様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９　（共通）

科学研究費助成事業　　研究成果報告書

令和　３ 年 １０ 月 ２０ 日現在

機関番号：１４４０１
研究種目：若手研究
研究期間：2018～2019
課題番号：１８Ｋ１４６９５

研究課題名（和文）Immune cell-to-cell communication studied through label-free microscopy combined with machine learning

研究課題名（英文）Immune cell-to-cell communication studied through label-free microscopy combined with machine learning

研究代表者

パヴィヨン　ニコラ（Pavillon, Nicolas）

大阪大学・免疫学フロンティア研究センター・特任助教（常勤）

研究者番号：８０６４４５２５

交付決定額（研究期間全体）：（直接経費）　　3,200,000円

研究成果の概要（和文）：先に開発した無標識顕微鏡を使い、定量位相イメージングとラマン分光法で計測する。生細胞の形態と分子の特徴を同時に測定し、機械学習と組み合わせることで免疫細胞の反応を検査できる。今まで細胞株を使ったが、以後はマウスプライマリ細胞が検査され、細胞株より不均質である。それでも免疫反応は９０％の精度で計測を果たした。その上に、普通の方法では難しいのにも関わらず、食細胞のサブタイプも検知できた。
なお、リンパ球,特にT細胞の反応と分化も検査し、９５％の精度で検知でき、無標識顕微鏡で見分けた。

研究成果の学術的意義や社会的意義
細胞を分析する標準的な方法は高感度を果たすが、信号の検出のために標識の必要がある。標識等は細胞反応を変わり、生細胞の場合は表面受容体しか測定できない。細胞内分子を検知できるために固定がある。
無標識顕微鏡は生細胞の計測ができ、直接に細胞内分子を測定できる。我々はこの方法と機械学習で細胞の繊細な変化を検出できた。免疫細胞の色々な種類、例えば食細胞やT細胞のリンパ球に、反応を検出できることを証明した。

研究成果の概要（英文）：We employ our previously developed label-free non-invasive imaging system based on the simultaneous acquisition of quantitative phase images and Raman spectroscopy, which are indicative of morphology and molecular content, respectively. We couple these measurements on live cells with machine learning to derive statistical models that describe immune activation.
We validate our approach on primary cells derived from murine samples, which are much more heterogenous than cell lines, and achieve over 90% accuracy. We also show that we can distinguish different cellular sub-types that are difficult to identify with standard methods.
We also investigate the activation and differentiation of T cell lymphocytes, and show that we can detect T cell activation with over 95% accuracy, and also distinguish different types of activation, namely TCR stimulation and chemical bypass.

研究分野：生細胞イメージング

キーワード：免疫反応　細胞コミュニケーション　無標識顕微鏡法　生細胞イメージング　食細胞　リンパ球　ラマン分光学　定量位相

様　式　C－１９、F－１９－１、Z－１９（共通）

１．研究開始当初の背景

(1) We previously developed a multimodal microscope that allows the simultaneous measurement of quantitative phase images (QPI) and single-cell Raman spectra. These measurements can readily be obtained non-invasively from live cells without further processing, as these techniques do not require any extraneous label for imaging contrast.
(2) We could use these measurements to derive indicators related to the cellular state and response to stimuli. These indicators are based on morphology (as extracted from QPI), or intracellular content (as retrieved from Raman spectra). We then coupled these indicators with machine learning algorithms to reliably detect the activation state of macrophage cells, and could show that our indicators display a dosage-dependent behavior (through morphology) and allow the detection of specific signaling pathways in the cellular immune response (through spectral measurements).
(3) Label-free measurements present an interesting complement to standard approaches in biological imaging, where staining dyes, mostly fluorescent, are extensively used. The endogenous contrast obtained in this case can enable the detection of targets for which reliable staining is not available, and allow the observation of live and completely unaltered samples.

２．研究の目的

(1) The purpose of this project is to develop the use of label-free microscopy for the detection of biological responses at cellular level, in particular in the context of immunological response.
(2) Specifically, it aims at deepening the understanding of the measurements derived from both imaging and spectroscopy that are then employed to characterize the cellular immune response. While reliable indicators could be obtained previously in the context of macrophage activation, their signi- ficance in the larger context of the immune system is not yet fully understood.
(3) The purpose of this project is therefore to study the response of different cell types with our label-free system, and also study the interaction between immune cells by taking advantage of the non-invasiveness of our imaging platform that enables the observation of live cells.

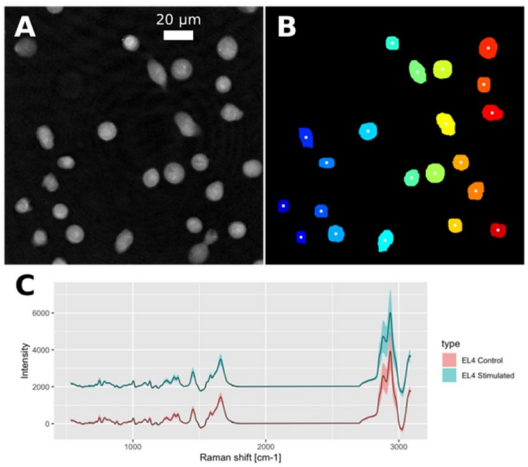

Figure 1: (A) Typical QPI image of EL4 T cells. (B) Automated segmentation of the objects in the field of view, where the center of each cells is used as an automatic target for the Raman laser excitation. (C) Typical average Raman spectrum of EL4 T cells with the standard deviation shown around the average (N=685).

３．研究の方法

(1) The research project is mainly based on the multimodal imaging platform described above, which enables the retrieval of label-free information based on both QPI images, related to the optical density of cells, and Raman spectra, which are indicative of the intracellular molecular content.
(2) This approach makes it possible to retrieve multivariate information on single-cell, where morphological indi- cators typically yield 300 variables related to shape, intensity and texture, while spectra lead to approximately 700 variables from different wavelengths. This provides a large vector on which statistical analysis can be performed, while ensuring large sample sizes at single-cell level, as thousands of cells can be measured with such a system.
(3) Exploratory analysis is typically performed with principal components analysis

(PCA), while the creation of models and machine learning is performed through penalized logistic regression. This method of classification presents the advantage of still enabling interpretation of the data as it provides a separation vector that identifies the features providing most of the separation between classes.
(4) A high throughput of measurements is ensured by recording one Raman spectrum representative of the whole cell content, by averaging the contributions from a whole region in the cell. During the project, we further increased the measurement throughput by automating the acquisition procedure, where cells can be automatically detected in the field of view of QPI images to determine the regions that should be targeted for measurements in the Raman modality.
(5) This process is illustrated in Fig. 1, where the QPI image (see Fig. 1A) is used to automatically segment cells, as shown in Fig. 1B. This allows the detection of the center of gravity (in white) that determines the center of the region to be measured.
(6) The purpose of this improved acquisition procedure is to ensure higher throughput that could be then applied to primary cells. We therefore developed and validated the protocols to extract different primary cells, and in particular peritoneal macrophages and lymphocytes (T and B cells) that could be used during our experiments. We also developed the stimulation protocols to study the immune response of these different cell types.

4. 研究成果

(1) The first part of the project aimed at further investigating macrophage cells activation, by validating our measurement approach on different samples. In our initial research [2], we demonstrated the ability of our label-free approach to detect activation on the Raw264 cell line, which provides quite homogenous populations, and is known to display some level of activation even in resting state.
(2) We therefore performed similar experiments with murine primary macrophages, extracted from the peritoneal cavity, which is known to host highly heterogenous cell populations. We extracted peritoneal cells from control mice (resident macrophages, RPM) or from specimens that were exposed to an immune stress which induces the recruitment of immune cells in the cavity (elicited macrophages, EPM). We then performed the measurements on the 3 cell types, and also stimulated cells *in vitro* with LPS.
(3) Our results show that it is possible to identify the activation state on the different macrophages, as shown in Fig. 2A with exploratory analysis (not yet classified). Furthermore, our non-invasive approach can also identify the cell type: Raw264, RPM, or EPM, as illustrated in Fig. 2B.

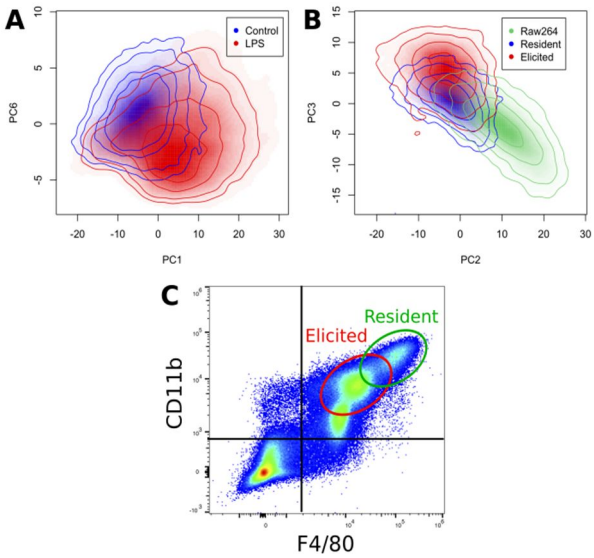

Figure 2: Exploratory analysis through PCA of macrophage cells (N≅20,000). (A) Distribution of cell either in resting or activated state, induced by exposure to LPS. (B) Distribution of the different cell types (adapted from [1]). (C) FACS plot of cells from the peritoneal cavity on two phagocytes related markers, F4/80 and CD11b, where RPM and EPM are identified.

(4) We could achieve over 90% accuracy for the classification of all 6 classes, namely the 3 cell types, either resting or activated [1]. This demonstrate the ability of our approach to detect the activation state on highly heterogenous cell populations such as peritoneal macrophages. Furthermore, it is known that the RPM and EPM are

functionally different as they originate from different progenitors; RPM are a self-renewed population within the peritoneal cavity, while EPM are differentiated from monocytes recruited from the blood. These populations are however hard to distinguish with standard methods such as fluorescence-activated cell sorting (FACS), as illustrated in Fig. 2C.
(5) Finally, we could show that our method is also sensitive to the specimen itself, as it can detect fine changes in the response of the mice to the stimulation [1]. This enables the detection of outlier behavior between experiments, and is an interesting lead for further investigation.

(6) In a second part of the project, the purpose was to investigate the influence of secreted cytokines on the activation state of cells. We first attempted to detect local effects by employing low dosages of lipopolysaccharide (LPS) for stimulation, and tried to correlate the detected activation state with the location of denser clusters of cells in the culture, where the cytokine concentration should be higher. This approach however did not yield significant differences.
(7) On the other hand, we employed co-cultures of macrophages with T cells from the murine cell line EL4. The lymphocytes were stimulated so that they would secrete high levels of cytokines and were co-cultured with Raw264 macrophage cells, which could then be observed as being activated. This shows that the activation purely based on cell secreted cytokines is possible.
(8) It is known that cytokines secreted by macrophages induce inflammation and migration of additional immune cells in the vicinity of injuries *in vivo*. We can hypothesize that the absence of significant differences in the case of mono-cultures is due to diffusion of cytokines in the media that leads to homogenous concentration, making *in vitro* models not suitable for these studies.
(9) In the last part of the research, we studied the influence of antigen-presenting cells (APC) on lymphocyte activation with our system. APCs bind to lymphocytes to create immune synapses through the T cell receptor (TCR), which induces lasting activation and differentiation in T cells.

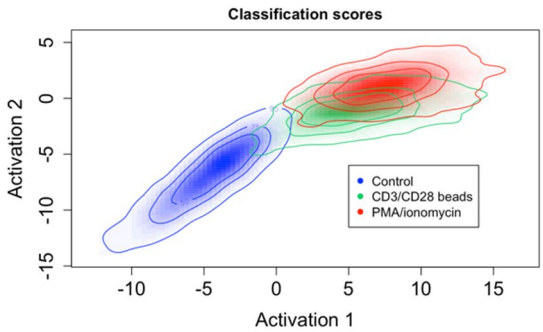

Figure 3: Population distribution after classification with Raman indicators (N=5000), where the large control vs. activated distinction can be seen, along with smaller differences between stimulation methods.

(10) To assess the effect of immune synapses on activation, we first employed artificial APCs, which are created by coating plastic beads with the required antigens that mimic the size and configuration of physiological APCs, while ensuring a high efficiency of activation. We compared this method with a known chemical way to stimulate T cells, based on a cocktail of phorbol 12-myristate 13-acetate (PMA) and ionomycin.
(11) We extracted naïve $CD4^+$ T cells from splenocytes, and exposed them to the two types of stimulations described above. We could then identify significant differences between the different conditions as illustrated in Fig. 3, and could reach over 95% in classification accuracy for the detection of T cell activation, again showing the ability of our approach to non-invasively detect cellular responses.
(12) Furthermore, the label-free indicators also make it possible to distinguish between the stimulation methods, with an accuracy of approximately 80%. The chemical stimulation PMA/ionomycin is known to bypass the standard signaling pathway to induce activation. This implies that the differences identified with our method between

chemical stimulation and artificial APCs originates mostly from the generation of the immune synapse and its subsequent signaling cascade.

(13) The measurements to study lymphocyte activation were performed on both cell lines (EL4) and primary cells (splenocytes, $CD4^+$), and showed that the measured responses are clearly stronger in the case of primary cells. This can be due to the fact that cell lines are commonly from tumor origins, which can affect both their resting state as well as their response to stimuli.

(14) Finally, we also obtained preliminary results in the use of physiological APC to generate the immune synapse and induce activation. In that case, we employed lymphocytes as APCs, by incubating them with superantigen molecules. These cells were then put in co-cultures with naïve T cells so that they can bind to the TCR and induce non-specific activation.

５．主な発表論文等

〔雑誌論文〕　計2件（うち査読付論文　1件／うち国際共著　0件／うちオープンアクセス　1件）

１．著者名 Nicolas Pavillon; Nicholas. I. Smith	４．巻 9
２．論文標題 Immune cell type, cell activation, and single cell heterogeneity revealed by label-free optical methods	５．発行年 2019年
３．雑誌名 Scientific Reports	６．最初と最後の頁 17054
掲載論文のDOI（デジタルオブジェクト識別子） 10.1038/s41598-019-53428-3	査読の有無 有
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 －

１．著者名 Nicolas Pavillon; Alison J. Hobro; Shizuo Akira; Nicholas I. Smith	４．巻 48
２．論文標題 生きた細胞の無標識的な顕微鏡法	５．発行年 2019年
３．雑誌名 日本光学会誌	６．最初と最後の頁 225
掲載論文のDOI（デジタルオブジェクト識別子） なし	査読の有無 無
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 －

〔学会発表〕　計6件（うち招待講演　3件／うち国際学会　6件）

１．発表者名 Nicolas Pavillon; Alison J. Hobro; Nicholas I. Smith
２．発表標題 Immune cell activation measured with multimodal label-free microscopy through morphology and spectral feature
３．学会等名 International Conference on Raman Spectroscopy (ICORS)（招待講演）（国際学会）
４．発表年 2018年

１．発表者名 Nicolas Pavillon; Alison J. Hobro; Nicholas I. Smith
２．発表標題 High-throughput analysis at single-cell level through multimodal label-free microscopy
３．学会等名 SPIE Photonics West（招待講演）（国際学会）
４．発表年 2019年

１．発表者名
Nicolas Pavillon; Alison J. Hobro; Nicholas I. Smith

２．発表標題
Single-cell immune activation detected through label-free microspectroscopy

３．学会等名
SPIE Photonics West（国際学会）

４．発表年
2019年

１．発表者名
Nicolas Pavillon; Nicholas I. Smith

２．発表標題
Single-cell immune activation detected with label-free imaging and spectroscopyy

３．学会等名
International Conference on Biology and Medical Sciences (ICBMS)（招待講演）（国際学会）

４．発表年
2019年

１．発表者名
Nicolas Pavillon; Alison J. Hobro; Nicholas I. Smith

２．発表標題
High-Throughput Multimodal Micro-spectroscopy for Live Cell Immune Activation

３．学会等名
International Conference on Advanced Vibrational Spectroscopy (ICAVS)（国際学会）

４．発表年
2019年

１．発表者名
Nicolas Pavillon; Nicholas I. Smith

２．発表標題
Label-free multimodal microscopy for the detection of cellular immune response

３．学会等名
Optics and Photonics Japan（国際学会）

４．発表年
2019年

〔図書〕　計0件

〔産業財産権〕

〔その他〕

Biophotonics Laboratory web page http://biophotonics.ifrec.osaka-u.ac.jp/node/2

６．研究組織

	氏名 （ローマ字氏名） （研究者番号）	所属研究機関・部局・職 （機関番号）	備考

７．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

８．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関