects of increasing methanol concentration on pSTAT5 staining of normal blood monocytes and granulocytes activated by GM-CSF

Untreated

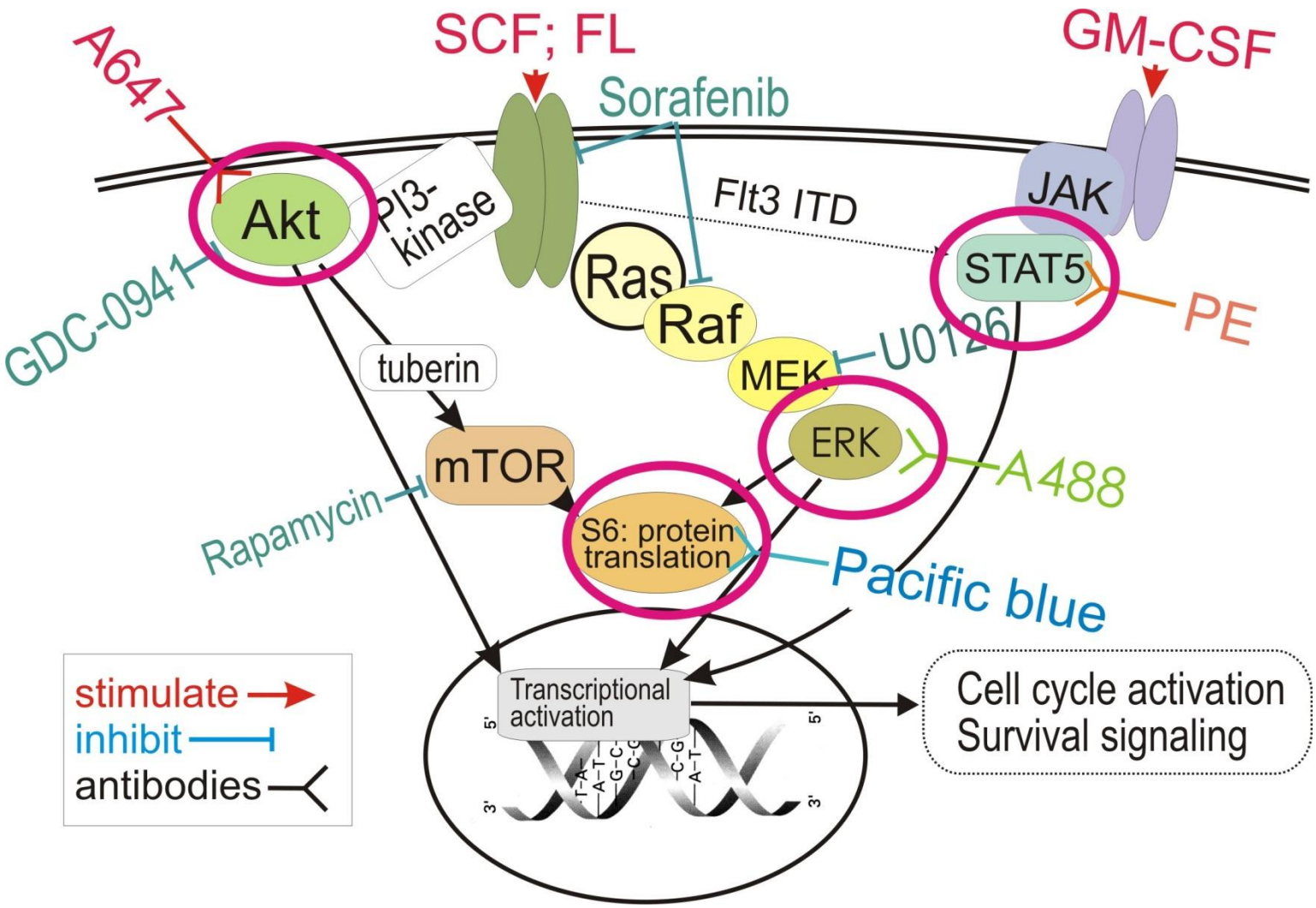
Methanol

Clinical application of cell signaling analysis by flow cytometry

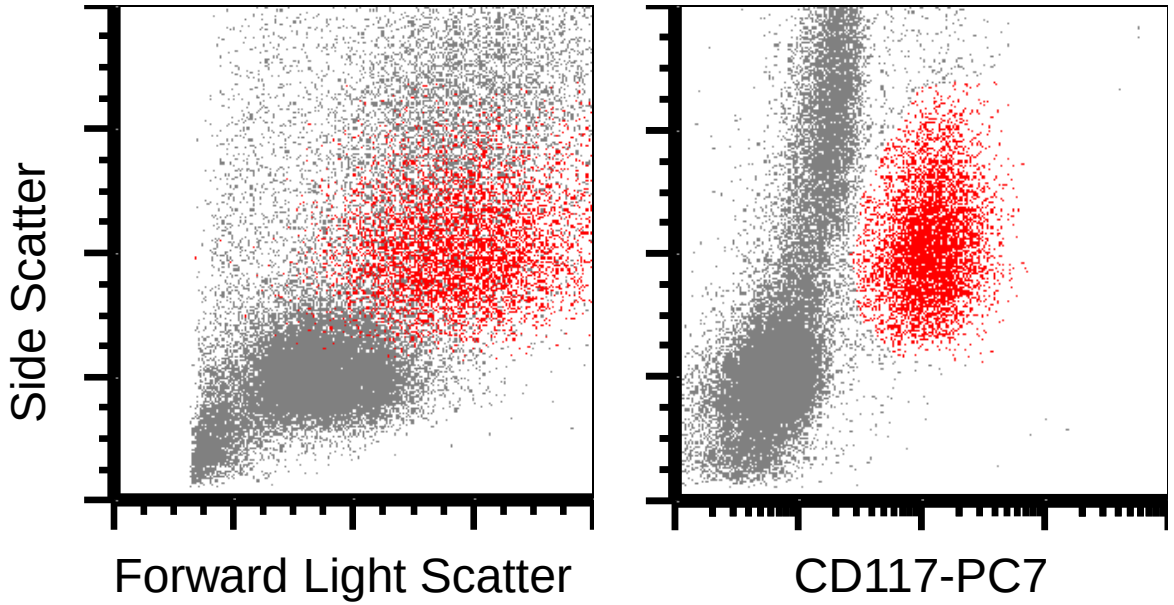
Why study cell signaling by flow?

- Ability to measure multiple activated signaling proteins allows dynamic study of basic science of cell signaling
- Techniques are readily applied to blood and bone marrow samples for clinical applications
  - detect abnormal signaling in leukemia
  - monitor effects during treatment
- Non-malignant clinical applications

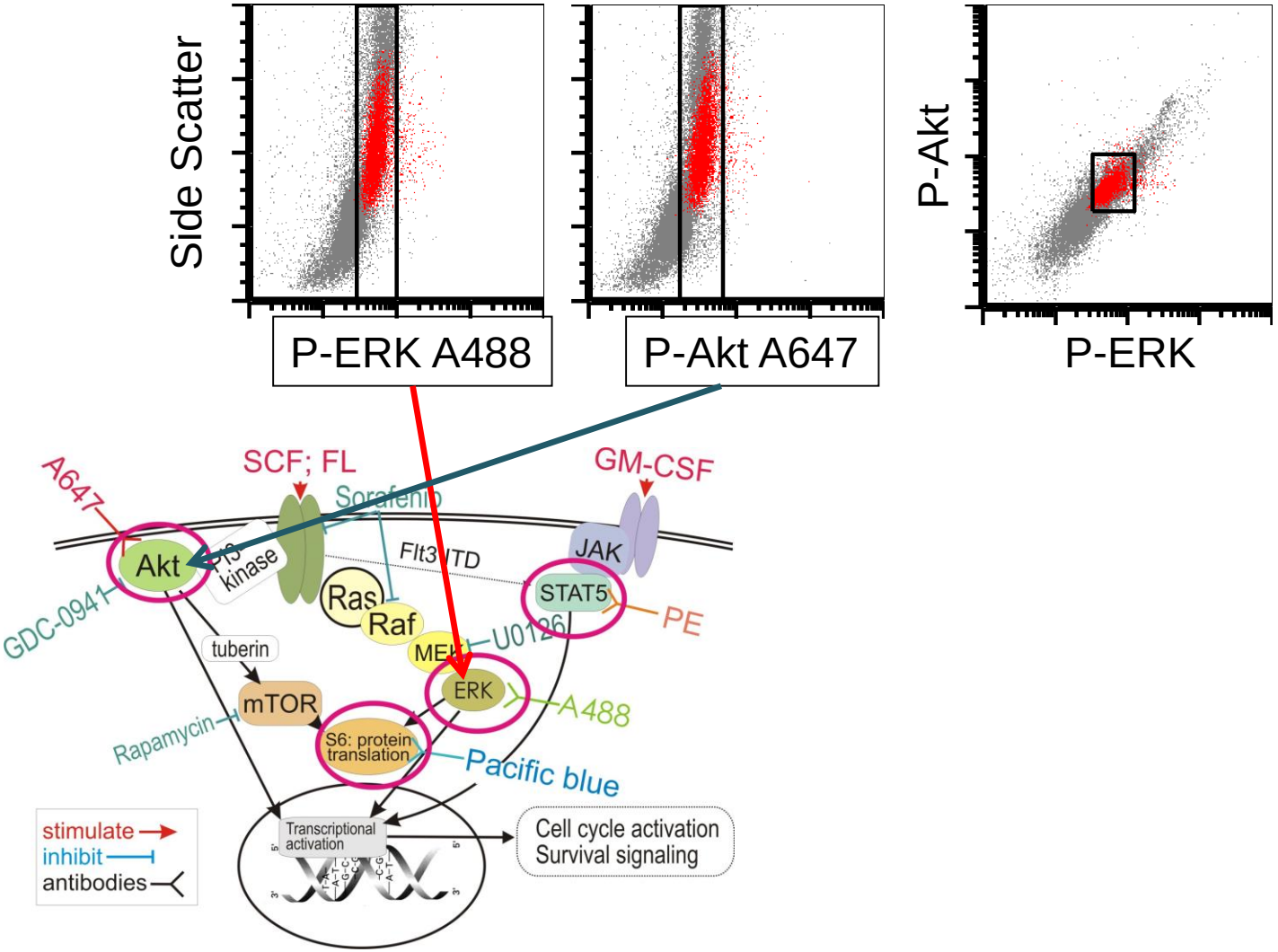
Four colour signaling panel to study the major alterations the occur in acute leukemia patients



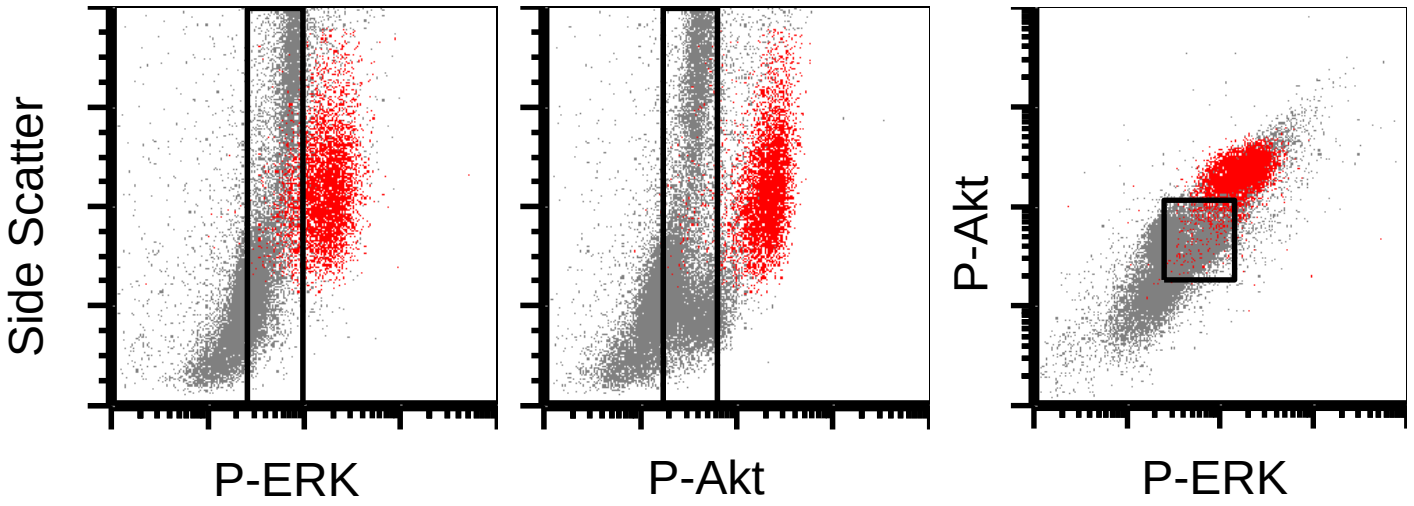
Signaling analysis of leukemia patient starts with identifying the leukemia cells in the blood sample, using light scatter and surface antigens. *The protocol that we teach in the lab gives excellent preservation of light scatter, and most surface markers*



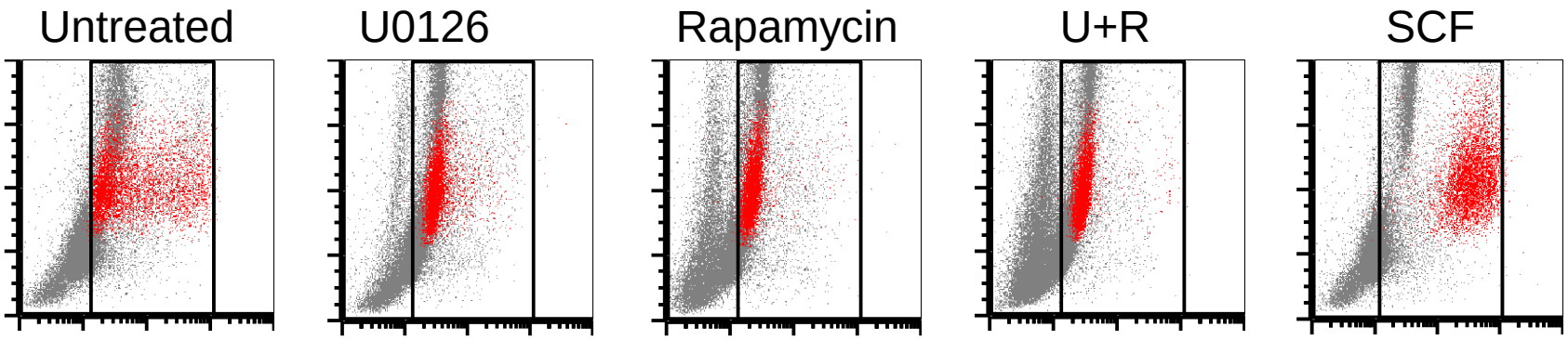
Dual staining for phosphorylated ERK and Akt shows that both are inactive in this patient sample …..



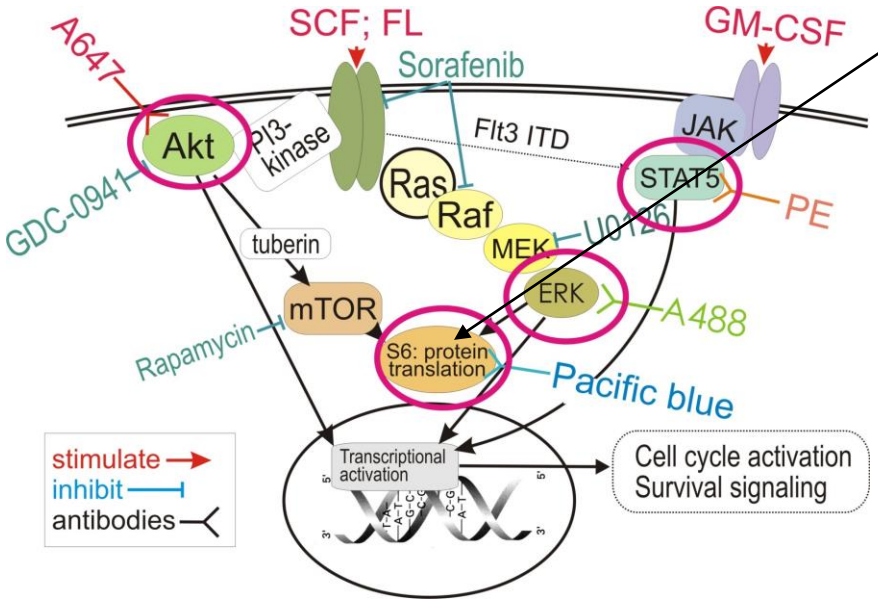
……. but ERK and Akt can be activated by the addition of the c-Kit (CD117) ligand Stem Cell Factor



Basal activation of S6 ribosomal protein, and effects of inhibitors



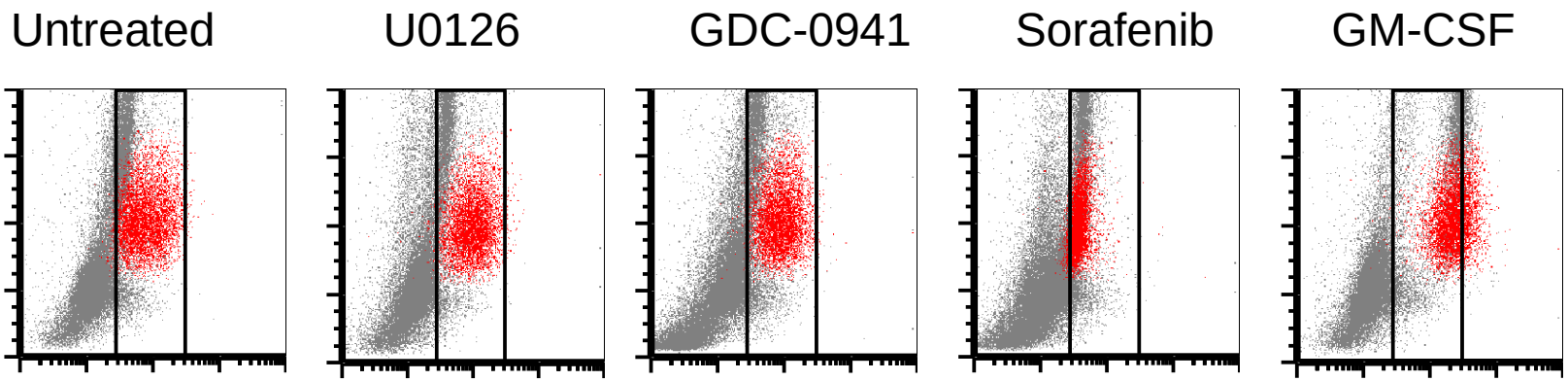
P-S6 Pacific Blue

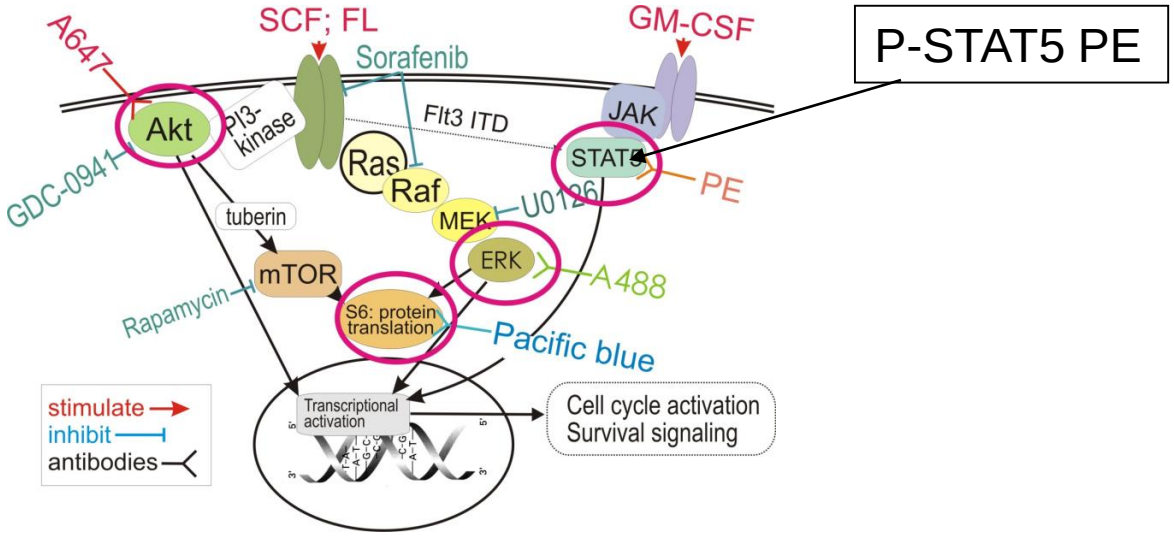


S6 is abundant intracellular protein. When activated it facilitates ribosome formation and protein synthesis. S6 phosphorylated by mTOR, and through ERK. As seen in this patient sample, some untreated blast cells have high levels of pS6. This is blocked by pathway inhibitors U0126 and Rapamycin. Treatment with Stem Cell Factor activates S6 in all blast cells

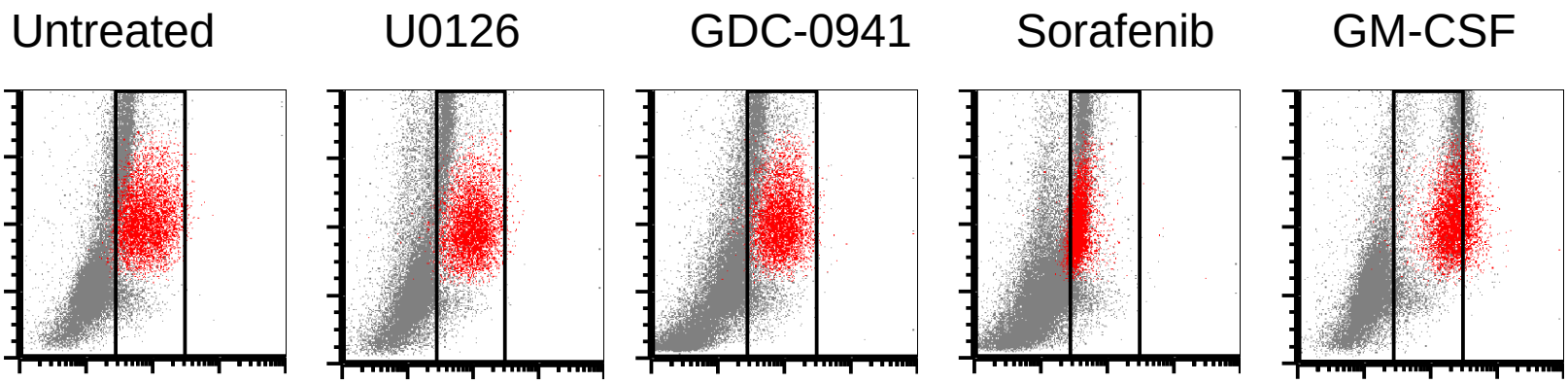
P-STAT5; sensitive to Flt-3 inhibitor Sorafenib but not to ERK and PI3-kinase inhibitors.

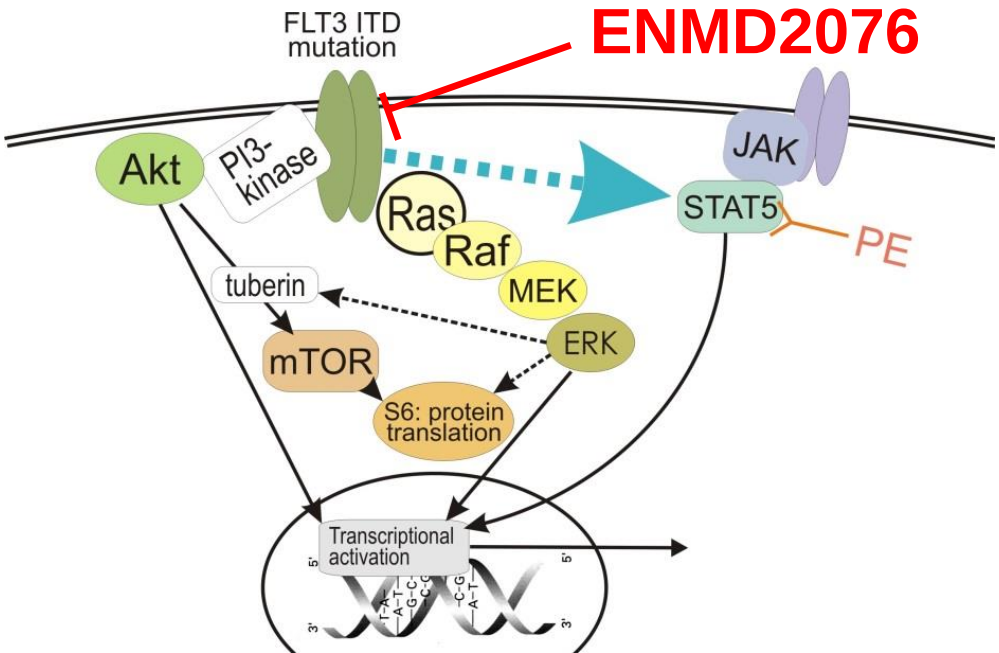
(Granulocytes respond to GM-CSF - positive control)



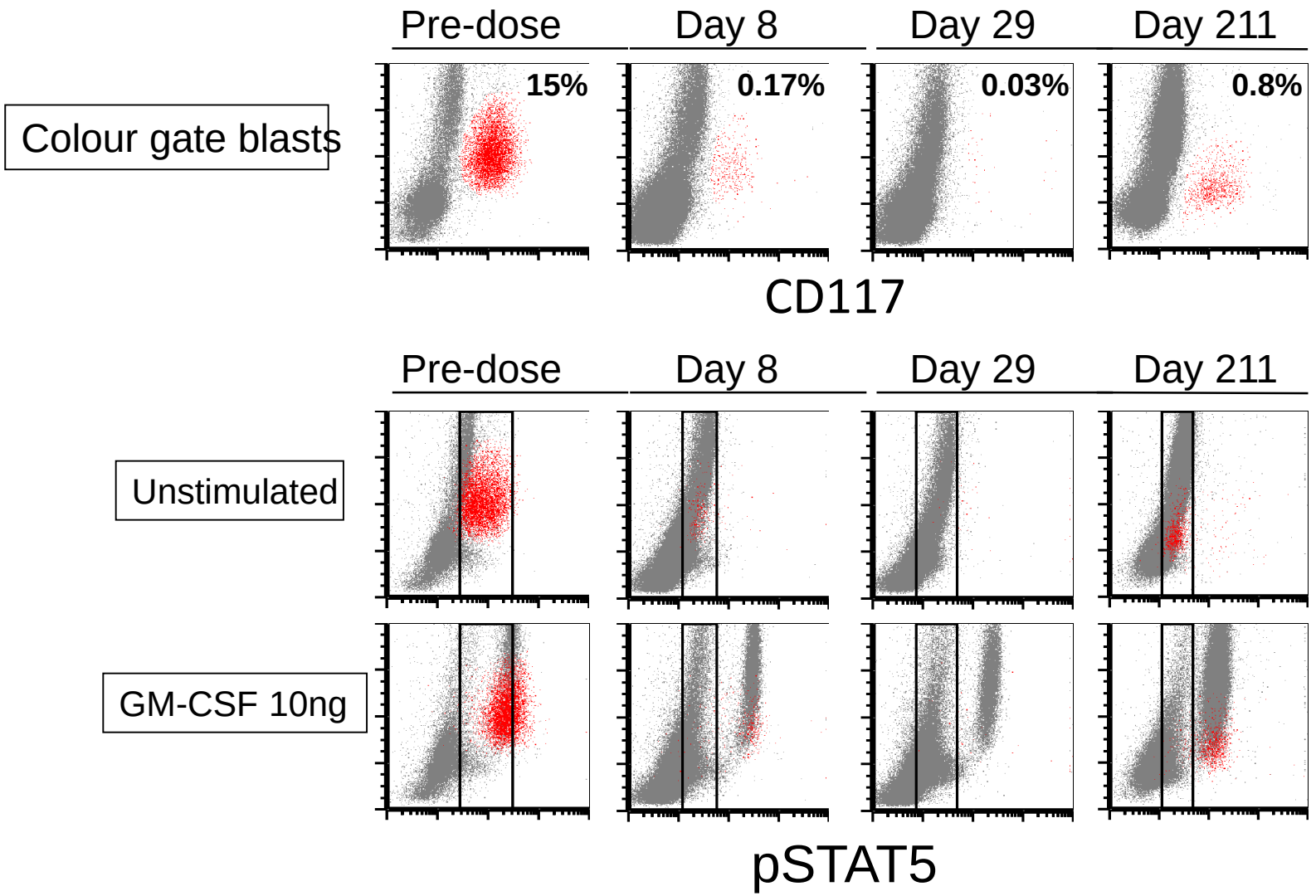


The patient was then treated with the novel Flt-3 inhibitor ENMD2076, and we monitored the response to treatment by flow cytometry





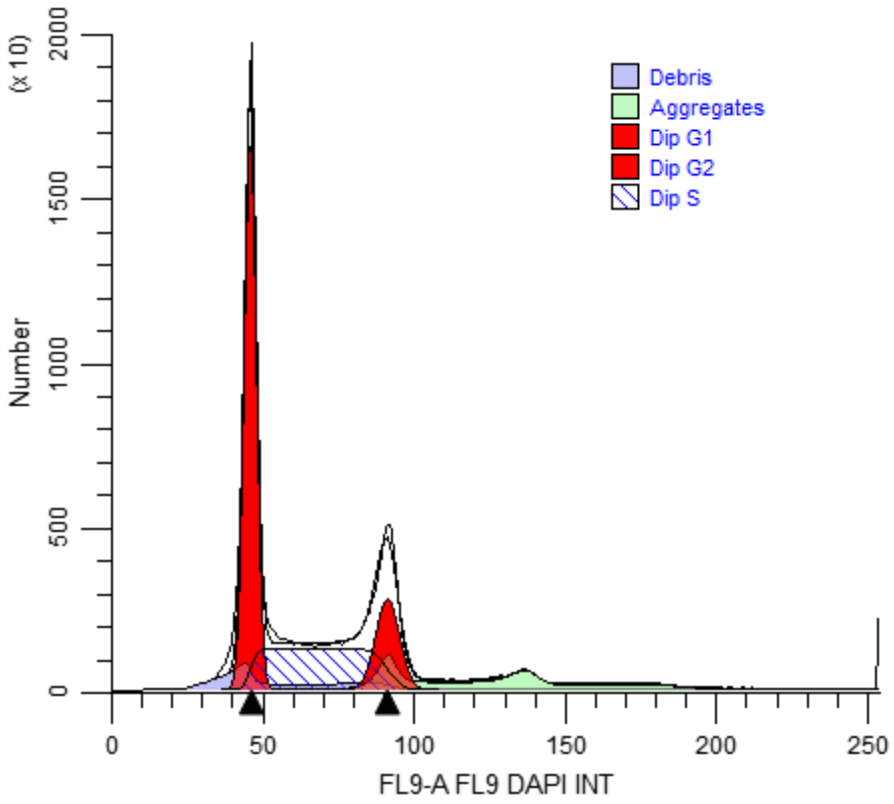
The patient responded to treatment with ENMD2076 (reduced CD117+blasts with dephosphorylated STAT5 on Day 8), but disease recurred on Day 211.



Cell cycle analysis by flow cytometry

- DNA content analysis
- Proteins that regulate cell cycle
  - cyclins; mitosis markers
- Incorporation of DNA precursors
  - thymidine analogues (BrdU; EdU)

Cells in G2 and mitosis have the same DNA content ……



Diploid: 100.00 %
Dip G1: 50.08 % at 45.55
Dip G2: 16.14 % at 91.10
Dip S: 33.78 % G2/G1: 2.00
%CV: 4.33

Total S-Phase: 33.78 %
Total B.A.D.: 10.57 %

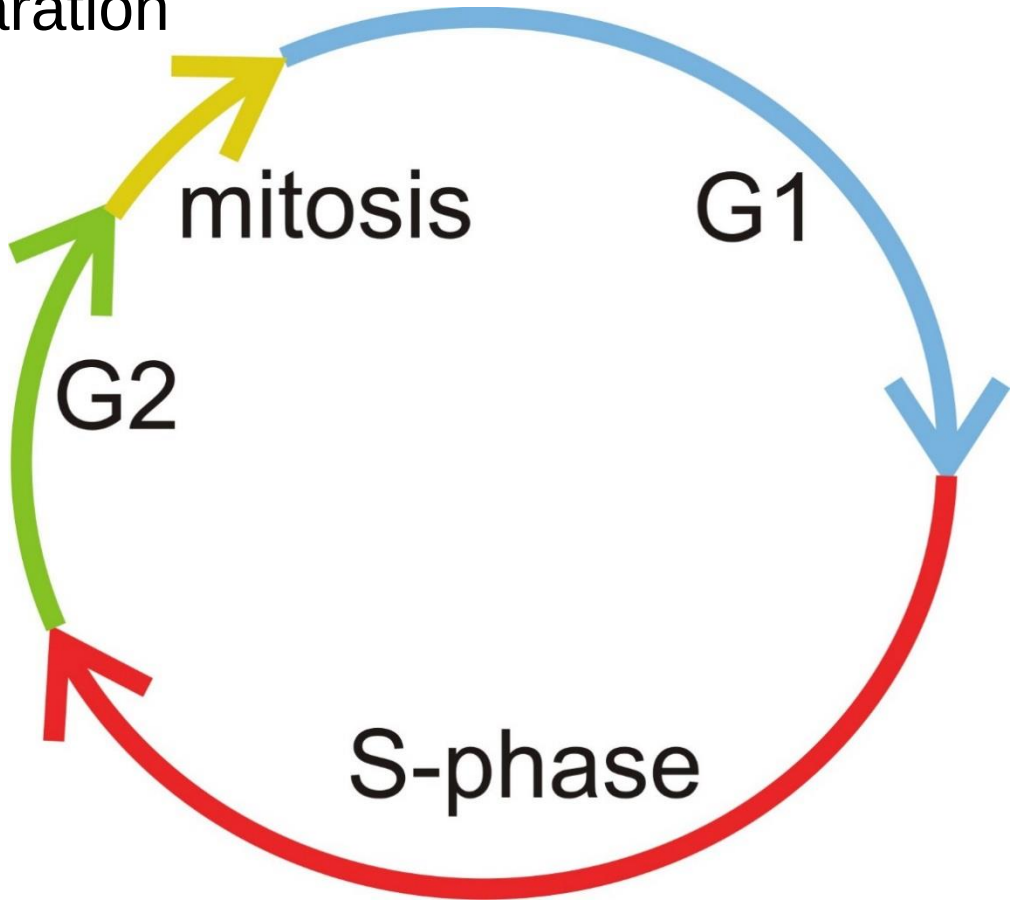
Debris: 9.18 %
Aggregates: 15.03 %
Modeled events: 224426
All cycle events: 170077
Cycle events per channel: 3654
RCS: 22.000

…… but are functionally very different!

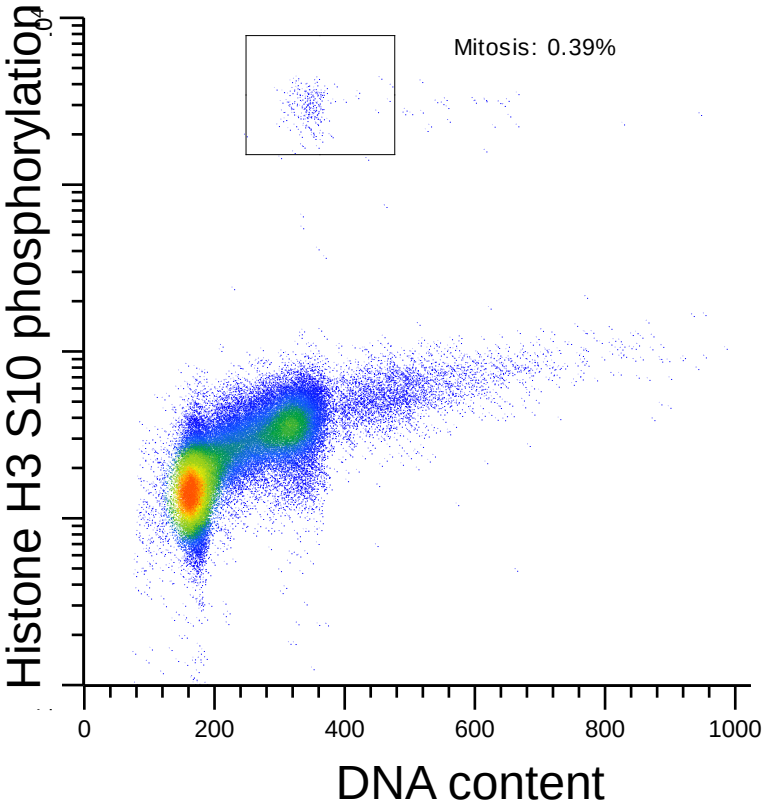
Continue to proliferate, or exit cell cycle:
- differentiate
- cell death or senescence
- quiescent (G0; stem cells)

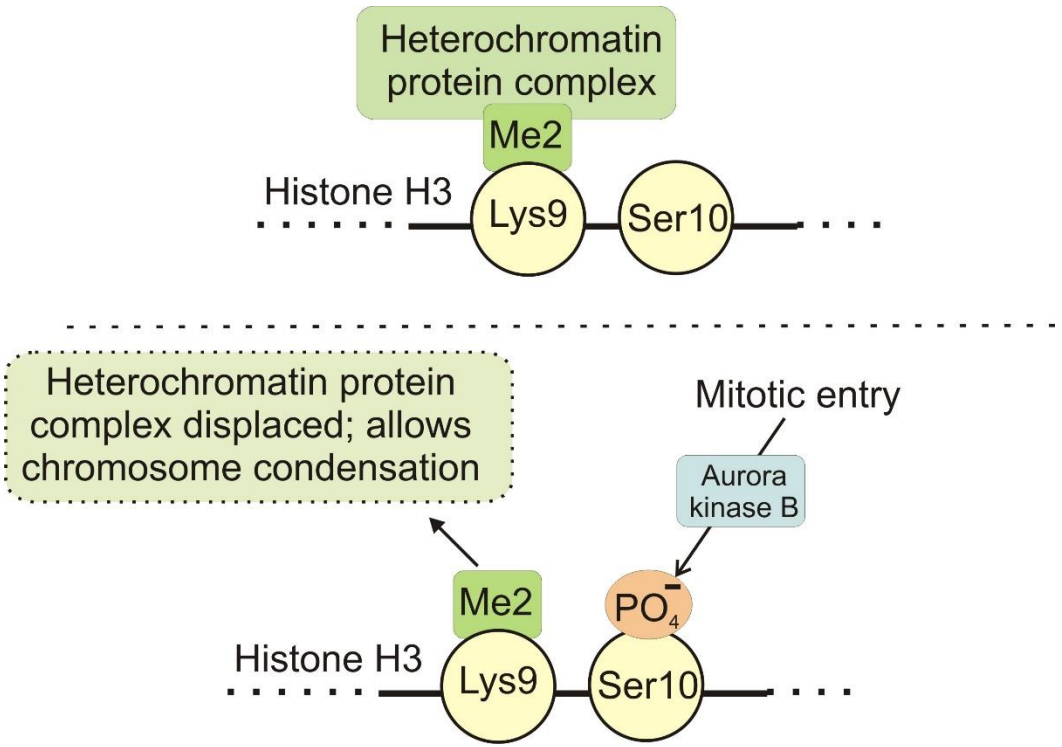
Chromosome separation
Cell division

Check for errors in DNA replication



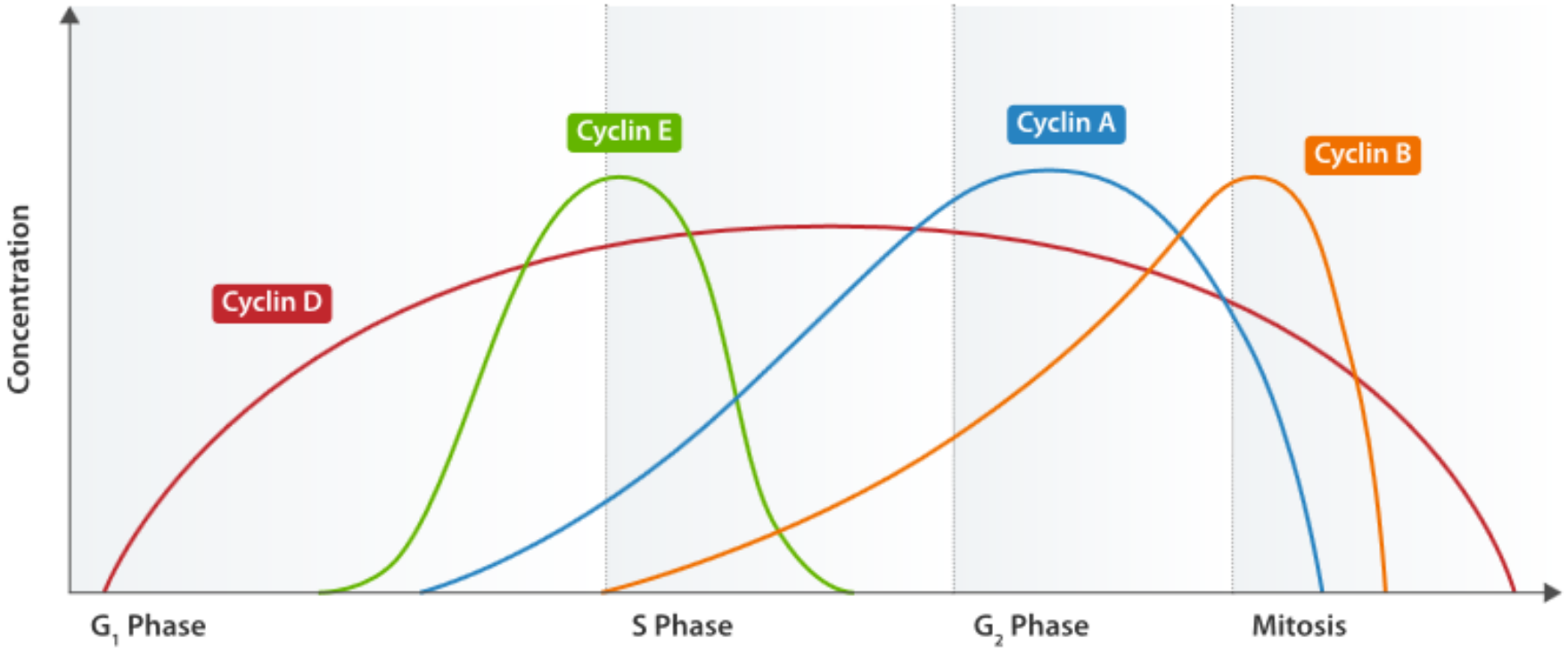
Separating G2 from mitosis: Serine 10 of histone H3 phosphorylated at entry into mitosis



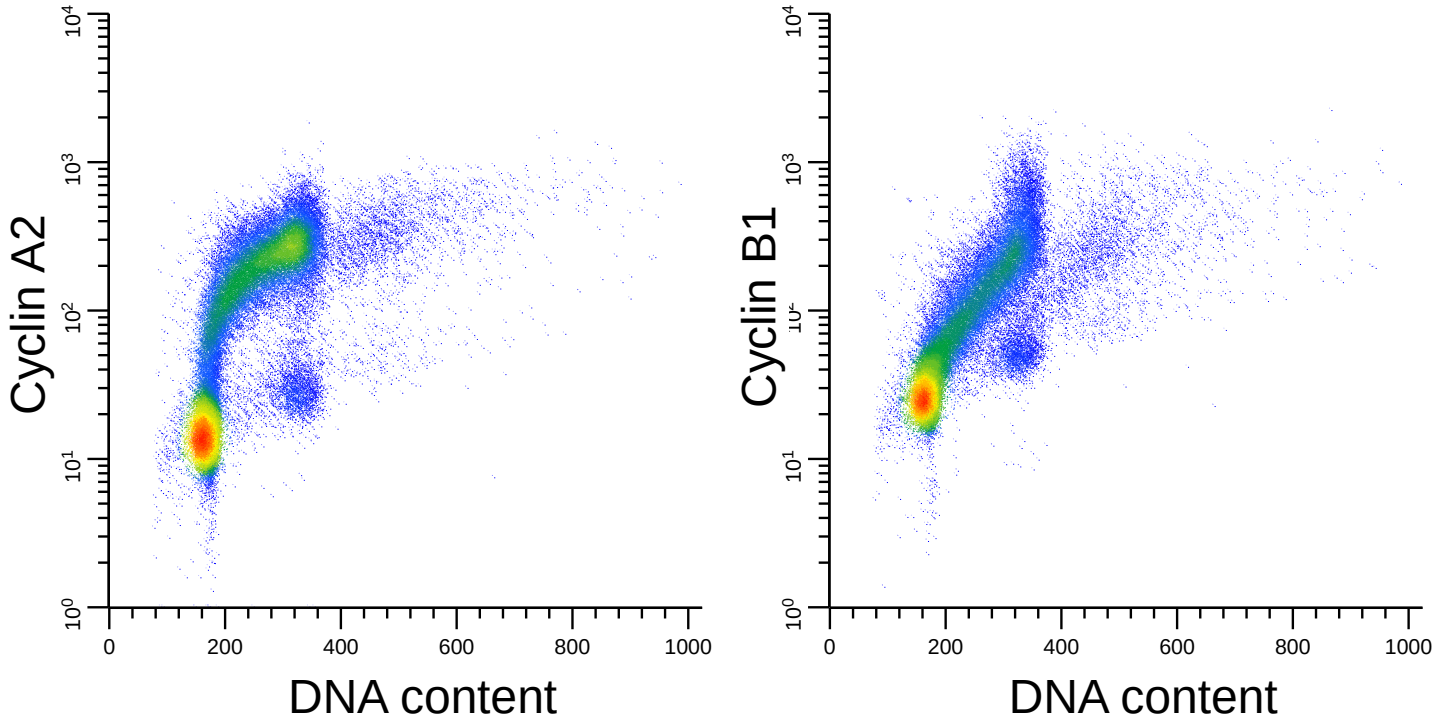


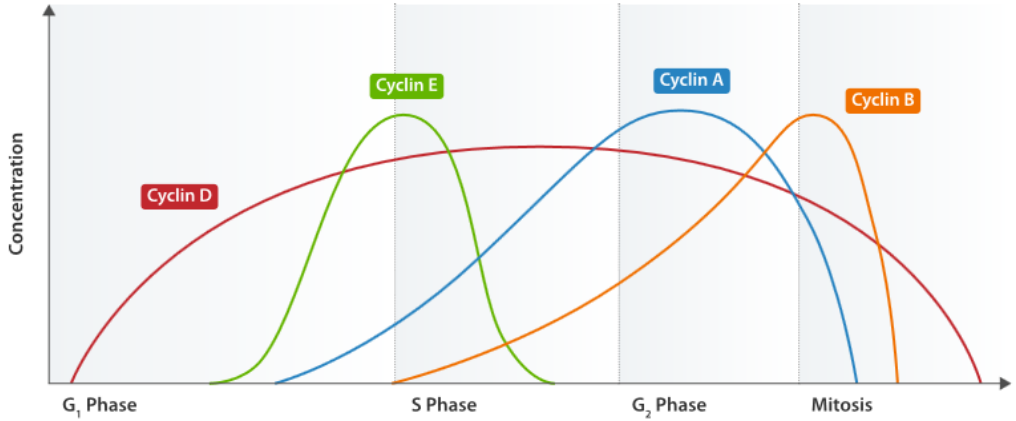
Basic science: Heterochromatin complex docks onto methylated histone H3 lysine 9, resulting in chromosomal condensation (heterochromatin), and gene silencing. Heterochromatin complex is displaced by the negative charge produced by Serine-10 phosphorylation, allowing the chromosomes to unwind, prior to entry into mitosis.

Progression through cell cycle driven by **cyclin-dependent kinases**. These are activated, and then inactivated, by the sequential synthesis and breakdown of cyclins.

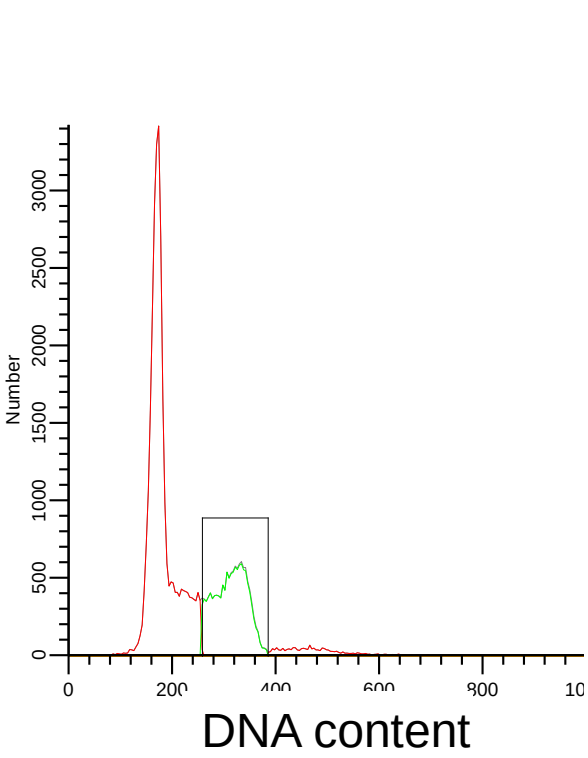


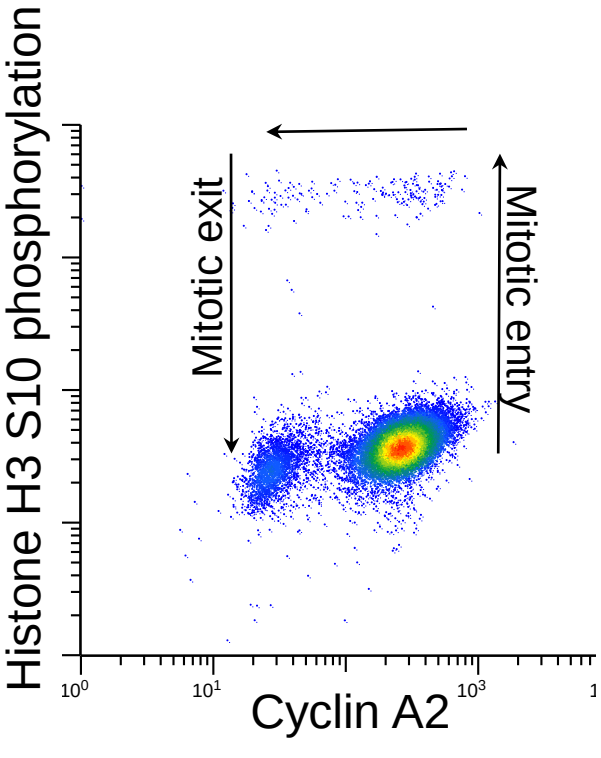
Differential expression of Cyclins A2 and B1 during the late cell cycle

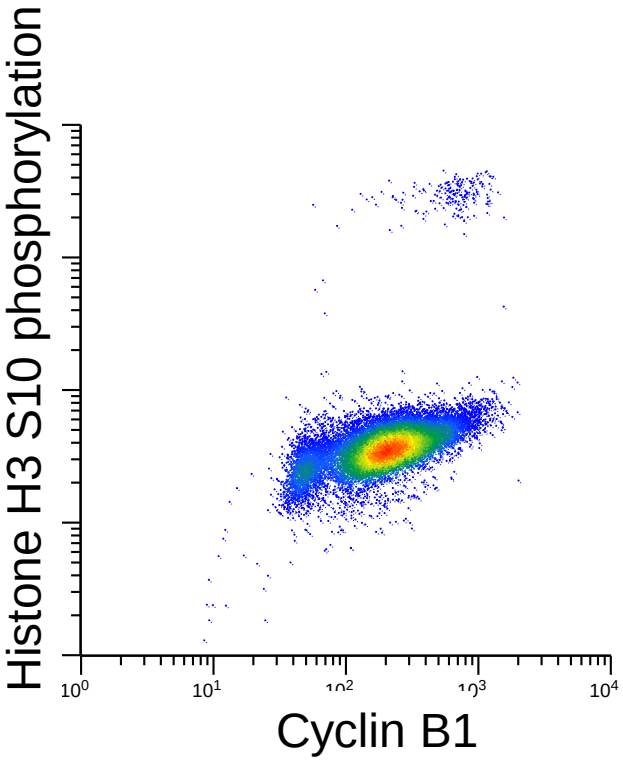




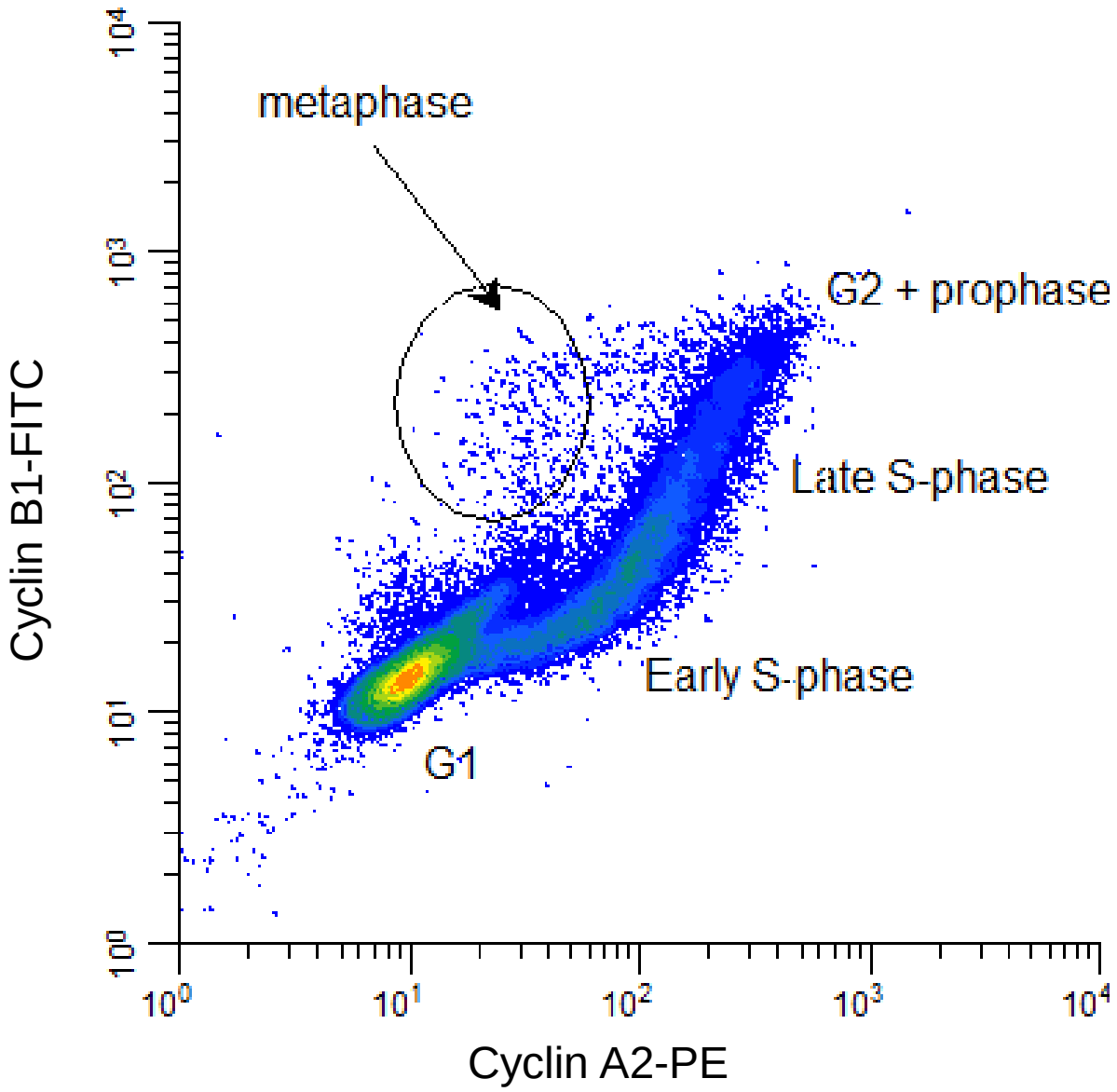
Cyclin A2 targeted for degradation during prophase; Cyclin B1 at entry into anaphase.





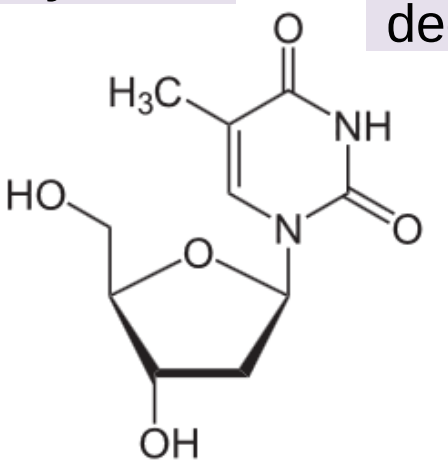


Defining the cell cycle with dual Cyclin A2 and B1 staining

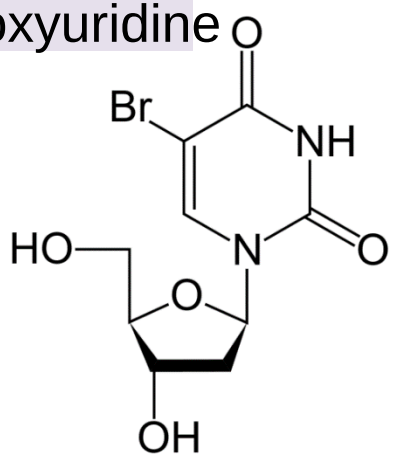


Thymidine modifications that incorporate into DNA during S-phase, and can be detected by flow cytometry

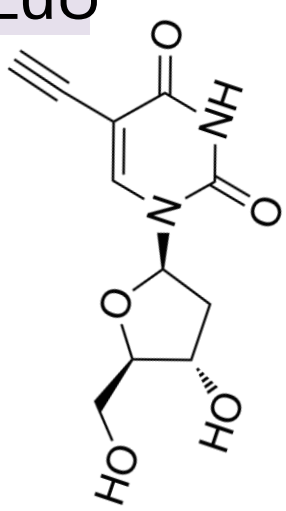
Thymidine



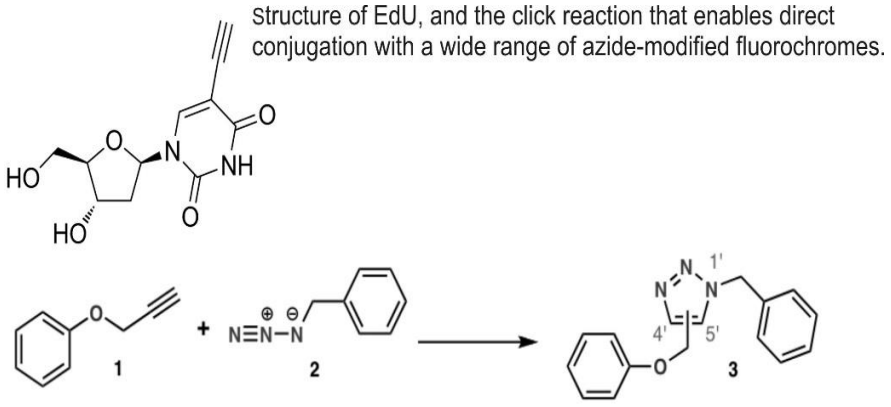
Bromo-deoxyuridine



EdU

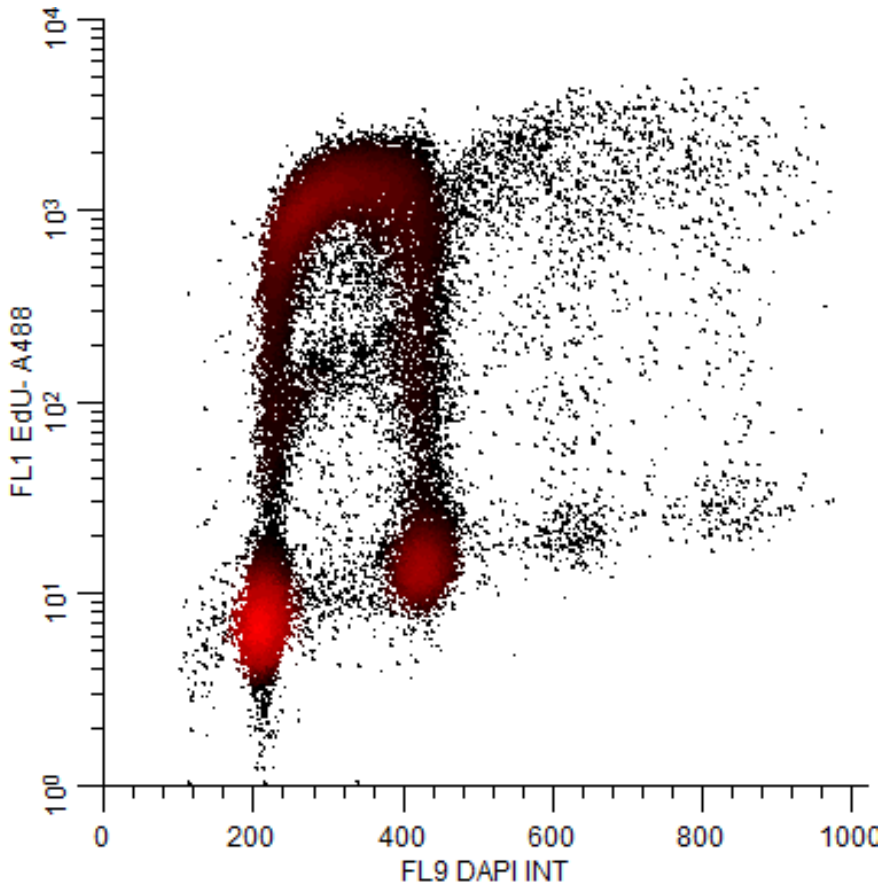


- Bromodeoxyuridine (BrdU) uses antibody detection
  - needs acid denaturation to separate DNA strands
- EdU uses copper-chelated click chemistry that covalently binds fluor directly onto incorporated EdU. So no acid denaturation



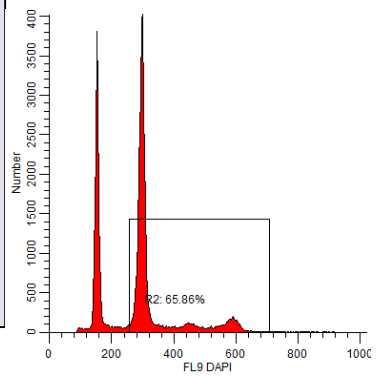
EdU vs. DNA content

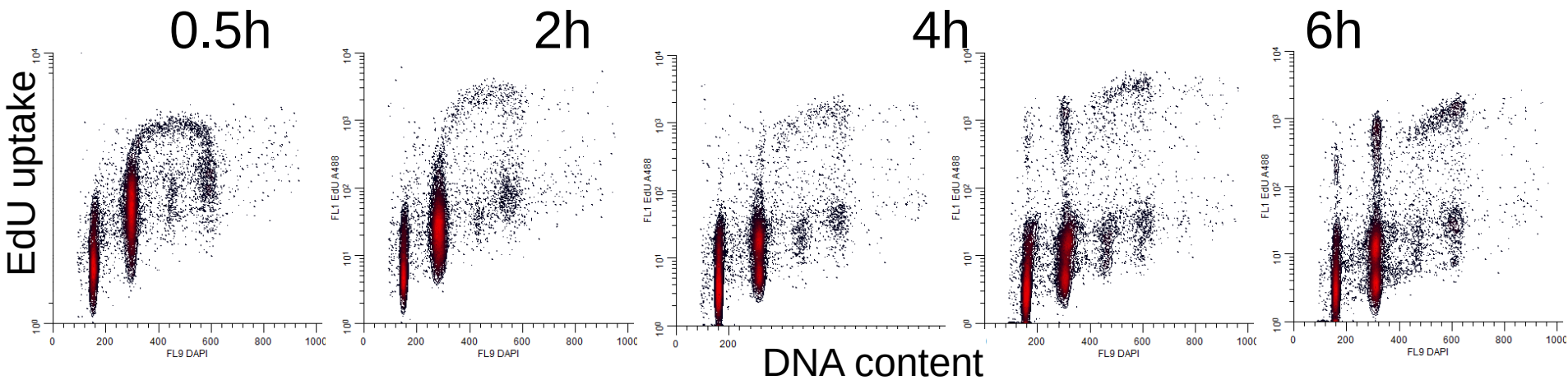
30 minute treatment, so only S-phase cells are EdU positive



Dynamic studies using EdU pulse/chase

Time course of movement of EdU-labeled cells through the cell cycle, using human tumour xenografts grown in immune-deficient mice, injected with EdU at time=0





Application of advanced cell cycle methods to cancer treatment

- Combining drugs that arrest cell division with chemotherapy drugs that kill dividing cells
- Effects of genomic instability on the progression of cancer cells through G2 and metaphase
- Test using cancer tissue from patients grown as xenograft in immune-deficient mice

Conclusions

- Detection of intracellular antigens, combined with preservation of light scatter and surface immunophenotype, allows the study of complex biological processes in heterogeneous cell populations.
- The study of cell cycle regulation and cell signaling discussed in this talk are just examples – there are many other possible applications that might allow you to discover something new!