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Quantitative *In Situ* Analysis of FoxP3+ T Regulatory Cells on Transplant Tissue Using Laser Scanning Cytometry

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Abstract

There is abundant evidence that immune cells infiltrating into a transplanted organ play a critical role for destructive inflammatory or regulatory immune reactions. Quantitative *in situ* analysis (i.e. in tissue sections) of immune cells remains challenging due to a lack of objective methodology. Laser scanning cytometry (LSC/iCys) is a recently developed methodology that utilizes fluorescence-based quantitative measurements on tissue sections or other cellular preparations at a single-cell level. In this study, we have developed a novel objective method for analysis of immune cells, including Foxp3$^+$ T regulatory cell (T reg), on formalin-fixed / paraffin embedded (FFPE) transplant biopsy sections using LSC/iCys.

The development of multiple immunofluorescent staining was established using FFPE human tonsil sample. The CD4/CD8 ratio and the population of T reg among CD4$^+$ cells were analyzed using LSC/iCys and compared with the results from conventional flow cytometry analysis (FCM).

Our multiple immunofluorescent staining techniques allow obtaining clear staining on FFPE sections. The CD4/CD8 ratio analyzed by LSC/iCys was concordant with those obtained by FCM. This method was also applicable for liver, small intestine, kidney, pancreas and heart transplant biopsy sections and provide an objective quantification of T regs within the grafts.

Keywords

T regulatory cell; Foxp3; laser scanning cytometry; transplant biopsy; quantification

Introduction

Recent improvements in the understanding of the alloimmune processes responsible for transplant rejection, available immunosuppressive medications, more sophisticated surgical techniques, and post-transplant intensive care have led to more successful organ

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transplantation. Although excellent short-term survival of most transplanted organs is currently achieved, late graft loss, organ shortage and the toxicities of chronic nonspecific immunosuppressive therapy remain major concerns in the field of transplantation.

T-cell-mediated immunoregulation is considered an important component of the cellular paradigm in transplantation and autoimmune disease (30,47,49). T regulatory cells (T regs) can promote peripheral self-tolerance by suppressing reactive immune effector cells (48). Sakaguchi et al described that $CD4^+CD25^+$T reg were hyporesponsive to T cell receptor stimulation and were capable of suppressing the proliferation and activation of both $CD4^+$ and $CD8^+$ T cells (46,48). Cytofluorographic analysis of $CD4^+CD25^+$ T reg in peripheral blood associated with various pathologic conditions, including transplantation, have shown clinical significance to the measurement of circulating T reg in peripheral blood (6,13,17,19,31,42,57).

Identification of putative T reg inside tissue by immunohistochemistry has been utilized since the discovery of the forkhead/winged helix transcriptional factor Foxp3 that is expressed in $CD4^+CD25^+$T cells (24). Using CD4 and Foxp3 expression as a marker for T reg, several studies revealed the importance of local (*in situ*) T regulatory cells in transplantation and other diseases by immunohistochemistry (54,56). These studies usually employ traditional manual counting of target cells, or semiquantitative grading schemes with a limited number of microscopic fields seen by examiners. To date, alternative analytical methods to define immune cell population including T reg population within tissue have not been described.

The Laser scanning cytometer (LSC/iCys®, CompuCyte, Cambridge, MA) is a recently developed instrument that utilizes fluorescence-based quantitative measurements on tissue sections or other cellular preparations at a single-cell level (1,20,21,41). Although LSC and FCM share some of the features to evaluate certain cell populations that are labeled with fluorescence, there are several advantages in LSC/iCys. One of the most distinct advantages of LSC/iCys is to allow objective quantification of large numbers of target molecules on the tissue section (21). Although this unique feature of LSC/iCys is potentially useful for clinical as well as research purposes, one of the obstacles is that LSC/iCys requires immunofluorescent labeling of target molecules. Immunofluorescent labeling is usually performed on frozen sections rather than on FFPE sections, but formalin-fixed/paraffin embedded (FFPE) tissue is more broadly available than frozen tissue. Despite many reports of immunofluorescent staining of FFPE sections, it has not been accepted as routine clinical or research modality (5,35,43). We have successfully developed multicolor immunofluorescent staining of CD4, CD8 and Foxp3 on FFPE sections optimized for analysis with LSC/iCys.

Using the combination of both techniques, we have demonstrated a novel method for *in situ* quantitative assessment of T cells on FFPE transplant biopsy sections from various organs. Our method potentially opens the door for detailed analysis of *in situ* immune cell populations in the grafts with these techniques, which allow for the profiling of the infiltrating immune cells and may be of assistance in understanding of local alloimmune responses in transplantation.

Materials and Methods

Development of *in situ* T reg analysis on FFPE biopsy was divided into the following three processes: (1) multiple immunofluorescent staining, (2) analysis using LSC/iCys and (3) application of this method for various transplant organ biopsies and example of *in situ* T reg analysis on intestinal allograft biopsy.

We utilized FFPE sections from human tonsil as a positive control for T regulatory cells. For the objective quantitative analysis of T reg, we aimed to calculate the ratio of $CD4^+$ to $CD8^+$ cells, the population of T reg ($CD4^+Foxp3^+$ cells) among $CD4^+$ T cells, and the population of T reg among the entire T cell population (a total of $CD4^+$ and $CD8^+$cells).

Protocol for multiple immunofluorescent staining on FFPE sections

All samples were fixed with 10% neutral buffered formalin for several hours, routinely processed by a rapid tissue processor (Tissue-Tek®Xpress®, Sakura, Torrance, CA) and embedded in the paraffin block. Two sections of 4μm in thickness from each block were prepared for staining.

One section was stained for CD4 and CD8. Sections were placed on the coated glass slide and baked in an oven for 30 minutes. Deparaffinization and rehydration were performed using xylene and ethanol. The antigen retrieval was one of the key processes for the successful multiple immunofluorescent staining on FFPE sections. We evaluated several antigen retrieval protocols (Table 1). Endogenous peroxidase activity was blocked by non-hydrogen peroxide formula (PeroxAbolish®, Biocare Medical, Concord, CA, USA) either before or after antigen retrieval. Antigen retrieval was performed using a pressure cooker (Decloaking Chammber Pro®, Biocare Medical, Concord, CA, USA) with 120°C for 10 minutes or with 125°C for 5 minutes, soaking sections in an antigen retrieval solution of high pH (pH 9.5: Borg Decloaker®, Biocare Medical, Concord, CA, USA) or low pH (pH 6.0: Target Retrieval Solution Citrate®, Dako, Carpinteria, CA, USA). Protein block was done by incubating 1% normal goat serum for 20 minutes. Anti human CD4 monoclonal antibody originated from mouse (clone BC/1F6, IgG1, Biocare Medical, Concord, CA, USA) and anti human CD8 polyclonal antibody originated from rabbit (abcam, Cambridge, MA, USA) were diluted by Van Gogh Yellow antibody diluent (Biocare Medical, Concord, CA, USA) and mixed with the final dilution being 1:25 and 1:50, respectively. Diluted primary antibodies were incubated overnight at 4°C. Labeling was performed by polymer horse radish peroxidase (HRP) and catalyzed signal amplification with CD4 and CD8 being labeled by Alexa 647® and Alexa 488®, respectively. Polymer HRP conjugated anti mouse secondary antibody (EnVision®, DAKO, Carpinteria, CA, USA) was incubated for 45 minutes at room temperature, and then Alexa 647® conjugated tyramide (Invitrogen, Carlsbad, CA, USA) was incubated for 10 minutes at room temperature for the labeling of CD4. Then, peroxidase activity was blocked by non-hydrogen peroxide formula (PeroxAbolish®, Biocare Medical, Concord, CA, USA) for 30 minutes at room temperature. Polymer HRP conjugated anti rabbit secondary antibody (EnVision®, DAKO, Carpinteria, CA, USA) was incubated for 45 minutes at room temperature, and then Alexa 488® conjugated tyramide (Invitrogen, Carlsbad, CA, USA) was incubated for 10 minutes at room temperature. Nuclear counter staining was performed by incubation of propidium iodide (PI) diluted 1:50 with antibody diluent (Invitrogen, Carlsbad, CA, USA) for 10 minutes. The stained slide was cover-slipped using mounting media (ProLong®, Invitrogen, Carlsbad, CA, USA) and stored in the dark at 4°C until analysis. Another section was stained for CD4 and Foxp3. Staining protocol was the same as described above except for the primary antibodies. Instead of the antibody to CD4 and CD8, we used anti human CD4 monoclonal antibody (clone BC/1F6, IgG1, Biocare Medical, Concord, CA, USA) with dilution of 1:25 and anti human Foxp3 polyclonal antibody (abcam, Cambridge, MA, USA) with dilution of 1:400. CD4 and Foxp3 were labeled with Alexa 647 and Alexa 488, and nuclear counter stain was done by PI.

The same staining procedure without primary antibody on tonsil sample was run as negative control. All stained slides were subjected to the analysis using LSC/iCys within 3 days after staining.

Protocol for analysis using LSC/iCys and validation in comparison with FCM

LSC/iCys equips three lasers, Violet Diode laser, Argon Ion laser and Helium Neon laser for the excitation of fluorescent dye and four filters, blue, green, orange and long red for the detection of emission. Two scanning passes are available at any of certain locations on the slide. We used, for the multiple fluorescent staining, Alexa 488, Alexa 647 and PI, and the following scanning protocol was considered to be optimal: (1) During the 1st scanning pass, Argon Ion laser was utilized for the excitation, and green and orange filter were used for the detection of Alexa488 and PI; (2) During the 2nd scanning pass, Helium Neon laser was utilized, and long red filter was used for the detection of Alexa647; (3) Slides were scanned at 40×, and nuclei were contoured using PI signal integral; (4) Area, x position, y position fluorescent integral and maximum intensity were recorded for all detection channels at a single-cell level; (5) A single cell population was identified, gating according to the PI signal integral area; (6) Each fluorescent integral of each single cell was shown on a histogram and a scatter gram; (7) CD4/CD8 ratio, the population of $CD4^+$ $Foxp3^+$ T reg among $CD4^+$ cells were analyzed with iCys® cytometric analysis software version 3 (CompuCyte, MA, USA) and the population of $CD4^+Foxp3^+$ cells among the entire T cells (a total of $CD4^+$cells and $CD8^+$ cells) were calculated.

To investigate the validity of the analysis using LSC/iCys, we compared the CD4/CD8 ratio between regular flow cytometry (FCM) and LSC/iCys. Eleven cases of human tonsil with varying CD4/CD8 ratios were selected for this validation study, which had FFPE sample and cytological examination of CD4/CD8 rate by FCM from the same location (i.e., Each of harvested tonsil was divided into two pieces; one was subjected to cytological examination of CD4/CD8 rate using FCM as a routine lymphoma working up and another one was processed to FFPE sample for a routine clinical histology). Several sections were obtained according to approved procedure at Miami Transplant Institute, University of Miami from each residual FFPE sample archived in our institute under informed consent. Those FFPE slides were prepared and analyzed for their CD4/CD8 ratio as mentioned above. The CD4/CD8 ratio obtained by LSC/iCys analysis was compared with those by regular FCM examination using paired t-test (SPSS version 11) and p-value less than 0.05 was considered statistically significant.

Applicability of this method on various transplant organ biopsies

This developed methodology was applied to investigate its applicability on the following transplant biopsy samples that were diagnosed as acute rejection: (1) liver biopsy with moderate acute rejection; (2) small intestine biopsy with mild acute cellular rejection (ACR); (3) kidney biopsy with acute T-cell-mediated rejection, type IIA; (4) pancreas biopsy with mild acute cell-mediated rejection; (5) endomyocardial biopsy with acute cellular rejection, grade 3R (Fig. 1). The CD4/CD8 rate, the population of $CD4^+Foxp3^+$ cells among $CD4^+$ cells and the population of $CD4^+Foxp3^+$ cells among the entire T cells (a total of $CD4^+$ and $CD8^+$ cells) were obtained.

Comparative analysis of T reg population within inflammatory infiltrate between early onset and late onset acute cellular rejection in intestinal transplantation

Ten cases of early onset acute cellular rejection (E-ACR), defined as FFPE biopsy sample from intestinal allograft showing histopathological ACR within 1 month after intestinal transplantation, and ten cases of late onset acute cellular rejection (L-ACR), defined as FFPE biopsy sample from intestinal allograft showing histopathological ACR beyond 6 months after intestinal transplantation, were compared regarding the histopathological features listed in Table 2 and analyzed T reg population within inflammatory infiltrate in lamina propria using *in situ* quantitative analysis of T reg. All the cases were morphologically compatible with grade 1 ACR. Median days from intestinal transplantation

to biopsy of E-ACR and L-ACR cases were 22 days, ranging from 10 to 27, and 228 days, ranging from 190 to 423, respectively. The histopathological features were blindly evaluated by two authors, HT and VD, using a semi-quantitative scoring system (Table 3) (45). Unpaired t-test, Mann-Whitney's U test, chi-square test were used for statistical analysis whenever appropriate. P-values below 0.05 were considered statistically significant.

Results

Multiple immunofluorescent staining on FFPE sections

Table 1 shows the results of immunofluorescent staining in different antigen retrieval protocols. Immunofluorescent staining for CD4 was the crucial part of multiple immunofluorescent staining (Table 1). When blocking of endogenous peroxidase activity after antigen retrieval was performed, we could not detect immunofluorescence for CD4 in our setting of multiple immunofluorescent staining on FFPE sections. We therefore determined that the optimal protocol for antigen retrieval was (1) using a pressure cooker with 120°C for 10 minutes, (2) soaking sections in an antigen retrieval solution of pH 9.5, and (3) blocking of endogenous peroxidase activity before antigen retrieval (Table 1). We employed this protocol for the further experiments in this study. Figure 2 shows the multiple immunofluorescent staining on tonsil sections and the analysis of $CD4^+Foxp3^+$ T reg. The staining quality of Alexa488 (CD8 or Foxp3) and Alexa647 (CD4) were satisfactory (Fig. 2A and 2C) for the analysis by LSC/iCys. Nuclear counter staining by PI was clearly identified and detected by LSC/iCys (Fig. 2C).

Comparison of CD4/CD8 ratio between conventional FCM and LSC/iCys and in situ quantitative analysis of T reg immunophenotyping

Figure 2B shows the analysis of CD4/CD8 rate on tonsil sections using LSC/iCys. Approximately 40,000 cells were recorded. Distribution of signal integral of Alexa488 (CD8) and Alexa647 (CD4) were visualized in a scatter gram and the CD4/CD8 rate was 0.38. A certain amount of cells were detected as double positive cells possibly due to the extensive overlapping of cells within a section. Figure 2D shows the distribution of signal integral of Alexa488, which represents the Foxp3 positive cells, among the cell population gated at $CD4^+$ cells. The population of $CD4^+Foxp3^+$ cells among $CD4^+$ cells was 8.5%. Thus, the population of $CD4^+Foxp3^+$ cells among the entire T cell population (a total of $CD4^+$ and $CD8^+$ cells) was calculated to be 2.3%. Figure 2E shows the comparison of CD4/CD8 ratios between FCM and LSC/iCys in eleven cases. There was no statistically significant difference between these two methods ($p = 0.393$).

Application of the method on various transplant biopsies

Figure 3 shows the application of *in situ* quantitative analysis of T reg on various transplant biopsies, corresponding to H&E stains in Figure 1. Figure 3A, 3B, 3C, 3D and 3E demonstrate the scattergrams, which represent CD8 (Alexa488, x axis) and CD4 (Alexa647, y axis) of liver, small intestinal, kidney, pancreas and endomyocardial biopsies, respectively. Note that double positive cells were less than those determined in tonsil sample because there is less intensive overlapping of inflammatory cells in graft biopsies; this allowed more precise detection of each fluorescent integral related to each single cell. Figure 3F, 3G, 3H, 3I and 3J demonstrate the histograms, which represent the distribution of signal integral of Alexa488 among the population gated at $CD4^+$ cells in liver, small intestine, kidney, pancreas and endomyocardial biopsies, respectively. Using this information, CD4/CD8 ratio, the population of $CD4^+Foxp3^+$ T reg among $CD4^+$ T cell population and the population of $CD4^+Foxp3^+$ T reg among the entire T cell population (a total of $CD4^+$ and $CD8^+$ cells) was calculated and summarized in table 4.

Quantitative analysis of T reg population within inflammatory infiltrate in intestinal transplantation; comparative analysis between early onset and late onset acute cellular rejection

Comparisons of histopathological features were summarized in table 2. All histopathological features, including average number of apoptotic bodies in 10 crypts as well as severity of inflammatory infiltrate, mucosal edema, blunting of villi, vascular congestion and red blood cell extravasation, failed to show statistically significant differences between E-ACR and L-ACR cases (Table 2). Whereas there were not statistically significant differences number of CD4 cells, CD8 cells and CD4/CD8 rate acquired by LSC/iCys, *in situ* quantitative analysis of T reg showed significantly decreased rate of T reg/CD4 and T reg/total T cell in L-ACR in comparison with those in E-ACR (Table 2).

Discussion

Recent intensive researches have revealed the importance of T regulatory cells (T reg) in autoimmune regulations (7,29,34,39,50,54,56). The mechanisms of suppressive function of T reg are not yet fully understood, but the following progression of events are thought to be involved: engagement of T reg TCR by antigen, direct cell interaction, local secretion and cytokine signaling, such as TGF-beta and IL-10, and inhibition of transcription of genes related to effector functions (24,32,40,55). Considering the potential regulatory mechanisms in T reg, the behavior of local T reg within graft tissue is critical for understanding local alloimmune regulations. Several reports have implied that the local T reg had more significant clinical relevance rather than the circulating T reg (14,15,44).

The specificity of Foxp3 expression for T reg function has been shown in mice and humans although Foxp3 can be expressed in some activated human CD4 or CD8 T cells that do not express CD25 constitutively (24). Recent reports showed immunohistochemical detection of Foxp3 mainly identified $Foxp3^{bright}$ cells by FCM, which largely corresponded to immunosuppressive T reg, while $Foxp3^{low}$ cells by FCM (including activated non T regs) remained below the detection threshold (4,37,52). Therefore, $CD4^{+}Foxp3^{+}$ cells on transplant biopsy section detected by our method may consist mainly of immunosuppressive T reg. However, $CD4^{+}Foxp3^{+}$ cells detected by our method need to be assessed regarding immunosuppressive function for the further investigation because our method employed signal amplification for immunofluorescent labeling and LSC/iCys for detection of Foxp3 positive cells; the detection threshold of Foxp3 may vary from other detection methodologies. Previous studies used CD4 and Foxp3 as a marker for local T reg and revealed its significance using quantification or semi-quantification of *in situ* T reg populations by manual counting under microscopic examination (54,56). Although this method is convenient, there is an inevitable issue of reproducibility. Moreover, it is impossible to compare the local T reg population with that in peripheral blood because there is no method to bridge the gap between manual quantification of local T reg and objective quantification of peripheral T reg by FCM.

Laser scanning cytometry is a recently introduced instrument which allows objective quantification of fluorescent labeled molecule on a glass slide (21). The details of instrumentation and analysis software were described elsewhere (3,11,18,20,21,25,27,28,36,53). Essentially, LSC/iCys utilizes an optical microscope with slide scanning system and fluorescent detection system, which allows the quantitative capabilities of FCM analysis. LSC/iCys is distinct from FCM in the following aspects: (1) LSC/iCys can utilize a section on slide, not requiring the special preparation for suspension of cells, with microanatomy of tissue being preserved, (2) LSC/iCys acquires and retains precise position data of each cell, allowing direct visualization of the target cells, repeated scanning, and potentially re-staining, (3) based on the microanatomical data, quantitative

analysis in a particular area, such as portal area in liver tissue, within section is possible, (4) LSC/iCys can obtain a detailed quantitative analysis using very few cells, making it ideally suited for small samples from biopsies. These features of LSC/iCys provide an opportunity for *in situ* objective quantitative analysis.

Despite the advantages of frozen sections in applying immunofluorescent staining and real time PCR, the majority of clinical samples are processed as FFPE ones and they are more commonly available than frozen tissue because of the superiority of FFPE sections to frozen sections regarding preservation of morphology. Indeed, most clinical samples of transplant biopsy are archived as FFPE samples in our institute. Therefore, to apply the LSC/iCys analysis in various clinical and research situations, the effective immunofluorescent staining on FFPE sections is quite important. Immunofluorescent labeling of FFPE samples has not yet been widely accepted although there have been many published reports that describe these techniques. One major reason is that fluorescent signals from FFPE sections are usually weaker when compared to frozen sections, and insufficient for precise examination. We utilized a catalyzed signal amplification combined with polymer HRP to increase the labeled fluorescent signals. The catalyzed signal amplification has been applied in various detection techniques, such as immunofluorescent staining and *in situ* hybridization, in which the peroxidase-mediated deposition of hapten- or fluorochrome-labeled tyramides is used to visualize target molecules with significant signal amplification (8,22,23,33) and several staining kits are commercially available. Moreover, we used polymer HRP, instead of ordinary HRP conjugated secondary antibody, because the HRP activity is higher in polymer HRP than in ordinary HRP conjugated secondary antibody and more intense deposition of fluorochrome labeled tyramides is expected (10). Another major issue of immunofluorescent staining on FFPE sections is autofluorescence (43). Actually, we experienced autofluorescence but it was minimal as we show in Figures 1A and C. In this study, autofluorescence did not cause significant effects on analysis partly because narrow band laser light illumination used in LSC/iCys minimizes the effect of undesirable autofluorescence. Moreover, LSC/iCys equips the compensation function to reduce the effect of autofluorescence in case autofluorescence affects the quality of analysis. Reproducibility of immunofluorescent staining is possibly influenced by the quality of FFPE sample, e.g. the fixation period in formalin. The antigen retrieval process is one of the key steps for the reproducible staining. We evaluated several antigen retrieval processes, including the use of a pressure cooker (Table 1). In this study, antigen retrieval after blocking of endogenous peroxidase using a pressure cooker with 120°C for 10 minutes, soaking sections in an antigen retrieval solution of pH 9.5, provided the most reproducible immunofluorescent staining. Although modifications on antigen retrieval protocol may be required for the optimization of multiple immunofluorescent staining on FFPE sections under each experimental circumstances, among CD4, CD8 and Foxp3, staining for CD4 was the most crucial and the important points for successful CD4 staining were (1) to block endogenous peroxidase activity before antigen retrieval and (2) to use high pH (i.e. pH 9.5) antigen retrieval solution. Also, the quality of FFPE sample may influence on the degree of signal amplification by a catalyzed signal amplification of the specific signals as well as the background signals. In case that increased background signal amplification impairs the quality of analysis, reduced incubation time of polymer HRP conjugated secondary antibody would decrease background signal amplification more than specific signal amplification, resulting in better signal-to-noise ratio and more reproducible analysis by LSC/iCys. Those processes are keys for obtaining reproducible multiple immunofluorescent staining and need to be optimized under the individual experimental conditions for the stable analysis by LSC/iCys.

Previous studies have described the concordance between LSC and FCM analysis (3,10). Our method showed concordance of the CD4/CD8 ratio between LSC and FCM, indicating

that the results from LSC/iCys and FCM can be seen equivalent. Direct comparison between local, i.e. *in situ*, and systemic, i.e. in peripheral blood, would be quite interesting. It has not been feasible to directly compare the result from manual quantification *in situ* and objective quantification by FCM because the nature of quantification is completely different. However, it is potentially possible to investigate the correlation between the quantification of *in situ* (e.g. intragraft) T reg by LSC/iCys and of circulating T reg in peripheral blood by FCM although further precise study is required to confirm the validity of direct comparison between LSC/iCys and FCM.

In this report, we introduced a protocol for multiple immunofluorescent staining and analysis with LSC/iCys, which was optimized for the quantitative *in situ* analysis of T reg on FFPE sample, and the data shown in this report were anecdotal just to evaluate the potential application of our protocol. Additionally, preliminary data from limited number of samples of intestinal allograft biopsy were shown regarding the differences of T reg population in inflammatory infiltrate between E-ACR and L-ACR. Although clinical significances of L-ACR were reported in various organ transplantations, its characteristics regarding immunological and molecular mechanisms have not been yet fully investigated (2,12,26,38). Our quantitative analysis successfully showed that significant differences of T reg population in inflammatory infiltrate between E-ACR and L-ACR cases in intestinal transplantation. Because of the small sample size of those preliminary data, clinical and immunological significance of the reduced number of T reg in L-ACR were not clear. However, those results indicated that even morphologically identical ACR cases might vary in terms of the nature of infiltrated inflammatory cells, which can be detected by our multiple immunofluorescent staining and quantitative analysis with LSC/iCys. The detailed analysis regarding the roles of *in situ* T reg associated with alloimmunity in the various setting of transplantation would be performed with a large number of samples in the future study. Although only data regarding T cells was shown in this study, there are numerous potential applications using the similar technique. Our method allows relatively complicated phenotypic analysis of memory/effector T cells, $CD8^+$ regulatory T cells, dendritic cells and macrophages (9,16). By combining intra-cytokine/chemokine staining, Th1/Th2 and Th17 T cells can be also quantitatively evaluated in the grafts (51). Moreover, we will be able to identify the cells specifically infiltrating tissue, combined with the staining of tissue specific markers. An ongoing clinical study using these techniques will be useful to further investigate how immune cells located in the graft site play an important role in immune reaction.

Our method has a disadvantage as well. Our multiple immunofluorescent staining is a time-consuming procedure. Moreover, the data acquisition by LSC/iCys is much slower than those from suspended cells by FCM. In the future, instrumental advances and/or modification of multiple immunofluorescent staining may make it easier to perform quantitative analysis with LSC/iCys.

In conclusion, LSC/iCys and multiple immunofluorescent staining with signal amplification allowed objective quantification of *in situ* $CD4^+/FoxP3^+$ T cell populations, with precise quantitative analysis at a single-cell level on FFPE sections. This objective method can provide essential quantitative analysis of T reg populations even in small biopsy tissues from various transplant organs. Detailed analysis of *in situ* T reg population in transplant biopsy samples may contribute to a better understanding of local alloimmune response in transplantation.

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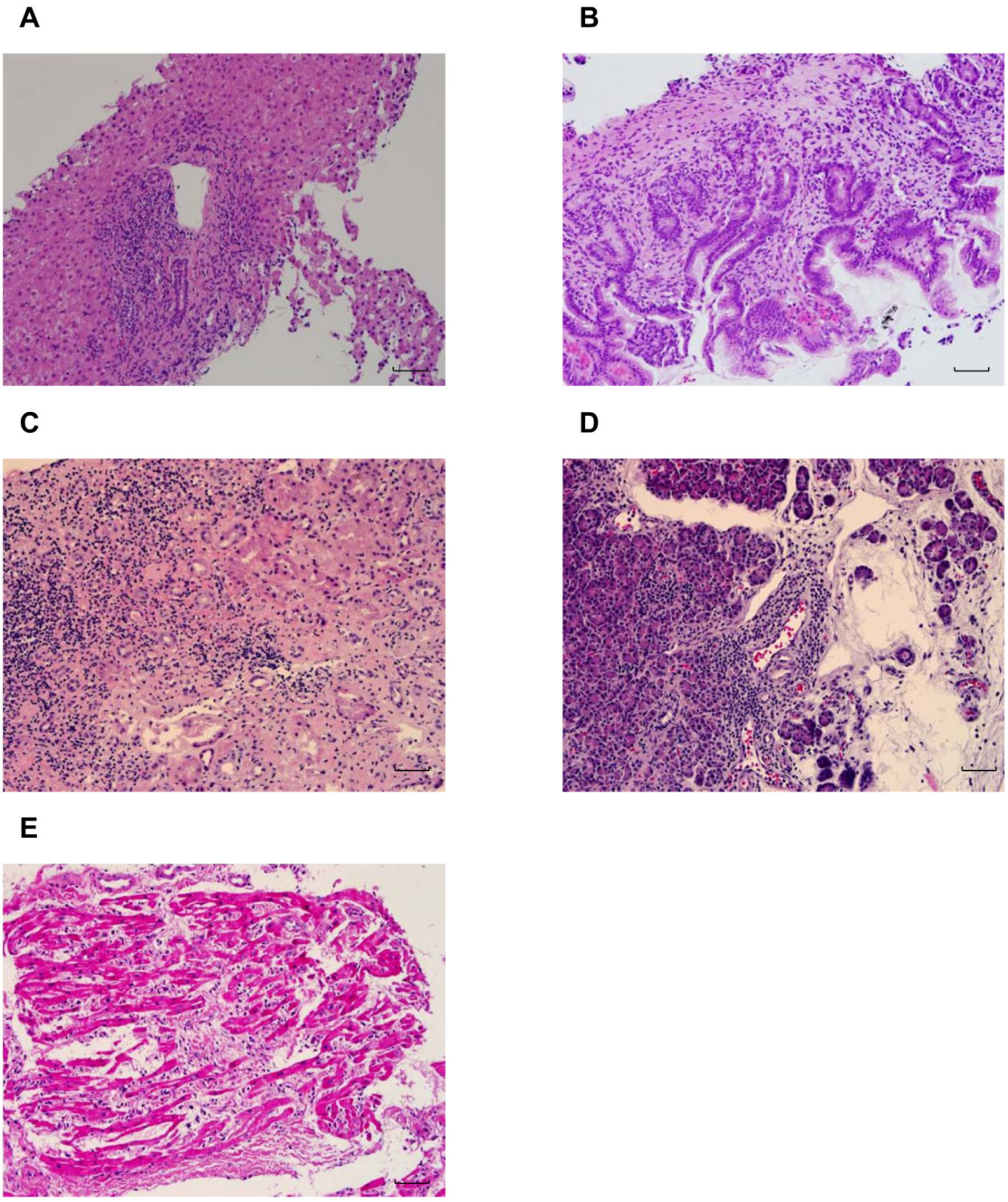
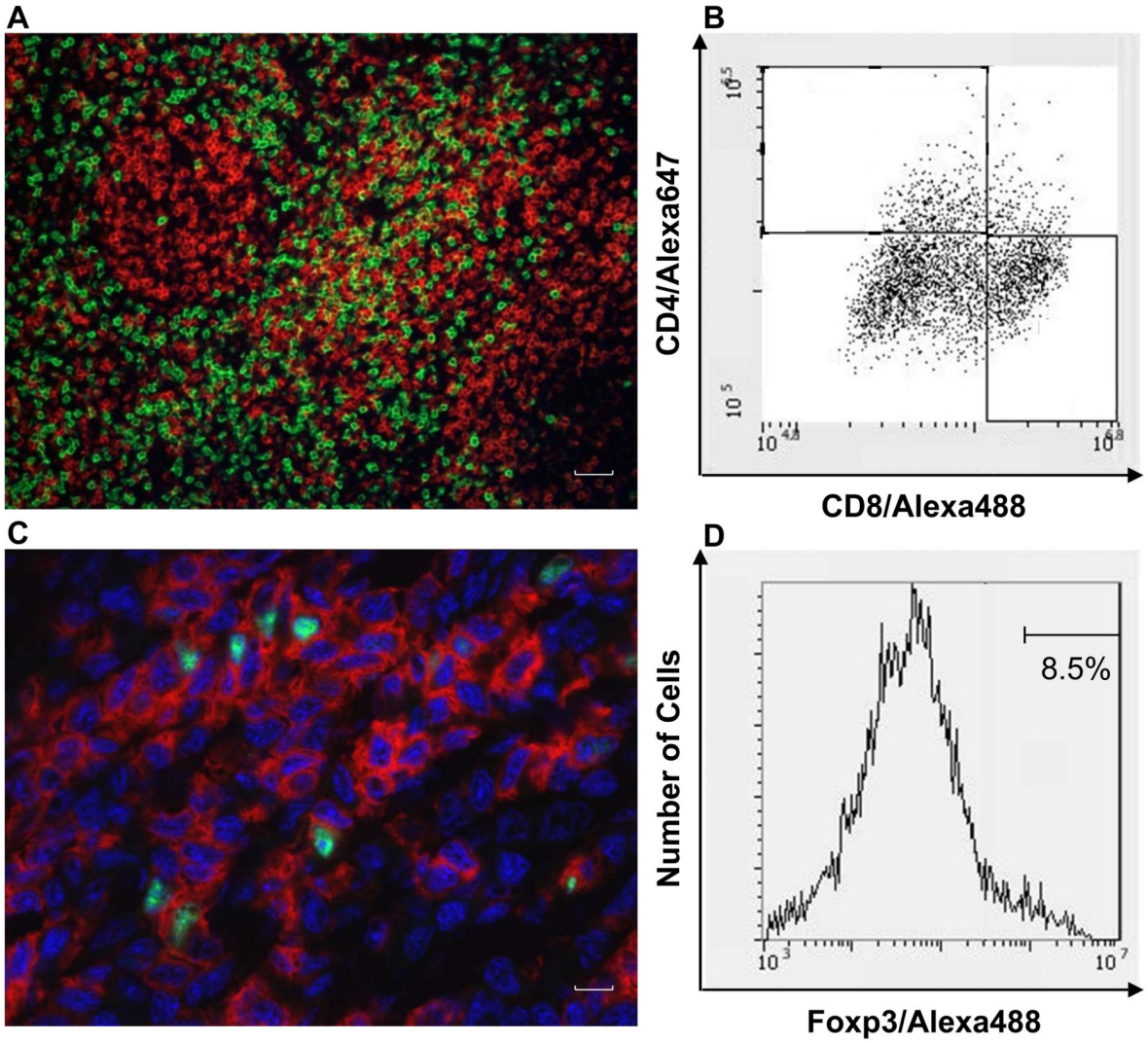


Figure 1.
H&E stains of transplant organ biopsies (scales indicate 250 μm). (A) liver biopsy with moderate acute rejection (×200); (B) small intestine biopsy with mild acute cellular rejection (×200); (C) kidney biopsy with acute T-cell-mediated rejection (×200), type IIA; (D) pancreas biopsy with mild acute cell-mediated rejection (×200); (E) endomyocardial biopsy with acute cellular rejection, grade 3R (×200).



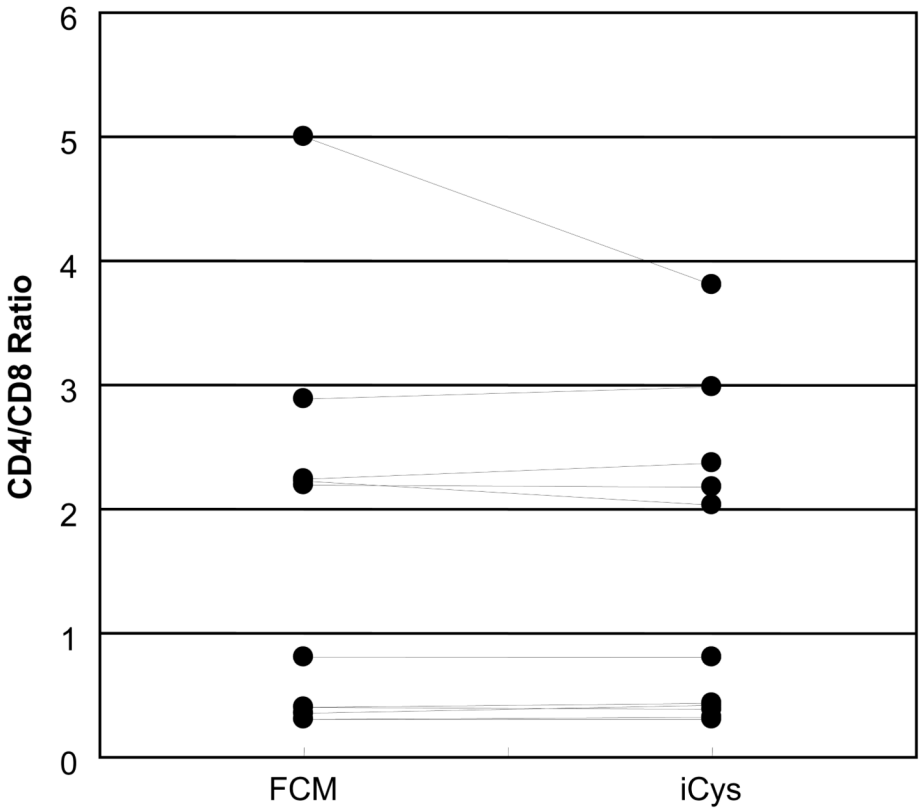


Figure 2.

Multiple immunofluorescent staining on FFPE human tonsil section and the analysis of $CD4^+Foxp3^+$ T reg. (A) FFPE human tonsil section stained for CD4 (Alexa647, red) and CD8 (Alexa488, green) (×200). Nuclear staining by PI was blanked. Scale in figure indicates 50 μm. (B) Distribution of signal integral of Alexa488 (CD8) and Alexa647 (CD4) obtained with LSC/iCys. The CD4/CD8 ratio on this sample was revealed 0.38. (C) CD4 (Alexa647, red) and Foxp3 (Alexa488, green) staining on the same tonsil section (×400). Nuclear staining was performed by PI (blue). Scale in figure indicates 25 μm. (D) Distribution of Alexa488 (Foxp3) gated at $CD4^+$ cell population. The population of T reg ($CD4^+Foxp3^+$ cell) was 8.5%. (E) Comparison of CD4/CD8 ratio between FCM and LSC. There was no statistically significant difference between them ($p = 0.393$).

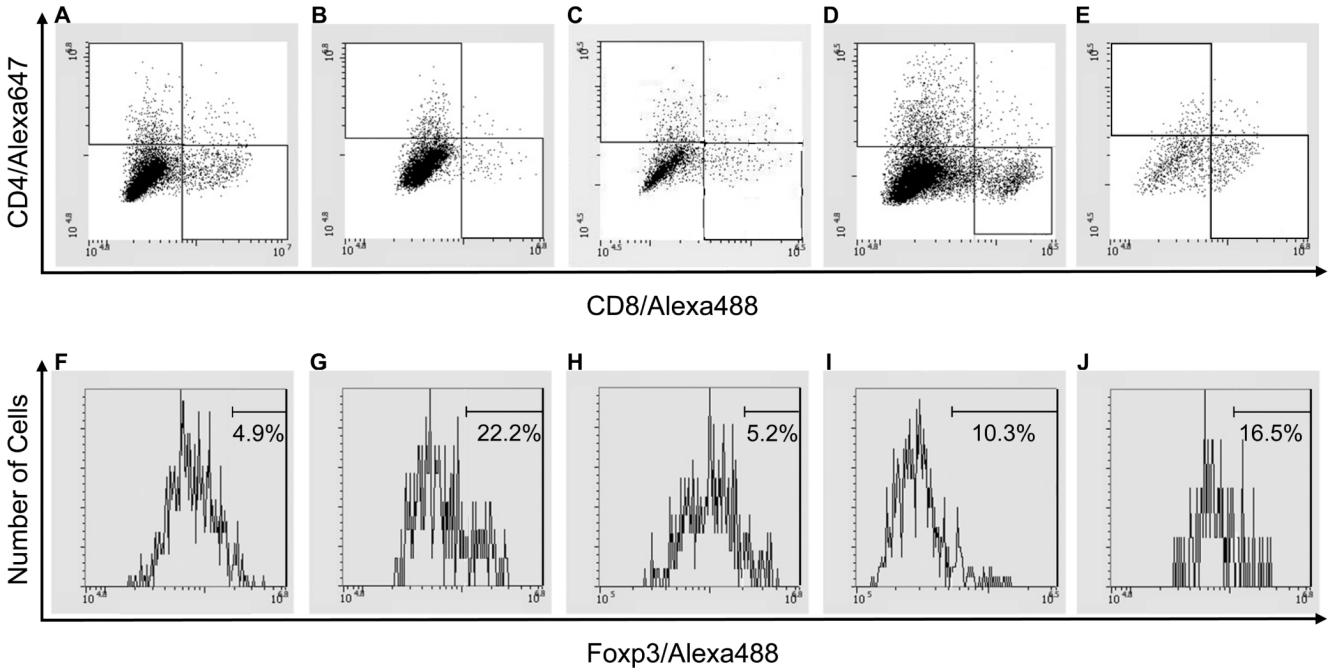


Figure 3.

In situ quantitative analysis of T reg on FFPE sections of various transplant biopsies. Figure 3A, 3B, 3C, 3D and 3E demonstrate the distributions of signal integral of Alexa488 (CD8, x axis) and Alexa647 (CD4, y axis) on liver, small intestinal, kidney, pancreas and endomyocardial biopsies, respectively. Note that double positive cells were less than those determined in tonsil sample because less intensive overlapping of inflammatory cells in graft biopsy allowed more precise detection of each fluorescent integral related to each single cell. Figure 3F, 3G, 3H, 3I and 3J demonstrate the distributions of signal integral of Alexa488 (Foxp3, x axis) among the cell population gated at $CD4^+$ cells in liver, small intestine, kidney, pancreas and endomyocardial biopsies, respectively.

Table 1

Results of immunofluorescent staining in different protocols for antigen retrieval.

Blocking of endogenous peroxidase activity		**before antigen retrieval**				**after aintigen retrieval**
use of pressure cooker		120°C for 10 min		125°C for 5 min		any
antigen retrieval solution		low pH (6.0)	high pH (9.5)	low pH (6.0)	high pH (9.5)	any
immunofluoresecent staining	CD4	**poor**	**good**	**poor**	**intermediate**	**none**
	CD8	**good**	**excellent**	**good**	**excellent**	**good**
	Foxp3	**excellent**	**excellent**	**good**	**good**	**intermediate**

Table 2

Summary of histopathological comparison and *in situ* quantitative analysis of T reg[1] in early onset acute cellular rejection and late onset acute cellular rejection in intestinal transplantation.

		Early (n = 10)	Late (n = 10)	p-value
[2]Number of apoptotic body (mean±SD)		6.30±1.252	6.8±0.789	n.s.
Inflammatory infiltrate				n.s.
Grade	0	1	1	
	1	3	8	
	2	5	0	
	3	1	1	
Mucosal edema				n.s.
Grade	0	1	5	
	1	6	4	
	2	3	1	
	3	0	0	
Blunting of villi				n.s
Grade	0	1	2	
	1	3	5	
	2	3	3	
	3	3	0	
Vascular congestion				n.s
Grade	0	2	0	
	1	7	9	
	2	1	1	
	3	0	0	
Red blood cell extravasation				n.s
Grade	0	4	5	
	1	5	3	
	2	1	2	
	3	0	0	
Number of $CD4^+$ cells		63.40±28.20	82.10±41.40	n.s
Number of $CD8^+$ cells		221.00±154.96	323.70±134.68	n.s
CD4/CD8		0.360±0.184	0.258±0.082	n.s.
[2]Treg/CD4 (%)		19.28±7.18	12.12±6.45	0.031
[2]Treg/total T cell (%)		4.74±2.41	2.62±1.61	0.032

[1]T reg is defined as FoxP3+ cells (see Materials and Methods).

[2]Number of apoptotic body: number of cryptepithelial cell apoptosis in 10 crypts.

Table 3

Semi-quantitative scoring system for histopathological changes in transplant biopsy.

Grade	Histopathological changes	
0	No significant or minimal	0-10% of tissue shows changes
1	mild	10-40% of tissue shows changes
2	moderate	40-70% of tissue shows changes
3	severe	70% or grater portion of tissue shows changes

Table 4

Summary of *in situ* quantitative analysis of T reg[1] on various transplant biopsies.

Graft	Histological Diagnosis	$CD4^+/CD8^+$ rate	Treg/$CD4^+$ (%)	Treg/total Tcell (%)
Liver	Moderate acute rejection	0.64	4.9	1.9
Intestine	Acute cellular rejection, mild	3.1	22.3	16.8
Kidney	Acute T-cell-mediated rejection, IIA	1.3	5.2	3.0
Pancreas	Mild acute cell-mediated rejection	1.0	10.3	5.2
Heart	Acute cellular rejection, 3R	0.30	16.5	3.8

[1] T reg is defined as FoxP3+ cells (see Materials and Methods).