Date: January, 2018
Institute Laboratories' Research Promotion Office RIKEN

Research Project Mid-term Evaluation Result

The following research project underwent a mid-term evaluation in accordance to Clause 10 and 11, Chapter 2 of the *Regulations for Research and Development Evaluations* (Regulation No. 74, October 1, 2003.)

Evaluation system:
Out of five reviewers, two experts from outside of RIKEN and three RIKEN Science Council Research Programs Committee members were appointed as reviewers for the following research project. The reviewers evaluated the project based on the reporting session held on November 9, 2017.

Reviewers list:
External experts (alphabetical order)
1) Toyoshi FUJIMOTO, Professor, Nagoya University Graduate School of Medicine
2) Shiroh FUTAKI, Professor, Institute for Chemical Research, Kyoto University

RIKEN Science Council Research Program committee member (alphabetical order)
3) Sidonia FAGARASAN, Team Leader, Laboratory for Mucosal Immunity, IMS
4) Yukishige ITO, Chief Scientist, Synthetic Cellular Chemistry Laboratory
5) Ken SHIRASU, Group Director, Plant Immunity Research Group, CSRS

Research project brief overview
Project name: Integrated lipidology
Project Leader： Yasushi SAKO
Project duration： April, 2014～March, 2019（5 years）
Budget allocated：Total of 432,980 thousand Yen（past 4 years）
Research overview：
The fundamental structure of biomembranes is a lipid bilayer. Although the bilayer can be reconstituted using a single lipid species, thousands of lipid species exist in our body. The reason of this diversity is still unknown. Many diseases such as atherosclerosis, heart disease and metabolic syndrome are considered lipid disorders. Metabolic disorders of lipid are called lipidosis, and our life fundamentally relies on lipid. To date, however, lipid research lags behind the protein- and nucleic acids research mainly for two reasons. Firstly, the water-insoluble property of lipids makes the experiments difficult. Secondly, lipids are not the primary products of gene expression. Recent scientific advances overcame these difficulties; we can now study metabolism, distribution and dynamics of individual lipids.
This project, “Integrated Lipidology” started in 2014 by employing highly original techniques of advanced lipid and biomembrane research, such as lipid imaging, single cell mass spectrometry, and single molecular dynamics measurements and simulation of lipids and proteins, to elucidate the

fundamental functions of lipids. We also develop diagnostic, curative and preventing methods useful in lipid disorders.

1. Comprehensive Evaluation (To be disclosed)

1) Evaluation on five-grade scale	S	A	B	C	D
(1) Research objective:	1	3	1	0	0
(2) Implementation of research plan:	0	4	1	0	0
(3) Research achievement:	0	5	0	0	0
(4) Future research plan:	0	4	1	0	0

S Outstanding / A Excellent / B Good / C Acceptable / D Not acceptable

2) Evaluation details (reviewer's number is different from the order of the above list)

＜Reviewer 1＞

(1) Research objective

The purpose of this project is to identify single lipid species and to study their metabolism, distribution, dynamics as well as functional role in vivo. The project is ambitious, given the difficulty in studying lipids and their complex roles. In order to advance the lipidomic studies, RIKEN assembled and supported a team of specialists able to tackle such problems in terms of technology development and application. The project is wisely organized into three groups, dealing with development of lipid visualizing probes (SP1 headed by Toshihide Kobayashi), studying lipid-protein interactions (SP2, headed by Yasushi Sako) and dynamic lipid physiology (SP3 headed by Yoshio Hirabayashi). They generated a collaboration network called integrated lipidology.

(2) Implementation of research plan and (3) Research Achievement

The achievements of the whole group are very impressive, and the number of the publications mind blowing. The SP1 group indeed contributed to the discovery of several highly specific lipid probes and also developed technologies aiming to visualize and study their function. Although a very unfortunate loss of one of its leader, progress and interesting results came from single cell mass spectrometry analyses. The metabolome characterization of single cells or even single organelle in for example extremely rare cells-such as solid tumor cells mobilized and circulating in blood-is indeed very promising in terms of diagnostic and therapeutics.

The SP2 group focusing on lipid-protein interactions on membranes made progress with imaging the movement, clustering and the consequences in terms of signal transduction. The SP3 group also made good progress with placing the glycolipids and sphingolipids or their receptors in the context of physiology-particularly the brain and adipose tissues-associated with neurodegenerative and autoimmune or metabolic diseases. The characterization of novel lipid mediator-Lyso-phosphatidylglucoside in neuron-glial cell interaction is particularly impressive. In addition the visualization by IMS of lipid molecules in brain in association with devastating

diseases is important. The studies related with Hikeshi will likely generate interesting new directions. Finally, the group of Koyasu makes an interesting and important bridge between the lipid studies and the innate immune responses associated with inflammatory metabolic diseases.

(4) Future research plan

The research plans written by each participant are well organized. Given the expertise and commitment to science of the researches involved in the project I also expect new exciting developments. Perhaps more synergy will develop between (especially SP2 group) and within the three subgroups.

＜Reviewer 2＞

(1) Research objective

It has been previously considered that cell membranes only act as hydrophobic barriers to separate the interior and the exterior of the cell. However, recent studies have been highlighting the importance of membrane conditions (including lipid compositions, curvature, tension, and fluidity) as decisive factors affecting cellular homeostasis. The membrane is also associated with membrane proteins, which include receptors and transporters that are involved in the transmission of biological signals into cells. Interestingly, it has also been elucidated that membrane conditions have a considerable role in the activation of these proteins. Alternatively, protein structural alterations of these membrane proteins also affect the packing states of the membranes, which may affect also the assembly state and activation of other membrane proteins. Moreover, disorders on membrane states are also closely related to occurrence of diseases.

Necessary technologies for achieving this goal are: (i) Identification and localization of lipid components in live-cell membranes; spatiotemporal analysis of lipid molecules should be ideal to understand membrane dynamics and resulting effects in live cells. Development of high-sensitivity mass spectrometry, synthetic methodology, and live cell imaging should contribute much to achieve this goal. (ii) Methodologies to analyze membrane-protein interactions are also indispensable. Biophysical approaches including single-molecule analysis of membrane proteins in live cells plays a role in this context. (iii) Roles of membranes, lipids, and the related molecules to maintain cellular conditions and their relationships with our life (including health, disease, and aging) are also an important aspect of studies in “lipidology”.

Therefore, the topic of this Pioneering Project, “Integrated Lipidology”, is timely and highly relevant in current life science researches. Findings in this project should bring conceptual breakthroughs for understanding cell functions and the mechanisms, having significant molecular cell-biological and therapeutic implications.

(2) Implementation of research plan

This Pioneering Project is comprised of three sub-projects (SPs), which includes Advanced Lipidomics (SP1), Molecular Protein Lipidology (SP2) and Dynamic Lipid Physiology (SP3).

The objective of SP1 is to develop research tools/techniques/database that are available for all project members and for the lipid society outside of RIKEN. Since Dr. Kobayashi left for CNRS, the leadership was transferred to Dr. M. Arita. However, the participation of Dr. Arita, while Dr. Kobayashi was still in the team, seemed to have greatly strengthened SP1 by achieving the development and application of novel lipid-specific probes, and the development of live single-cell mass spectrometry and multi-lipidomecs, and so on.

SP2 has six major research topics which includes the following: (i) single-molecule measurements of lipid-protein interactions; (ii) molecular dynamics simulation of lipid-protein structures and interactions; (iii) X-ray crystallography of membrane proteins; (iv) cryo-EM and XFEL of lipid-protein complexes; (v) solid-state NMR analysis of lipid-protein interactions; and (vi) image correlation spectroscopy of lipid-protein assembly. Dr. Sako, the leader of SP2 (succeeded the leader position of the project), has been pursuing single-molecule imaging of EGFR, with tight collaboration with Dr. Kobayashi (SP1). This work has a considerable impact on the understanding of behaviors involved in the activation of receptor tyrosine kinases (RTKs) and related molecules; these proteins have critical roles in growth and tumor progression.

SP3 has been focusing on identifying new lipid metabolites and on understanding their physiological functions, in relation to lipid abnormalities and the onset, progression and severity of diseases. Dr. Y. Hirabayashi predominantly contributed in identifying novel glucosylated lipids, and in the elucidating physiological significance of these lipids, while preparing animal models having lipid metabolic disorders. The roles of GPCR in fatty acid-induced insulin resistance and energy homeostasis were studies in collaboration with with Dr. Sako of SP2. However, collaborations among SP3 may not be very apparent at current stage.

Overall, this Pioneering Project “Integrated Lipidology” is operated in good collaboration between three SPs. However, enhanced collaboration within and among SPs should yield synergic effects and the more fruitful accomplishments in the end of the research project.

(3) Research achievement

One of the major and outstanding accomplishments in this Pioneering Project is the development of lipid-specific probes. Although heterogeneity in lipid composition is crucial to understand the biological and physiological roles, very few means are available to observe this in real time on live-cell membranes. Especially, importance of lipid rafts on signal transmission into cells (e.g., by controlling receptor structures and aggregation states), nutrition uptake, and viral infection have been reported. Detailed understanding of the formation and dynamics of raft should give us profound information on cell biology, and on its pathological and therapeutic implications. Dr. Kobayashi and his collaborators found several important and unique protein/peptide probes that can specifically recognize membrane lipids. Especially one of the probes, named “Nakanori”, can be utilized as a powerful tool to identify lipid rafts. Using these probes, novel findings on heterogeneities of lipid distribution has been elucidated. It should be also noted that these probes are now available through RIKEN BRC. Further development of these probes should not only

contribute to this Pioneering Project but to the studies on lipid biology and pathology. I therefore greatly expect progress in this particular aspect of lipid visualization on live-cell membranes with the help of mass analysis by Dr. Arita of SP1 and in collaboration with SP2 (live-cell observation of raft/receptor) and SP3 (pathological relationship) members. Development of live single-cell mass spectrometry and of multi-lipidomics should also be of great help to identify and study physiological contributions of various lipids. It should also be noted that the relationship of EGFR activation and raft fragmentation is currently being elucidated (by Dr. Sako), as well as the identification of minor lipids acting as ligands of orphan GPCR (by Dr. Hirabayashi); these findings are highly important and can be breakthroughs in molecular cell biology.

It is amazing that this project resulted to almost 250 publications in the past 3.5 years. Most notably, this also includes 20 collaboration works among the research groups. However, I feel that the researchers in related projects should not be too pressured to increase numbers of publication for evaluation, but allow to spare time and energy for deepening their research.

(4) Future research plan

Future plans proposed are based on the accomplishments and progresses up to now, considering should-be-solved problems and challenges, and are thus reasonable. Along with research progress, greater collaboration within and among SPs can be expected. Since lipidology is a steadily emerging research field, many of serendipitous findings can also be expected in the near future form this Pioneering Project “Integrated Lipidology”.

＜Reviewer 3＞

(1) Research objective

Lipids are essential components of all living systems and their biological roles are numerous. Along with their ubiquitous presence, structural diversity and interesting physical properties of lipids allow them to provide exciting subjects for a wide range of researchers, including biologists, chemists, and physicists. Accordingly, there is little reason to doubt importance of study that focus on biological functions of lipids, especially at research institution as RIKEN that is going to cover fundamental areas of natural sciences. As such, concept of this project fits well with the philosophy of “Pioneering Project”. The project is indeed going to address a number of fundamental problems required for deepening understanding biological function of lipids, through exploiting various techniques available in laboratories of project members. Selection of research subjects is mostly appropriate.

In spite of these, I feel that objectives of the whole project need to be explained more clearly. Whereas collaborative studies between member’s laboratories and subprojects are actively conducted, progress report as well as presentations gave me an impression that the project is a mixture of focused studies conducted by individual members. In this context, strong message from the leading researcher as for direction and objective of the project is strongly desired.

(2) Implementation of research plan / (3) Research achievements

I believe, the project plan has been implemented well in accordance with original plan. It is very nice to see that the project has been able to attract a number of researchers to lipid area and successful in increasing its interdisciplinary character. As a matter of fact, a number of exciting results have been made, including 1) development of research tools for detecting distribution and clustering membrane lipids, 2) high sensitivity analysis of lipid components that is going to enable analysis even at single cell level, 3) imaging of membrane bound proteins, 4) protein function regulation by lipids, and 5) discovery, biosynthetic pathways and physiological activities of novel lipids.

Given these, I observed that the research plan has been executed well. Research outputs are satisfactory, in terms of both quality and quantity.

The leading researcher would be advised to consider following issues, in order to make the project even more successful.

- The leading researcher might take stronger leadership to drive the project. I feel that his own view on lipidology could have been explained better.
- There is imbalance of budget distribution among sub-projects. It is seen that more than half of the budget is consumed by the SP2, despite its relevance as major component of Integrated "Lipidology" may not be maximum. This imbalance might be improved in coming years.
- There are several subjects which do not seem to be entirely suitable as parts of lipidology research. It should be clarified how they can contribute to understand lipid functions.

(4) Future research plan

Future plan for each subject is quite convincing. The project leader may wish to think more deeply how results from all subprojects and laboratories will be integrated to understand more deeply about lipids. As AMED-CREST lipid project started a few years ago, strong message would be required in order to assert feature of Integrated Lipidology project.

＜Reviewer 4＞

(1) Research objective

This project aims to elucidate fundamental functions of lipids in organisms by developing new methodologies and tools to visualize and quantify lipid metabolism/distribution/dynamics. Lipids are fundamentally important molecules in living organisms as providing lipid bilayers for compartmentalization and serving as signaling components. However, lipid research is not yet as advanced as other molecules such as nucleic acids or proteins due to its low solubility in water and its highly complicated metabolism. Thus the overall research objective is well thought-out and quite timely.

(2) Implementation of research plan

The project consists of three sub-projects, SP1: The advance lipidomics, SP2, The molecular protein lipidology, SP3: The dynamic lipid physiology. SP1 aims to develop lipid-visualizing probes and screen inhibitors/regulators of lipid metabolism / distribution / dynamics. SP1 also aims to analyze lipids by single cell mass spectrometry (MS) and non-target MS to create a lipidomics database. SP2 focuses more on individual lipid-protein interactions by structural and biophysical analyses. SP3 aims to discover novel lipid species and their physiological function in cells and tissues. The sub-projects were very well organized within each category. But I believe that the integration between each sub-project can be still improved.

(3) Research achievement

The project developed seven new highly useful lipid-binding proteins and peptides that specific lipid molecules such as sphingomylein / cholesterol, ceramide phosphoethanolamine, diacylglycerol. Various imaging tools such as high-speed atomic force microscopy, Raman spectroscopy, darkfield microscopy were also developed. These new tools were nicely used to examine cell surfaces. In addition, two dimensional fluorescence lifetime correlated spectroscopy and live single-cell mass spectrometry were also developed. The lipid mass data bank was built by multi-lipidomics achieved by combining targeted and non-targeted analyses. Using this lipidomics pipeline, a novel acidic glycolipid class in plant was discovered. Thus technology development was highly successful. Lipid-based regulation of membrane proteins were also nicely studied using structural and biophysical analyses. Main focuses were on membrane proteins such as EGFR, FGFR, GPCR, ion pomp NiE:NOR complex, and ABC transporter. Detail atom-to-atom analyses reveal biophysical properties of various lipids. For physiological studies, specific cell lines and model animals were used to discover novel functions of various lipids. For instance, Lyso-phosphatidylglucoside was discovered to mediate neuron-glia interaction. Also β-cholesterylglucodie was found in vertebrate brain. In addition, FABP7 involvement in schizophrenia is quite interesting and I hope that we can learn more about it.

(4) Future research plan

Future research plans are basically continuation of the current research. The most of plans seem achievable within the project time line.

＜Reviewer 5＞

(1) Research objective

It has become increasingly clear that lipids are involved in many biological phenomena. In comparison to nucleic acids and proteins, however, there remain many uncertainties about how lipids function and how their functions are regulated. Recent progress in lipidomic analyses is providing a solid technical basis for research on lipid mediators, which functions as individual molecules. However, methods to study lipids in complex systems, such as biomembranes, that work as a multi-molecular entity are still limited. In view of this status quo of lipid research and

also prevalence of lipid-related diseases, e.g., metabolic syndrome, atherosclerosis, etc., it is timely that the “Integrated Lipidology” project was planned and started, aiming to elucidate the fundamental properties of lipids through developing original and highly advanced techniques. The objectives of the three subgroups, i.e., development of basic methodology, analysis of lipid–protein interactions, and understanding of lipid functions in neurons, immune cells, and adipocytes, are all very important. Actually they are foci of intensive research worldwide.

(2) Implementation of research plan and (3) Research achievement

The objective of the first subgroup is to develop basic methodology for lipid analysis, such as lipid-specific probes, single-cell mass spectrometry, and multi-lipidomics. This plan has been implemented effectively and the research group has obtained many important results. The Kobayashi group introduced a number of new probes that specifically bind to different kinds of lipids in biological membranes. The probe named as Nakanori is notable because it binds to cholesterol–sphingomyelin clusters. This probe should be useful to understand lipid rafts, which still harbor many riddles after being debated for the past two decades. The Masujima (Shimizu) group developed live single cell mass spectrometry and is now trying to extend it to a single organelle level. The Makoto Arita group implemented multi-lipidomics approach by a combination of targeted and non-targeted analyses. All of these are methods of the cutting edge and have a potential to lead lipid research to a new dimension.

The second subgroup focuses on lipid–protein interactions and utilizes various biophysical techniques, such as single molecule imaging, solid state NMR, and molecular simulation, to obtain new perspectives. The Sako group found that acidic phospholipids, phosphatidylserine and phosphatidylinositol 4,5-bisphosphate, induce dimerization of the juxtamembrane domain of epidermal growth factor receptor (EGFR) and that phosphorylation of an amino acid residue desensitizes EGFR to this lipid effect. The group also studied fibroblast growth factor receptor and found that binding of acidic phospholipids to its juxtamembrane domain affects the orientation of the transmembrane domain. Importantly, this conclusion was confirmed by the Sugita group by using a novel method of molecular dynamics simulation. It remains difficult to understand how lipids in the membrane affect the structure and function of integral membrane proteins, but the above study presented an example that lipid–protein relationship in the membrane can be elucidated by a combination of biophysical methods.

The third subgroup studies physiological aspects of lipids at a systemic level, especially in the nervous system; the study includes function of newly found lipids, especially those bearing a glucose residue in the head group. The Hirabayashi group discovered a new lipid mediator, lyso-phosphatidylglucoside, and its receptor GPR55, and found their importance in neural development. The Yoshikawa group found a link between polyunsaturated fatty acid deficiency and schizophrenia. These as well as other results obtained by this subgroup suggested that lipids, both known and hitherto unknown ones, may exert critical functions in development of various organ systems in vivo. Further studies on such systemic lipid functions should contribute to our

understanding on the physiological process and may also lead to discovery of pathogenetic mechanism.

Many parts of the research have been carried out by collaboration between team members, not only within the same subgroup but also between different subgroups, whose specialties are quite diversified. The achievement obtained by this multidisciplinary approach has been well accepted by the international research community, as exemplified by the number of papers published in high-rank journals and awards given to team members, including many young researchers.

(4) Future research plan

The projects listed in the future research plan are well founded and scientifically attractive, and in view of the achievement up to this point, it is more than likely that members of this research project will continuously produce important results. It is natural that each researcher will conduct studies according to his/her own curiosity and those studies are related to lipids in different ways. The target of individual research projects, however, may not be lipids per se. To integrate such heterogeneous studies and to achieve a higher goal, it appears important that studies on lipids themselves will continue to be placed in the center of the project. By doing so, the chance of inventing novel methodologies and tactics for lipid research will increase and a quantum jump in this field may be more likely to happen. Considering that RIKEN has many top scientists in the lipid field, this reviewer hopes that the current research project will develop further and become a basis to establish a nation-wide research center.

RIKEN Science Council Research Programs Committee

Counter argument report:

018.1.22

Re: Research Project Mid-term Evaluation Result

Thank you reviewers for evaluation of our project “Integrated Lipidology”.

Most of the comments raised by the reviewers are invaluable for us. We would like to have a notice on the comment form reviewer 3 about the imbalance of budget (p.6).

In the tables of budged appeared in the midterm report, especially after FY2015, budget for SP2 looks much larger than those for SP1 and 3. It is caused by the fact that budget for personnel employed in this project is included in the budget for SP2 headed by the lead researcher (Sako). We have 5 postdocs and 3 and 1 of them are working in SP1 and SP3, respectively. In addition, budget for the research in Kobayshi’s group in SP1 is counted in the budget of SP2 after 2015, because he became a senior visiting scientist of Sako’s laboratory in SP2 after his movement to CNRS. Considering these things, distribution of the budget in effect, for example at FY2017, is about 40,000, 31,000 and 20,000 thousand yen for SP1 (7 laboratories), 2 (5 laboratories), and 3 (4 laboratories), respectively. We do not think this distribution is so much imbalanced as reviewer 3 pointed out.

Yasuhi Sako
Lead researcher
“Integrated Lipidology”