IMMUNOHAEMATOLOGY BULLETIN

Bulletin of The National Institute of Immunohaematology
(I.C.M.R.)

Vol. 44; No. 3

Sep - Dec 2013

Workshop on “Karyotyping” as a part of Hematocon-2013

Workshop on “Advanced Applications of Flowcytometry: Diagnosis of PID”

CELLULER SIGNALING USING MULTI PARAMETRIC FLOW CYTOMETRY : A LEARNER'S GUIDE

Swati Garg

Summary : Most of the vital cellular functions are regulated by a network of signaling molecules intricate in cell signaling cascades. They control important aspects of cells including proliferation, differentiation, communication and apoptosis. Aberration in cell signaling pathways result in pathological conditions, for example; defect in signal transducer and activator of transcription (STAT) 3 is correlated with Hyper IgE syndrome. Over expression of STAT-5 as a result of increased bcr-abl kinase activity is correlated with poor prognosis in chronic leukemia. Transient phosphorylation of important amino acid residues remains to be the most important action mechanism by which these cell signaling pathways propagate. Assays employing flow cytometry for cell signaling are now well established and ready for their application in research as well as clinical settings. This review speaks about development of flow cytometry based cell signaling assays, their technical details and finally recent advancements and applications.

Cells respond to subtle changes in their immediate environment through a cascade of molecules including ligands, receptors, channels, enzymes, transcription factors, genes and proteins [1]. Cell proliferation, differentiation, apoptosis and response to cytokines is regulated by intricated signal transduction cascades. These cascades in turn are meticulously regulated by transient phosphorylation of intracellular signaling proteins, controlled by assortment of kinases and phosphatases [2]. In a typical case of signal transduction mediated by growth factor; binding to its cell surface receptor triggers a series of events which initiates with phosphorylation of the receptor and eventually propagates through secondary messengers resulting in alteration of gene expression [3]. Different molecular circuits of signal transduction pathway distinguish, amplify, transduce and integrate diverse signals to bring about changes in gene expression, enzyme activity and other effector mechanisms [4]. Overview of a cell surface receptor mediated signal transduction pathways is illustrated in figure1.

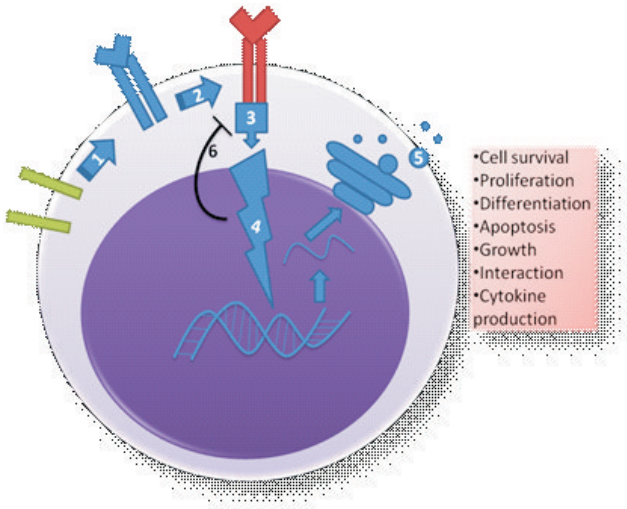

Figure1 : Overview of signal transduction mediated by cell surface receptor :

1. Detection of stimuli generally results in dimerization / multimerization of receptor which increases the receptor-lignad affinity, **2**. Upon binding of ligand to its receptor, auto or cross phosphorylation occurs at specific domains of receptor which provide docking site for other kinases and phosphatases (eg. Janus Kinase), **3.** These protein kinases activate secondary messengers, **4.** These either translocate to nucleus (eg. STAT) or activate other molecules to effect the gene expression (eg. Akt), **5.** It brings about specific changes in cell biology and **6.** Another cascade of events activates feedback loop similarly to ensure system equilibrium.

Swati Garg, Senior Research Fellow,

Department of Paediatric Immunology and Leaucocyte Biology, National Institute of Immonohaematology (ICMR)

13th Floor, NMS Building, KEM Hospital, Parel, Mumbai 400 012, India

These phoshorylation events are transient and reversible where phosphorylated state most often corresponds to active form of protein [5]. Edmond H. Fischer and Edwin G. Krebs were awarded Nobel Prize in physiology and Medicine in the year 1992 after showing that reversible protein phosphorylation affects the structure, function and activity of proteins that are responsible for regulation of nearly all aspects of cell biology [6]. Evaluation of phosphorylation provides information about activation of signaling cascade by specific stimuli, its kinetics and downstream targets [7]. Conventional methods to measure these events include western blotting-immunoblotting and ELISA from cell extracts. However, with development of new analytical techniques, flow cytometry provides rapid, quantitative and multi parametric, single cell based analysis of cell signaling events [8]. The first report on flow cytometry based cell signaling assay has been described in 1990 by Fierring et al [9]; a group led by father of FACS: Prof. Herzenberg. Muller et al [10] were the first one to deploy multiparametric flow cytometry for analysis of phospho tyrosine levels in hematopoietic cells of human origin. They devised flow cytometry based methodology to estimate phospho tyrosine levels in circulating peripheral blood system cells post granulocyte colony stimulating factor (G-CSF) therapy in patients. On the other hand Fleisher et al [11] used multi parametric flow cytometry assay for detection of intracellular phosphorylated STAT-1 in peripheral blood monocytes upon stimulation with interferon gamma. Expression of phosphorylated STAT-5 in normal peripheral blood mono nuclear cells after interleukin-2 (IL-2) stimulation and post IL-2 immunotherapy in immune cell subset of patients with malignant melanoma and renal cell carcinoma was shown by Varker et al [12]. Simultaneous measurement of multiple active phosphorylated states of proteins belonging to Mitogen Activated Protein kinase (MAP), / AKT-Protein kinase B (PKB) and T cell activation pathways were described by Perez et al [13]. Multiple phospho proteins in different populations of peripheral blood mono nuclear cells were measured using multi parametric flow cytometry by Montag et al [14-15] where they focused on molecules in Janus Kinase (JAK)/STAT and MAPK/ Extracellular signal Regulated Kinase (ERK) pathways. Firaguay et al [16] devised protocol for analyzing signaling events by dynamic flow cytometry where they analyzed effects of different inhibitors on Akt signaling in cell lines. There are many remarkable studies in the field of cell signaling involving tyrosine kinase mediated responses and phospho protein levels in different subsets of leukocytes, cell signaling in normal and cancer cells and many others. A comprehensive analysis of various cell fixation and permeabilization protocols was done by Krutzik et al [17]. Subsequently, the same group had established flow cytometry based fluorescence cell bar-coding protocols which allowed high throughput analysis of cell signaling profile [18]. Chow et al [19] had established whole blood fixation and permeabilization protocol for evaluating intracellular phospho proteins in leukocyte subpopulations by compiling the data from various experiments to obtain maximum signal upon stimulation with cytokines and growth factors. Recently, Woost et al. [20] have described high resolution kinetics of cytokine signaling in normal human bone marrow CD34+/CD117+ cells. Their efforts had enlightened the approach to kinetics of cytokine stimulated cell signaling based assays. Following that, Marvin et al. [21] had studied normal bone marrow cell signaling profile where they described cell signaling profile in seven immunophenotypically distinct populations in normal human bone marrow when subjected to stimulation by growth factors and cytokines and by measuring their downstream response of phosphorylated molecules. Later on, Bendall et al. [22] employed mass cytometry for simultaneous analysis of 34 parameters in single cells based immunoassays across human hematopoietic continuum.

With advances in flow cytometry and commercial approach to cell signaling based research, these kinds of assays are welcomed warmly, but before one starts their experiment there are certain dos and don'ts of these assays which are made handy in this review. Important aspects of a cell signaling experiments are as follows:

1. **Signaling pathway** : It is important to know the signaling cascade very well for planning a cell signaling experiment. This includes:

a. Knowledge of pathway activator or inducer, specific inhibitor [23], important downstream molecules with their phosphorylation site, state and cross talk with other members of cell signaling network.

b. Choice of phospho specific antibody depends on pathway in interest, for example while working with signal transducer and activator of transcription, maximal transcription activity requires phosphorylation of serine 727 in STAT1 and STAT3 [24]. In a study involving comparison of AKT phosphorylation level on threonine (Thr) 308 and serine 473 in 58 acute myeloid leukemia patients, it was identified that patients with high Thr308 AKT were presented with high-risk cytogenetics and poor overall survival [25]. A few examples of pathway activators, important downstream signaling molecules and inhibitors are given in Table 1.

c. Phospho protein analysis by flow cytometry employs the use of phospho specific monoclonal antibody against a known epitope. Here lies a fact that flow cytometry will not be very useful for searching a novel phospho protein or a site. Conventionally this work is done by biosynthetic labeling, immunoprecipitation , phosho amino acid analysis and protein kinase assays [26] . However, effect of stimuli, drugs or inhibitors on a particular molecule or its phospho state can be easily observed using this tool.

d. In cases where a little is known about these effects, assay controls become important wherein a same or different cell model with expected or known outcomes to a particular treatment can be used For example; HUT78 and U937 cell lines can be used for negative and positive controls respectively for phospho AKT estimation [16].

e With redundancy being a common biological phenomenon, pathway cross talk becomes important when one wants to observe effects coming from a single cascade specifically. In this situation knowledge of specific inhibitors or agents for switching off the cross talking pathway or using pathway agonists instead of biological activator can assist in analyzing the responses. Mendoza et al. [27] has reviewed cross talk of RAS-ERK and Phospho inositol 3 (Pi3)-Mammalian Target of Rapamycin (mTOR) pathways. In this review; cross inhibition, cross activation and pathway convergence of these two pathways at different points has been described.

2. **Sample source :** Source where from the cells of interest are derived should always be a concern since there are many important factors which rely on it, such as :

a. While analyzing response to a cytokine, one should see that cells of interest express the receptor for that particular cytokine. Work done by Marvin et al. [21] suggests a correlation between expression of GCSF receptor and phospho ERK stimulation. In case of transfected cell models for over or under expression of receptor; results should be drawn in light of the initial state of cells.

b. Source of cells becomes an important aspect for overall experimental protocol. Preparation of single cell suspension from solid tissues should not affect signaling experiment. Standard protocols for preparation of single cell suspension from glioblastoma multiforme and subsequent use in phospho flow analysis has been described by Kostianovsky et al [28].

Table 1: Examples of pathway activators, important downstream molecules, their activation / phosho specific sites and inhibitors

S.No.	Pathway	activators	Signaling molecule	site for activation	Inhibitors
1	**MAP kinase pathway**	Growth factors, mitogens	Ras	G protein (NRAS, H RAS, K-RAS) [42]	Farnesyltransferase inhibitors [43] Tipifarnib, Ionafarnib [44]
			Raf	A Raf: S432, T452,T455; B Raf: S579, S602, T599; -C Raf:T491, S494, S471 [45]	Sorafenib, PLX4032, plx 4037 [45]
			MEK	S217, S221 [46]	U0126, Selumitinib [47]
			ERK 1/2	T202, Y204 [48]	FR148083, hypothemycin [48]
		Stress factors, UV	JNK	tyr-thr in thr-pro-tyr motif [49]	SP600125 [50]
		Cell damaging agents, infections	p38	TGY motif [51]	sb202190 [51]
2	**PI3 /AKT**	Growth factors, insulin	RTK	Domains: TK, JM and c terminal (eg. IGF1 autophosphorylation on Y1162, Y1158, Y1163) [52]	Imatinib, Sumitinib, gefitinib[52]
			PI3 kinase	p110 kinase, p85 adapter subunit [53]	LY294002, PWT-458, CAL-101 [53,43]
			PDK1	Catalytic and PH domian [54]	UCN-01, OSU-03012 [43]
			m-TOR	catalytic domain (K2187-F2421) [55] phosphoryaltion sites: T2446, S2448, S2481, S1261 [56]	Rapamycin, WAY -600, PP242 [56]
			AKT	T308, S473, Y315, Y326 [57]	A-443654, GSK690693, BI 69A11 [57]
3	**JAK/STAT**	Growth factors, cytokines	JAK	JH1-JH7 domain (JH1:kinase domain) (Y980, Y981 in JAK3 activation loop) [58]	AFN941, Pd173955 [58] CMP-6, CP-690 [59]
			STAT	STAT1 (S727, S701) STAT3 (S727) STAT4 S(721) STAT5 (S725, S730, S779) [60], Stat1: Y701 [61]	WP-1034 [62]

c. Cell loss in manual assays is a common phenomenon, so one should be starting their experiment with enough number of cells of interest. In case of low cell counts, one might need to concentrate cell suspension in order to achieve final events which are amenable to analysis.

d. Cell clumping becomes a practical problem while working with high density cell suspensions. So checking cell count, appropriate dilution and adding certain protein such as serum albumin for blocking in wash solutions while being careful to reactivity of phospho antibodies can be helpful.

e. While working with cultured cells, one should consider the effect of media components and culture conditions on molecule of interest. Effect of calcium, serum, glucose and other components on cascade of interest should be well calculated. Glucose, amino acids, oxygen, growth factors and stress are known to regulate mTOR complex 1 [29].

3. Assay protocol : Enormous literature is available in the field of flow cytometry that is dedicated to optimization of experimental protocol for phospho protein analysis [19-21]. Generally, an assay of this kind will consist of series of streamline events: cellular activation/ inhibition followed by fixation-permeabilization, immuno staining, data acquisition and analysis. Commercial kits such as IntraPrep™ (Beckman Coulter), BD Cytofix /Cytoperm™ are provided with their standard operating protocol which has made these experiments very easy. The following points with which one should be familiar while seeking to perform these assays are enlisted follows:

a. Concentration and time are important aspects of cellular activation or inhibition and should be well optimized for assay system. Phosphorylation of proteins is a rapid and reversible event where after a fraction of time negative loop comes into play for system equilibrium. In other situation, a molecule might be degraded by an assembly in its phosphoryalted state unless a signal induces its conversion to non phospho state such as for beta catenin in Wnt signaling. Work done by Woost et al. [20] is an example of intensive time point based phospho protein estimation.

b. Assays including time dependent analysis, should be started with longest time point first so at that the shortest time point can come at the end and one could simultaneously fix cells in different tubes/wells.

c. Fixative agent could be a solution which can provide aldehyde or alcohol groups for protein cross linking. It can be formaldehyde, glutaraldehyde, methanol solution or something from commercially available kits used at an appropriate concentration and time [5, 19]

d. Fixation should be quick and enough to fix the state of protein molecules. Repeated pipetting or thorough vortex after addition of fixative is generally recommended. Insufficient mixing and excessive concentration of fixative solution cause aggregation of cells and clumping and harm scatter properties of cells. Generally formaldehyde solution can be added up to 1.6-4% of final concentration [19].

e. Permeabilization reagents can be divided into mild and harsh depending upon ingredient such as soap based or alcohol based respectively. Generally, cytoplasmic molecules such as phospho Erk is susceptible to harsh permeabilizing agents and can lose its epitope whereas signal from STAT proteins could be missed easily if harsh permeabilization is not employed.

f. Mostly cells are incubated in perm reagents for 30min to an hour. However, there are differences in opinion about temperature at which permeabilization should be performed. Largely, soap based permeabilization can be done at room

temperature or at 37^{0}C preferably if followed by formaldehyde fixation. However, while using methanol it is better to incubate cells on ice to achieve sufficient dehydration with minimum loss of scatter properties. Again at this point proper mixing of cells is recommended [5, 19 -20].

g. At the end of permeabilization, reaction should be stopped with appropriate buffer. Generally phosphate buffered saline with serum albumin is used which also helps in blocking non specific binding of intracellular antibodies.

h. Staining with intracellular phospho specific antibody can be done at room temperature for 30 -60 min. It is important to stain untreated tube as well which do not receive any stimuli but is processed similar to other tubes in order to estimate baseline fluorescence of antibody.

I. In case where unlabeled primary phospho specific antibody is used, choice of secondary antibody should be based on its origin and reactivity with blocking solution. For instance, while working with gelatin or bovine serum albumin based washing/blocking solution; use of a secondary antibody raised in swine or bovine model respectively can give certain degree of non specific binding or background (our unpublished data). Similarly, while working with cells derived from mice model, secondary should not be from same species. For example, if rabbit anti mouse phospho specific MAb is used then secondary can be goat anti rabbit or donkey anti rabbit but not mouse anti rabbit.

4. **Data acquisition and analysis :** As a rule in flow cytometry, a minimum of ten thousand cells of interest should be acquired, where cell counts are less or cells are rare a proper cluster of live cells should be analyzed. Signal to noise ratio is a well adopted method for data analysis in signaling experiment where normally ratio of fluorescence intensity of stimulated tube is divided by that of untreated one and fold change is calculated [14-17, 19-23,30].

Application of flow cytometry based cell signaling assay: Estimation of phospho protein levels by flow cytometry is an easy, rapid and robust approach to study signal transduction pathways. With availability of wide range of reagents and standard protocols signaling pathways can be studied at an ease in clinical settings as well. A few examples of clinical application of these assays are enlisted below :

Leukemia Research : Leukemogenesis in part is a result of mutant or deregulated signal transduction pathways where abnormality can be either in receptor, secondary messenger or transcription factor and\associated proteins. Mutations in important genes for receptors such as fms-related tyrosine kinase-3 (FLT3), c-kit; cell signaling mediators such as NRAS (neuroblastoma RAS viral (v-ras) oncogene homolog),KRAS; transcription factors as CEBPA (CCAAT/Enhancer-Binding Protein Alpha) and translocations involving kinase such as Abl are known to play crucial role in different types of hematological malignancies. Effect of various chromosomal translocations and gene mutations on import signal transduction pathways are known and extensive research is in progress to device treatment strategies based on it. Few examples where flow cytometric phospho protein estimation techniques have been used in leukemia research are enlisted here. Translocations involving Philadelphia chromosome is associated with unfavorable cytogenetics in leukemia. Kinase inhibitors such as Imatinib, sunitinib and many new agents are used for treatment; here these new agents can be evaluated in vitro for their action mechanism. Jacobberger et al. [31] had devised a\ flow cytometry based assay for evaluating bcr-abl kinase activity in K562 cell line using formaldehyde fixation and methanol permeabilization. They measured bcr-abl kinase activity by evaluating levels of tyrosine phosphorylated STAT5.

Chow et al. [32] had employed flow cytometry protocol for elucidating phosphorylation of S6 ribosomal protein in peripheral blasts of acute leukemia patients. Hedley et al. [33] employed flow cytometry based phospho protein level estimation protocol to show dose-dependent inhibition of c-Kit during a phase I clinical trial treating acute leukemia patients with sorafenib. Later, the same group had established a comprehensive standard design for cell signaling assay with core protocol of whole blood fixation, lysis and permealization [34].

Evaluation of new immunosupressants :

Transplantation medicine involve immunosuppressive agents for narrowing down possibilities of GVHD and graft rejection on the other hand, amphetamines are important agents in neurological medicines. New molecules can be analyzed in vitro by using phopsho flow techniques based on their effect on signal transduction pathways. In a recent study, Bredholt et al. [35] incorporated phospho flow techniques to analyze effects of amphetamines and derivatives on signal transduction pathways in leukocyte populations. They evaluated effect of immunosuppressive agents on phosphorylation of c-Cbl. ERK1/2, p38, AKT, NF-kB, p53, STAT1 and STAT6 in normal peripheral blood leukocytes.

Solid tumor research : Conventionally, flow cytometry is mainly used as a tool for blood and bone marrow based research. However, with advances in sample preparation techniques and in vitro research, it is possible to use this tool for research based on solid tumors as well. In a recent study, effect of microRNA-34a (mir-34a) on cell death of human gastric cancer cell line (SGC-7901) and cisplatin-resistant cell lines (SGC-7901/DDP) was elucidated by analyzing phospho AKT levels using multiparametric flow cytometry [36]. Varkar et al. [12] used flow cytometry for evaluation of phospho Stat 5 levels in immune cells of patients with renal cell carcinoma and metastatic melanoma before and after interleukin-2 immunotherapy.

Stem cell research : Most of the vital functions of a cell are critically regulated by signal transduction pathways. Cell quiescence, proliferation, differentiation and apoptosis are synchronous play of various signal cascades. Stem cell research is an important field for its obvious immediate application in transplantation medicine. Flow cytometry based cell signaling assay is a potent tool for stem cell research where stem cells from different sources can be subjected to various agents and functional assays can be performed at an ease and speed. Barbosa et al. [37] showed that PLCγ2 and PKC are important upstream signals in regulation of myelopoiesis through cytokines. They also characterized differences in downstream signaling by M-CSF and G-CSF mediated by ERK1/2 and STAT3 respectively which contribute to myeloid lineage commitment and differentiation. Recent application of flow cytometry based phospho protein estimation protocol was described by Chandesris et al [38].

Diagnosis of primary immunodeficiencies: Primary immune deficiencies are rare genetic disorders which create an immune compromised state in affected individuals. Mostly their onset starts early in life, affecting children and often are life threatening. Molecular basis of these disorders are certain mutations which ultimately effect signal transduction pathways critical in immune response mechanisms. Some of these disorders can be promptly diagnosed by evaluating activity of cell signaling molecules in immune cells of the affected individual. A few examples where multiparametric flow cytometry based cell signaling assays are used as a diagnostic tool in primary immune deficiency disorders are described in here.

The main genetic etiology of hyper immunoglobin E syndrome is an autosomal dominant deficiency of STAT-3. In a study involving 60 patients of Hyper IgE syndrome, STAT-3 tyrosine phosphorylation (Y705) levels were significantly lower than normal in response to IL-6, IL-10 and IL-21 [38]. X-Linked

Hypogammaglobulinemia can be detected by using flow cytometry based assay utilizing monoclonal antibody against Bruton tyrosine kinase. Peripheral blood monocytes of patients with this condition show lack of expression of BTK [39]. Orieji et al. [40] had described application of flow cytometry in diagnosis of primary immune deficiencies.

Likewise, flow cytometry based cell signaling assays can be very useful in devising strategies for diagnostic and therapeutic purpose both in clinical and laboratory settings when signal transduction pathways come into question. Recently, [41] Tanqri et al had compiled extensive data on validation of cell based fluorescence assay in form of practice guidelines wherein, information right beginning from selection of fluorochrome, instrument setup and tracking permeabilzation protocol, data acquisition, analysis and visualization is provided exquisitely.

In conclusion, signal transduction pathways can be studied conveniently using flow cytometry based single cell phospho protein estimation protocols both in research as well as clinical settings. Its rapid and efficient mode of operation definitely gives advantage over conventional tools such as western blot and

Hypogammaglobulinemia can be detected by using flow cytometry based assay utilizing monoclonal antibody against Bruton tyrosine kinase. Peripheral blood monocytes of patients with this condition show lack of expression of BTK [39]. Orieji et al. [40] had described application of flow cytometry in diagnosis of primary immune deficiencies.

Likewise, flow cytometry based cell signaling assays can be very useful in devising strategies for diagnostic and therapeutic purpose both in clinical and laboratory settings when signal transduction pathways come into question. Recently, [41] Tanqri et al had compiled extensive data on validation of cell based fluorescence assay in form of practice guidelines wherein, information right beginning from selection of fluorochrome, instrument setup and tracking permeabilzation protocol, data acquisition, analysis and visualization is provided exquisitely.

In conclusion, signal transduction pathways can be studied conveniently using flow cytometry based single cell phospho protein estimation protocols both in research as well as clinical settings. Its rapid and efficient mode of operation definitely gives advantage over conventional tools such as western blot and

ELISA. Various instrumental up gradations such coupling of flow cytometry with confocal microscopy or Atomic Force Microscopy (AFM) and mass spectrometry has enhanced the potential of this tool to manifold. This technical review can help beginners in the field for assay designing and trouble shooting. It can also come handy as a gateway to literature of cell signaling experiments where experts have provided enormous information related to these assays and their applicability.

Utilisation of these protocols at National Institute of Immunohaematology :

Diagnosis of Primary Immunodeficiency involves expression analysis of various cell signaling related molecules for example expression of Bruton Tyrosine Kinase (BTK) on monocytes and IL-2 induced expression of phospho STAT-5 on T-cells for confirmatory diagnosis of SCID. These assays are routinely being used for PID diagnosis as well as in the research project where they are being used to study cell signaling pathways in acute myeloid leukemia (AML). Constitutive expression of various cell signaling molecules in myeloid blasts of leukemia patients was correlated with poor response to the treatment and overall disease outcome.

References :

1. Sawyer TK, Shakespeare WC, Wang Y, et al. Protein phosphorylation and signal transduction modulation: chemistry perspectives for small-molecule drug discovery. Med Chem 2005; 1:293-319.
2. Cohen P. The regulation of protein function by multisite phosphorylation--a 25 year update. Trends Biochem Sci 2000; 25:596-601.
3. Shawn Li. Mechanisms of Cellular Signal Transduction. Int J Biol Sci 2005; 1: 152.
4. Berg JM, Tymoczko JL, Stryer L. Biochemistry. 5th edition. New York: W H Freeman; 2002. Chapter 15, Signal-Transduction Pathways: An Introduction to Information Metabolism. Available from : http://www.ncbi.nlm.nih.gov/books/NBK21205
5. Suni MA, Maino VC. Flow cytometric analysis of cell signaling proteins. Methods Mol Biol 2011; 717:155-69.
6. Krebs EG, Fischer EH (1956). The phosphorylase b to a converting enzyme of rabbit skeletal muscle. 1989. Biochim Biophys Acta, 1000, 302-309.
7. Krutzik PO, Irish JM, Nolan GP et al. Analysis of protein phosphorylation and cellular signaling events by flow cytometry: techniques and clinical applications. Clin Immunol 2004 ;110:206-21.
8. Jacques A. Nunes, Guylene Firaguay and Emilie Coppin . Analysis of Cellular Signaling Events by Flow Cytometry. Flow Cytometry - Recent Perspectives, M.Sc. Ingrid Schmid (Ed.). Available from: http://www.intechopen.com/books/flow--cytometry-recent-perspectives/analysis-of cellular-signaling-events-by-flow cytometry.
9. Fierring S, Northrop JP, Nolan GP et al. Single cell assay of a transcription factor reveals a threshold in transcription activated by signals emanating from the T-cell antigen receptor. Genes Dev 1990; 4:1823 -34.
10. Müller C, Bonmann M, Cassens U et al. Flow cytometric analysis of protein phosphorylation in the hematopoetic system. Leuk Lymphoma 1998; 29:351-60.
11. Fleisher TA, Dorman SE, Anderson JA et al Detection of intracellular phosphory lated STAT-1 by flow cytometry. Clin Immunol 1999; 90:425-30.
12. Varker KA, Kondadasula SV, Go MR et al. Multiparametric flow cytometric analysis of signal transducer and activator of transcription 5 phosphorylation in immune cell subsets in vitro and following interleukin-2 immunotherapy. Clin Cancer Res 2006; 12:5850-58.
13. Perez OD, Nolan GP. Simultaneous measurement of multiple active kinase states using polychromatic flow cytometry. Nat Biotechnol 2002; 20:155-62.
14. Montag DT, Lotze MT. Successful simultaneous measurement of cell membrane and cytokine induced phosphorylation pathways [CIPP] in human peripheral blood mononuclear cells. J Immunol Methods 2006; 30:48-60.
15. Montag DT, Lotze MT. Rapid flow cytometric measurement of cytokine-induced phosphorylation pathways [CIPP] in human peripheral blood leukocytes. Clin Immunol 2006; 121:215-26.
16. Firaguay G, Nunès JA. Analysis of signaling events by dynamic phosphoflow cytometry. Sci Signal. 2009; 2 :86.
17. Krutzik PO, Nolan GP. Intracellular phospho-protein staining techniques for flow cytometry: monitoring single cell signaling events. Cytometry A. 2003; 55:61-70.
18. Krutzik PO, Nolan GP. Fluorescent cell barcoding in flow cytometry allows high-throughput drug screening and signaling profiling. Nat Methods 2006; 3:361-68.

19. Chow S, Hedley D, Grom P et al. Whole blood fixation and permeabilization protocol with red blood cell lysis for flow cytometry of intracellular phosphorylated epitopes in leukocyte subpopulations. Cytometry A. 2005; 67:4-17.
20. Woost PG, Solchaga LA, Meyerson HJ et al. High-resolution kinetics of cytokine signaling in human CD34/CD117-positive cells in unfractionated bone marrow. Blood 2011; 117:131-41.
21. Marvin J, Swaminathan S, Kraker G et al. Normal bone marrow signal-transduction profiles: a requisite for enhanced detection of signaling dysregulations in AML. Blood. 2011; 117:120-30.
22. Bendall SC, Simonds EF, Qiu P et al. Single-cell mass cytometry of differential immune and drug responses across a human hematopoietic continuum. Science 2011; 332:687-96.
23. Chow S, Patel H, Hedley DW. Measurement of MAP kinase activation by flow cytometry using phospho-specific antibodies to MEK and ERK: potential for pharmacodynamic monitoring of signal transduction inhibitors. Cytometry 2001; 46:72-78.
24. Wen Z, Zhong Z, Darnell JE Jr. Maximal activation of transcription by Stat1 and Stat3 requires both tyrosine and serine phosphorylation. Cell 1995; 82:241-50.
25. Gallay N, Dos Santos C, Cuzin L et al. The level of AKT phosphorylation on threonine 308 but not on serine 473 is associated with high-risk cytogenetics and predicts poor overall survival in acute myeloid leukaemia. Leukemia 2009; 23:1029-38.
26. Chen RH, Sarnecki C, Blenis J. Nuclear localization and regulation of erk- and rsk-encoded protein kinases. Mol Cell Biol 1992; 12:915-27.
27. Mendoza MC, Er EE, Blenis J. The Ras-ERK and PI3K-mTOR pathways : cross-talk and compensation. Trends Biochem Sci 2011; 36:320-28.
28. Kostianovsky AM, Maier LM, Anderson RC etal. Astrocytic regulation of huma monocytic/microglial activation. J Immunol 2008; 181:5425-32.
29. Sengupta S, Peterson TR, Sabatini DM. Regulation of the mTOR complex 1 pathway by nutrients, growth factors, and stress. Mol Cell 2010; 40:310-22.
30. Kalaitzidis D, Neel BG. Flow-cytometric phosphoprotein analysis reveals agonist and temporal differences in responses of murine hematopoietic stem/progenitor cells. PLoS One. 2008; 3:e3776.
31. Jacobberger JW, Sramkoski RM, Frisa PS et al. Immunoreactivity of Stat5 phosphorylated on tyrosine as a cell-based measure of Bcr/Abl kinase activity. Cytometry A. 2003; 54:75-88.
32. Chow S, Minden MD, Hedley DW. Constitutive phosphorylation of the S6 ribosomal protein via mTOR and ERK signaling in the peripheral blasts of acute leukemia patients. Exp Hematol 2006; 34:1183-91.
33. Hedley DW, Chow S, Goolsby C et al. Pharmacodynamic monitoring of molecular-targeted agents in the peripheral blood of leukemia patients using flow cytometry. Toxicol Pathol 2008; 36:133-39.
34. Hedley DW, Chow S, Shankey TV. Cytometry of intracellular signaling: from laboratory bench to clinical application. Methods Cell Biol 2011; 103:203-20.
35. Bredholt T, Ersvær E, Erikstein BS et al. Distinct single cell signal transduction signatures in leukocyte subsets stimulated with khat extract, amphetamine-like cathinone, cathine or norephedrine. BMC Pharmacol Toxicol 2013; 14:35.
36. Cao W, Yang W, Fan R et al. miR-34a regulates cisplatin-induce gastric cancer cell death by modulating PI3K/AKT/survivin pathway. Tumour Biol. 2013. 13277:1171-7.

37. Barbosa CM, Bincoletto C, Barros CC, Ferreira AT, Paredes-Gamero EJ. Ca^{2+} signaling proteins PLCγ2 and PKC are important to myeloid lineage commitment: Differences in signaling triggered by M-SCF and G-CSF. 2014 ;115:42-51
38. Chandesris MO, Melki I, Natividad A et al. Autosomal dominant STAT3 deficiency and hyper-IgE syndrome: molecular, cellular, and clinical features from a French national survey. Medicine (Baltimore). 2012; 91: 1-19.
39. Futani T, T Miyawaki, S. Tsukada et al. Deficient expression of Bruton's tyrosine kinase in monocytes from X-linked agammaglobinemia as evaluated by a flow cytometric analysis and its clinical application to carrier detection. Blood 1998. 91: 595–602.
40. Orieji C. Illoh. Current Applications of Flow Cytometry in the Diagnosis of Primary Immunodeficiency Diseases. Archives of Pathology & Laboratory Medicine: 2004, Vol. 128, pp. 23-31.
41. Tanqri S, Vall H, Kaplan D, Hoffman B et al.; on behalf of ICSH/ICCS Working Group.Validation of cell-based fluorescence assays: Practice guidelines from the ICSH and ICCS - part III - analytical issues. Cytometry B Clin Cytom 2013; 84:291-308.
42. Goodsell DS. The molecular perspective: the ras oncogene. Oncologist 1999; 4:263-64.
43. Kloog Y, Cox AD. RAS inhibitors: potential for cancer therapeutics. Mol Med Today 2000; 6:398-402.
44. Chappell WH, Steelman LS, Long JM et al. Ras/Raf/MEK/ERK and PI3K/PTEN/Akt/ mTOR inhibitors: rationale and importance to inhibiting these pathways in human health. Oncotarget 2011; 2:135-64.
45. Takashima A, Faller DV. Targeting the RAS oncogene. Expert Opin Ther Targets 2013; 17:507-31.
46. Roskoski R Jr. RAF protein-serine/threonine kinases: structure and regulation. Biochem Biophys Res Commun 2010; 399:313-17.
47. Alessi DR, Saito Y, Campbell DG et al. Identification of the sites in MAP kinase kinase-1 phosphorylated by p74raf-1. EMBO J 1994; 13:1610-19.
48. McCubrey JA, Steelman LS, Chappell WH et al. Ras/Raf/MEK/ERK and PI3K/PTEN/Akt/ mTOR cascade inhibitors: how mutations can result in therapy resistance and how to overcome resistance. Oncotarget 2012; 3:1068-111.
49. Roskoski R Jr. ERK1/2 MAP kinases: structure, function, and regulation. Pharmacol Res 2012; 66:105-43.
50. Nishina H, Wada T, Katada T. Physiological roles of SAPK/JNK signaling pathway. J Biochem 2004; 136:123-6.
51. Karin M, Gallagher E. From JNK to pay dirt: jun kinases, their biochemistry, physiology and clinical importance. IUBMB Life 2005; 57:283-95.
52. Roux PP, Blenis J. ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. Microbiol Mol Biol Rev. 2004; 68:320-44.
53. Lemmon MA, Schlessinger J. Cell signaling by receptor tyrosine kinases. Cell. 2010; 141:1117-34.
54. Fan QW, Knight ZA, Goldenberg DD et al. A dual PI3 kinase/mTOR inhibitor reveals emergent efficacy in glioma. Cancer Cell 2006; 9:341-49.
55. Komander D, Fairservice A, Deak M et al. Structural insights into the regulation of PDK1 by phosphoinositides and inositol phosphates. EMBO J 2004; 23:3918-28.

56. Chang-Chih Wu, Po-Chien Chou, Estela Jacinto (2012). The Target of Rapamycin: Structure and Functions, Protein Kinases. Gabriela Da Silva Xavier (Ed.), ISBN: 978-953-51-0640-1, InTech, DOI: 10.5772/37927. Available from: http://www.intechopen.com/books/protein-kinases/the-target-of-rapamycin-structure-and-functions.
57. Watanabe R, Wei L, Huang J. mTOR signaling, function, novel inhibitors, and therapeutic targets. J Nucl Med 2011; 52:497-500.
58. Madhunapantula SV, Mosca PJ, Robertson GP. The Akt signaling pathway: an emerging therapeutic target in malignant melanoma. Cancer Biol Ther 2011; 12:1032-49.
59. Boggon TJ, Li Y, Manley PW et al. Crystal structure of the Jak3 kinase domain in complex with a staurosporine analog. Blood 2005; 106:996-1002.
60. Williams NK, Bamert RS, Patel O et al. Dissecting specificity in the Janus kinases: the structures of JAK-specific inhibitors complexed to the JAK1 and JAK2 protein tyrosine kinase domains. J Mol Biol 2009; 387:219-32.
61. Chindler C, Levy DE, Decker T. JAK-STAT signaling: from interferons to cytokines. J Biol Chem 2007; 282:20059-63.
62. Lemmon MA, Schlessinger J. Cell signaling by receptor tyrosine kinases. Cell 2010; 141:1117-34.
63. Faderl S, Ferrajoli A, Harris D et al. A novel JAK-STAT inhibitor, with proapoptotic and antileukemic activity in acute myeloid leukemia (AML). Anticancer Res 2005; 25:1841-50.

NIIH Happenings

Dr K Ghosh, Director

1. Invited to deliver a lecture on Hepatitis B at Haffkine Institute on 25th September 2013.
2. Elected as President (Medical) of Hemophilia Federation of India on 28th September 2013.
3. Invited as an Expert Member for the Scientific Advisory Committee Meeting of Span Diagnostic held at Surat on 5th October 2013.
4. Invited as a Member of Project Review Committee Meeting at ICMR, New Delhi on 5th November 2013.
5. Attended the Technical Resource Meeting at ICMR, New Delhi on 4th November 2013.
6. Attended the HAEMATOCON 2013 held at Mumbai from 7th to 10th November 2013.
7. Attended the CME programme of Haemophilia Chapter at Chennai from 16th-17th November 2013
9. Invited as an Expert for the Tribal Research Group Meeting at ICMR on 5th December 2013.
10. Attended the Thalassaemia Diagnostic Kit release function at ICMR, New Delhi on 17th December 2013
11. Invited as an External Examiner to conduct MD Transfusion Medicine exams at PGIMER, Chandigarh on 9th and 10th December 2013.
12. Attended the Foundation stone laying ceremony for Centre of Hemoglobinopathies at Chandrapur on 24th December 2013.

Department of Hematogenetics

Dr Roshan Colah, Scientist F

1. Invited as a guest faculty to organize a workshop on PCR for prenatal diagnosis of sickle cell disorders and seminar on hemoglobinopathies and to participate in public forum meeting and delivered a talk on "Prenatal diagnosis of sickle cell disorders in India" at Indira Gandhi Medical College, Nagpur from 19th to 21st September, 2013.
2. Invited as a Guest Speaker to talk on "Experiences with newborn screening for sickle cell disease in India" at the "8th Asia Pacific Regional Meeting of Newborn Screening" held at AIIMS, New Delhi from 27th to 29th Sep'2013.
3. Invited to deliver a lecture on "HPLC – A Versatile tool for identification of Hb Variants in the multi-ethnic Indian Population" at the Hematocon Conference 2013 held at Mumbai from 7th to 10th November, 2013
4. Attended a meeting in connection with RDB kits at ICMR, New Delhi on 12th November, 2013.

Dr Malay Mukherjee, Scientist D

1. Awarded ICMR BGRC Silver Jubilee Oration Award for the year 2010.
2. Attended the Foundation stone laying ceremony of the Centre for Hemoglobinopathies at Chandrapur on 24th December 2013.

Dr Anita Nadkarni, Scientist D

1. Attended 2nd National Congress of Indian Society of Transfusion Medicine held at Bangalore from 13th to 15th Sept 2013 and received Best Poster Award on a poster entitled "Effect of the hemochromatosis mutations on iron overload among the Indian β thalassemia carriers"

Dr P S Kedar, Technical Officer

1. Attended and presented two papers at 54th Annual Conference of Indian Society of Hematology and Blood Transfusion held at Mumbai from 7th to 10th November 2013.

i. Red cell membrane pathology in hereditary spherocytosis in India.

ii. Automated high speed micro fluidic chip based capillary electrophoresis technique for the identification of the red cell membrane protein defect.

Dr Prashant Warang, Technical Assistant

1. Attended 54th Annual Conference of Indian Society of Hematology and Blood Transfusion held at Mumbai from 7th to 10th November 2013 and presented a paper entitled "Effect of UGT1A1 and hemoxygenase gene polymorphisms on serum bilirubin levels of patients with hereditary spherocytosis".

   Following students have attended the 54th Annual Conference of Indian Society of Hematology and Blood Transfusion held at Mumbai from 7th to 10th November 2013 and made oral/poster presentation;

Ms Dipti Upadhye

1. Influence of single nucleotide polymorphisms in the BCL11A gene on Hb F levels and the clinical presentation of sickle cell disease in central India

Ms Vrushali Pathak

1. Major blood group antigens and growth of *P. falciparum* in *invitro* culture.

Ajay K Chaudhary

1. Role of Matrix Metalloproteinases and its inhibitors in hematological malignancies.

Department of Transfusion Medicine

Dr Ajit Gorakshakar, Scientist E

1. Attended 38th Annual Conference of Indian Society of Blood Transfusion and Immunohematology held at Surat from 18th to 20th October 2013 and delivered a talk entitled "Molecular Blood Group Typing in Immunohematology Laboratory" in the plenary session on "Future Transfusion Medicine".
2. Attended 54th Annual Conference of India Society of Hematology and Blood Transfusion held at Mumbai from 7th to 10th November 2013 and delivered a talk on " Iron Status in Regular Blood Donors : An Emerging Problem."
3. Won First Prize for Dr Homi Bhabha Science Essay Competition in Hindi, 2012,Organised by Hindi Vigyan Sahitya Parishad, BARC, Mumbai, on an Essay entitled " Kya Aap Jante Hai Ki Aap Thalassemia ke Wahak Ho Sakate Hai?".

Dr Swati Kulkarni, Scientist C

1. Invited to attended 2nd National Conference of Indian Society of Transfusion Medicine held at Bangalore from 13th to 15th September 2013 and delivered the following lectures :

i. Non-invasive fetal blood group typing.
ii. Blood Grouping by molecular methods.

2. Attended Pre Conference CME on Basic and Advance in Immunohaematology held at Surat from 16th to 17th November 2013 and delivered a lecture on "Genetics of Rh Blood group system & significance of detecting partial D".
3. Attended 38th Annual conference of Indian Society of Blood Transfusion and Immunohaematology held at Surat from 18th to 20th November 2013 and received Best Oral Presentation award on a paper entitled "Quality assurance of reagent red blood cells by DNA analysis".

Department of Hemostasis

Dr Shrimati Shetty, Scientist E

1. Invited to deliver a lecture on "how I detect factor VIII inhibitors?" in the 54th Annual Conference of Indian Society of Hematology and Blood Transfusion held at Mumbai from 7th to 10th November 2013.

Dr Bipin P. Kulkarni, Scientist B

1. Attended 54th Annual Conference of Indian Society of Hematology and Blood Transfusion held at Mumbai from 7th to 10th November 2013 and received 3rd Best Poster Award for a poster entitled "A Covalent Reverse Dot Blot (CRDB) hybridization assay for quick detection of arginine hot- spot mutations in von Willebrand Disease".

Following students have attended the 54th Annual Conference of Indian Society of Hematology and Blood Transfusion held at Mumbai from 7th to 10th November 2013 and presented the papers;

Ms Rucha Patil :

1. Can microparticles be a marker for heparin dose adjustment in women with recurrent pregnancy loss?

Ms Patricia Pinto :

1. Prevalence of Inhibitors in Indian Haemophilia patients and was awarded 2nd Best Oral Presentation.
2. A Study of F8 Intron 22 and Intron 1 Inversions as Predisposing Risk Factors for Inhibitor Development in Indian Severe Haemophilia A patients.

Ms Preethi S. Nair :

1. Molecular pathology of haemophilia A in patients from Western India.

Ms Priyanka Kasatkar :

1. Challenges in the molecular diagnosis of von Willebrand disease.

Department of Paediatric Immunology and Leukocyte Biology

Dr Manisha Madkaikar, Scientist E

1. Invited to deliver a lecture on ' Spectrum of hematological disorders' in the 3rd conference of the Indian Society for histocompatibility and immunogenetics 2013 held at All India Institute of Medical Sciences, New Delhi from 7th to 9th October 2013.
2. Invited to deliver a lecture on 'Primary Immunodeficiency disorders' in 14th Indo-US Flow Cytometry Workshop and clinical symposium held at Medanta, The Medicity, Gurgoan on 18th and 19th October 2013.
3. Invited to deliver a lecture on 'Diagnostic approach to Primary Immunodeficiency Disorders' in PHOCON 2013 conference held at Delhi on 19th October 2013.
4. Invited to conduct 'Meet the professor: Common Variable Immunodeficiency' at 54th Annual Conference of Indian Society of Haematology & Blood Transfusion held at Mumbai from 7th to 10th November 2013.
5. Invited to deliver a talk on 'How to Diagnose PID' in the CME on PID organized by Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow on 10th November 2013.
6. Invited to deliver a talk on 'Approach to Primary Immunodeficiency Disorders' in Seth G S Medical College, Mumbai on 17th December 2013.

Following staffs have attended the 54th Annual Conference of Indian Society of Hematology and Blood Transfusion held at Mumbai from 7th to 10th November 2013 and presented the posters;

Ms Anju Mishra

1. Mutation analysis of ITGβ_2 gene in patients with Leukocyte adhesion deficiency.

Ms Maya Gupta

1. Flow cytometry based prenatal diagnosis of Primary Immunodeficiency disorders.

Ms Aparna Dalvi

1. Characterization of CD40LG gene in patients with X-Linked Hyper IgM syndrome.

Ms Snehal Mhatre

1. Development of rapid and cost-effective flow cytometry based whole blood assays for NK cell function evaluation for diagnosis of Hemophagocytic Lymphohistiocytosis (HLH) and was awarded J C Patel award.

Ms Khushnooma Italia

1. Hydroxyurea – a new iron chelator on the horizon.

Department of Cytogenetics

Dr V. Babu Rao, Scientist D

1. Appointed as a member of the selection committee the interview the candidates for the Ph.D programme at NIRRH, on 22nd and 23rd October 2013.

2. Organized a workshop on "Karyotyping" as a part of Hematocon-2013 on 6th November 2013.
3. Delivered a guest lecture on "Study of major Fanconi anemia gene FANCA mutations in Indian population" in the Hematocon-2013 conference held at Mumbai from 7th to 10th November 2013.
4. Appointed as Chairman for the selection committee to interview the junior research fellow at NIRRH on 4th December 2013.

Ms Seema Korgaonkar, Technical Officer A

1. Attended Hematocon 2013 held at Mumbai from 7th to 10th November 2013 and presented a poster entitled "Cytogenetic and molecular study of Myelodysplastic syndrome in Indian Population"

   Following students have attended the 54th Annual Conference of Indian Society of Hematology and Blood Transfusion held at Mumbai from 7th to 10th November 2013 and presented the papers;

Ms Shantashri Vaidya

1. Understanding the interplay between BCR/ABL dependent and independent mechanisms of imatinib resistance.

Ms Dolly Joshi

1. Telomerase activity and telomerase gene mutation in aplastic anemia patients.

Library

Mr Vijay Padwal, ALIO

1. Attended a National Seminar on "Convergence of Resource, Technologies and Services : Trends and Challenges" held at Tata Memorial Hospital, Parel from 21st to 22nd October 2013.
2. Attended a meeting of Federation of All India ICMR Employees held at National Institute for Research in Reproductive Health, Mumbai on 28th November 2013.
3. Attended a training program on E-publishing and E-procurement of National Informatic Centre held at National Institute for Research in Reproductive Health, Mumbai on 12th December 2013.

Pledge ceremony during observance of vigilance awareness week 2013

Dr Malay Mukherjee, Scientist D receiving ICMR BGRC Silver Jubilee award from Shri Ghulam Nabi Azad, Hon'ble Minister, Health & Family Welfare, Govt. of India

Scientific Advisory Committee Meeting of the Institute

EDITORIAL BOARD

Immunohaematology Buttetin is Brought out by :

NATIONAL INSTITUTE OF IMMUNOHAEMATOLOGY

13th Floor, NMS Building, KEM Hospital, Parel, Mumbai 400 012. (INDIA) Web : www.niih.org.in