ELSEVIER

Journal of Immunological Methods 305 (2005) 67–74

JIM
Journal of
Immunological Methods

www.elsevier.com/locate/jim

Research paper

Detecting antibodies with similar reactivity patterns in the HLDA8 blind panel of flow cytometry data

M.P. Salganik [a,*], D.L. Hardie [b], B. Swart [c], G.W. Dandie [c], H. Zola [c], S. Shaw [d], H. Shapiro [e], K. Tinckam [f], E.L. Milford [f], M.P. Wand [g]

[a] *Department of Biostatistics, Harvard School of Public Health, 665 Huntington Avenue, Boston, MA 02115, USA*
[b] *MRC Centre for Immune Regulation, Institute of Biomedical Research, University of Birmingham, Birmingham B15 2TT, UK*
[c] *Child Health Research Institute, Women's and Children's Hospital, 72 King William Road, North Adelaide 5006, Australia*
[d] *Experimental Immunology Branch, National Cancer Institute, National Institute of Health, Bethesda, MD 20892-1360, USA*
[e] *The Center for Microbial Cytometry, West Newton, MA 02465, USA*
[f] *Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, USA*
[g] *Department of Statistics, School of Mathematics, University of New South Wales, Sydney 2052, Australia*

Accepted 20 June 2005
Available online 2 August 2005

Abstract

The blind panel collected for the 8th Human Leucocyte Differentiation Antigens Workshop (HLDA8; http://www.hlda8.org) included 49 antibodies of known CD specificities and 76 antibodies of unknown specificity. We have identified groups of antibodies showing similar patterns of reactivity that need to be investigated by biochemical methods to evaluate whether the antibodies within these groups are reacting with the same molecule. Our approach to data analysis was based on the work of Salganik et al. (in press) [Salganik, M.P., Milford E.L., Hardie D.L., Shaw, S., Wand, M.P., in press. Classifying antibodies using flow cytometry data: class prediction and class discovery. Biometrical Journal].

Keywords: Antibodies; Classification; Cluster of differentiation; CD; Flow cytometry; Blind panel; Reactivity pattern; Kernel smoothing

1. Introduction

In the early HLDA workshops (Bernard et al., 1984; Reinherz et al., 1986; McMichael et al., 1987; Knapp et al., 1989; Schlossman et al., 1995; Kishimoto et al., 1997), proving that two independent antibodies bound to the same antigen with a novel molecular structure was both sufficient and necessary to designate a new CD specificity. Panels of flow cytometry data (conventionally called "blind panels") have been used to characterize the reactivity pattern of antibodies across cell populations of different lineages. It was shown that similarity in the reactivity patterns of newly discovered antibodies was a strong predictor of

* Corresponding author. Tel.: +1 617 432 3689; fax: +1 617 432 3755.
E-mail address: salganik@hsph.harvard.edu (M.P. Salganik).

0022-1759/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.jim.2005.07.002

identity between their antigens. Blind panels were used to identify the groups of antibodies showing similar patterns of reactivity. Potential identity of specificity within these groups of antibodies was then evaluated by biochemical and molecular biological techniques, resulting in the assignment of a CD number to the antigen and the use of the same CD number to describe antibodies that exhibited specificity to that antigen. However, antigen cloning has rendered obsolete the requirement of having at least two independent antibodies of the same specificity to establish a new CD molecule. It is now considered appropriate (Mason et al., 2002) to establish a CD designation for a molecule if its gene has been cloned and at least one specific monoclonal antibody has been studied in the workshop. The role of flow cytometry data in the process of discovering new CDs has therefore decreased. Nevertheless, detecting similarity in the reactivity patterns of antibodies across a broad range of cell populations may still lead to the identification of new CDs and to assigning new antibodies to known CD clusters. This follows in part from the difficulty and unpredictability of molecular analysis, as it is more economically attractive to only invest in such analyses (Western blotting, immunoprecipitation) when several antibodies have the same apparent specificity. Furthermore, an analysis of antibody reactivity with antigenic post-translational variants (e.g. carbohydrate epitopes) provides a necessary and useful complement to biochemical and molecular analysis.

Biochemical and molecular characterization of antigens provides valuable background information, but users of antibodies against CD molecules are primarily interested in their reactivity with cells and tissues, which may vary in different applications. Flow-cytometric analysis of antibody reactivity ceased to be a major tool for discovering new molecules, but it continues to be central to HLDA workshop analysis and biological investigation.

This paper describes the application of a recently developed statistical methodology (Salganik et al., in press) to detecting groups of antibodies of similar reactivity in a panel of flow cytometry data collected by HLDA8 participating laboratories.

The development of statistical methodology for the design and analysis of blind panel experiments attracted only limited attention from researchers (e.g. Spiegelhalter and Gilks, 1987; Gilks et al., 1989; Gilks and Shaw, 1995; Hallam et al., 1997; Salganik et al., in press). Our approach to the analysis of the blind panel data is similar in spirit but differs in important implementation details (see also Salganik et al., in press) from the approaches used by Shaw et al. (1995) and Hallam et al. (1997). The novelty of our approach is that an automatic algorithm preselects a small subset of antibody pairs with "unusually high" similarity scores for subsequent visual inspection of the paired fluorescence staining profiles by a data analyst. This approach is similar to that used when searching for information on the Internet, where rapid search engines help focus the user's attention on the subset of possibly relevant objects. Previously used algorithms relied on summaries of the fluorescence intensity distributions (e.g. means and percentages of values above the threshold) in their evaluation of similarity between the antibodies. The matrix of similarity scores was used for hierarchical clustering of antibodies, and the summary of the resulting clusters was displayed in the form of a dendrogram. A data analyst used the dendrogram to identify antibodies that were clustered together and may therefore have identical specificity. However, the usefulness of this approach is bounded by the well-known deficiencies of hierarchical clustering. The structure of a dendrogram is often too sensitive to the intercluster similarity definition (i.e. the choice between group average, nearest neighbor and further neighbor methods) and small changes in the data. In addition, it is often too difficult to identify clusters based on visual inspection of the dendrogram or even to estimate the number of clusters in a dataset. Furthermore, important information may be lost when the distribution of log-fluorescence is summarized by the mean or mean and standard deviation. The partially automated approach to the detection of similarity in reactivity patterns, described herein, overcomes some of these limitations.

The analysis of the similarities in the staining patterns of HLDA8 antibodies guided the subsequent immunohistochemical and Western blot experiments as described by Swart et al. (2005).

2. Materials and methods

The HLDA8 blind panel of flow cytometry data included 49 antibodies of known CD specificity, 76

antibodies of unknown CD specificity and 3 negative controls. Each antibody was tested against 47 cell populations producing more than 6,000 flow cytometric assays. Each individual assay consisted of fluorescence measurements from thousands of cells (stained by an antibody or negative control) from a particular cell population. The description of the cell populations and antibodies is presented by Swart et al. (2005).

Similarity in the pattern of distributions of fluorescence intensities across different cell populations observed for a pair or group of antibodies suggests a possible identity in their antigens. The goal of our analysis was to detect pairs or groups of antibodies with "unusually high" similarity in reactivity patterns.

The automated algorithm identified the small subset of pairs formed by the 76 antibodies of unknown CD specificity with the "unusually high" similarity in reactivity patterns and then we re-evaluated this assignment of similarity by the visual comparison of the densities of log-fluorescence distributions.

Fig. 1(a) and (b) provide an example of such a comparison. The figures show the estimated probability density functions (or "densities" for short) of log-fluorescence distributions for $p=47$ cell populations (U397,. . ., CBM) stained by the fluorescently labelled antibodies 038 and 039 from the HLDA8 blind panel and the distributions of a baseline log-fluorescence observed with the negative control (unreactive) anti-

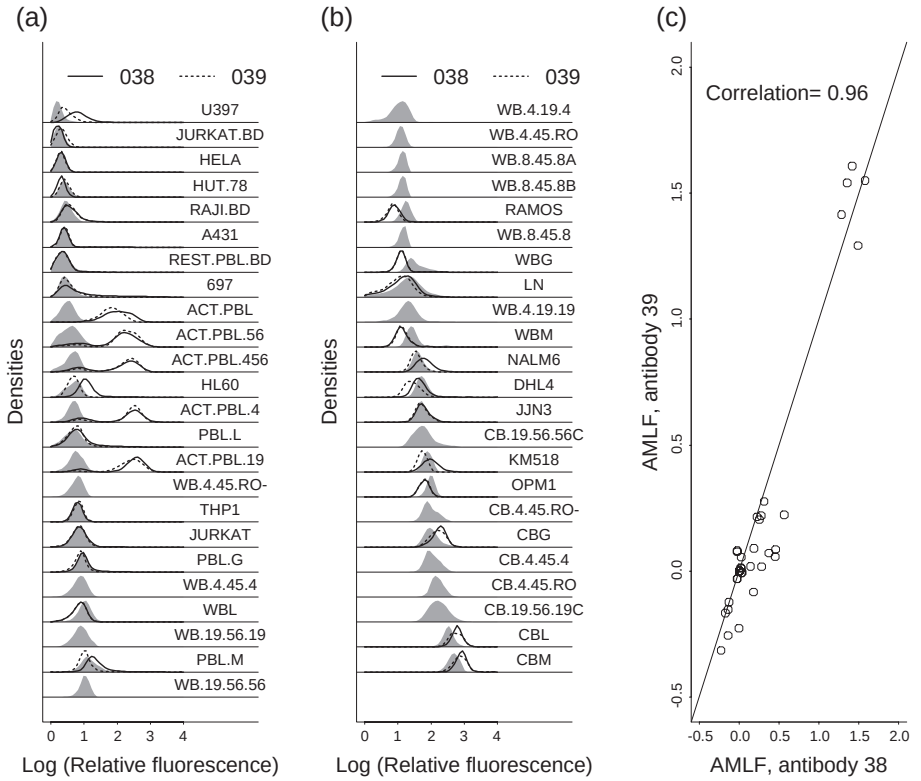

Fig. 1. (a, b) Density estimates of log-fluorescence intensity distributions for negative control-stained cell populations (gray polygons) and cell populations stained by antibodies (solid lines, antibody 038 and dashed lines, antibody 039). The density curves are scaled so that the maximum value for each of the cell populations is equal to one. Experiments with 16 cell sub-populations were intentionally not performed because they were expected to be negative (e.g. analysis of CD4 when T cells were shown to be negative with the antibody in question). The meaning of the labels of the cell populations and antibodies is explained in Swart et al. (2005). (c) A comparison of the baseline adjusted values of mean log-fluorescence (AMLF). Each symbol shows the values for the particular cell population stained by antibodies 038, 039. The line corresponds to equal fluorescence values.

body. The fluorescence of 5 of the 32 cell populations (ACT.PBL, ACT.PBL.56, ACT.PBL.456, ACT.PBL.4, ACT.PBL.19) increased dramatically after staining with the antibodies, while the increase of fluorescence associated with staining was negligible for the other cell populations. This similarity in the expression profiles suggests that antibodies 038 and 039 may bind to the same antigen.

A visual comparison of the densities of log-fluorescence required an evaluation of the significance of the observed differences in their features. We discuss the details of our approach to this analysis in Appendix A.

The pairs of antibodies with "unusually high" similarity in reactivity patterns were identified by their elevated values of a sample correlation coefficient r (or Pearson's product moment correlation coefficient) between the baseline-adjusted mean values (AMLF) of a log-fluorescence. The value of AMLF for a particular antibody and cell population quantifies the difference between the mean value of log-fluorescence observed in this cell population after its staining by the antibody and the mean value of a baseline log-fluorescence observed in the unstained cell population. The mean value of baseline log-fluorescence was estimated by averaging the mean log-fluorescence values observed in the control experiments. Fig. 1(c) shows the AMLF values for antibodies 038 and 039. The high value of the sample correlation coefficient ($r=0.96$) quantifies a substantial similarity in the reactivity patterns exhibited by the antibodies, which can be confirmed by a visual analysis of densities.

We designed the blind panel experiments so that the panel included a "training set" containing 49 antibodies of known CD specificities. These antibodies formed 1176 pairs of which 102 pairs were of identical specificity. We used this part of the panel for an empirical estimation of a correlation threshold value r_T which would then focus the visual analysis of densities on only a small subset of antibody pairs with correlation scores exceeding this threshold. The threshold value ($r_T=0.721$) was chosen as a 99% quantile of the distribution of the correlation scores for the 1074 pairs formed by the antibodies of known different CD specificity. We assumed that the "training set" of 1176 pairs was representative of the general population of these sets and therefore expected that the approach described should lead to a high specificity of detection for pairs of unknown antibodies with identical reactivities. Similar to our previous experience with other datasets (Salganik et al., in press), we found that the correlation scores for the antibody pairs formed by the antibodies of identical CD specificity were on average much higher than the correlation scores among the pairs with different CD specificity. We classified as "unusually similar" the reactivities of the pairs of antibodies with correlation scores exceeding the value $r_T=0.721$ of the quantile-based threshold. This classification rule correctly identified all of the pairs formed by the antibodies of known identical specificity in the "training set" as "unusually similar". We expected that application of this automatic algorithm utilizing the quantile-based threshold to the detection of the "unusually similar" pairs amongst the 2850 pairs formed by the antibodies of unknown CD specificity would similarly lead to reasonably high values of sensitivity and specificity. Fig. 2(a) shows the distributions of the correlation scores for the pairs formed by the antibodies.

Fig. 2(b) shows an image of the correlation scores for the panel of 125 antibodies of interest. We reordered the antibodies and showed only the correlations exceeding the threshold r_T. The first 49 antibodies (of known specificity) were re-indexed by their known CD specificities and are displayed in the following order: 8 antibodies from CD3, 7 antibodies from MHCII, 7 antibodies from CD45RA, 6 antibodies from CD14, 4 antibodies from CD19, 3 antibodies from CD15, 3 antibodies from CD113, 3 antibodies from CD45, 2 antibodies from RAI, 2 antibodies from APT4, 1 antibody each from CD44, CD45RB, BTCHAIN, FMLP receptor). The remaining 76 antibodies (of unknown specificity) were re-ordered to be consistent with the order on the dendrogram representing the results of hierarchical clustering (single linkage method) based on the dissimilarity scores $d=1-r$. The clustering-based reordering of objects (e.g. Hastie et al., 2001) increases the similarity between the objects with adjacent indices and thus improves the interpretability of the correlation image.

Some experiments in the panel were intentionally not performed, because they were thought to be redundant. It was assumed that if the experiment

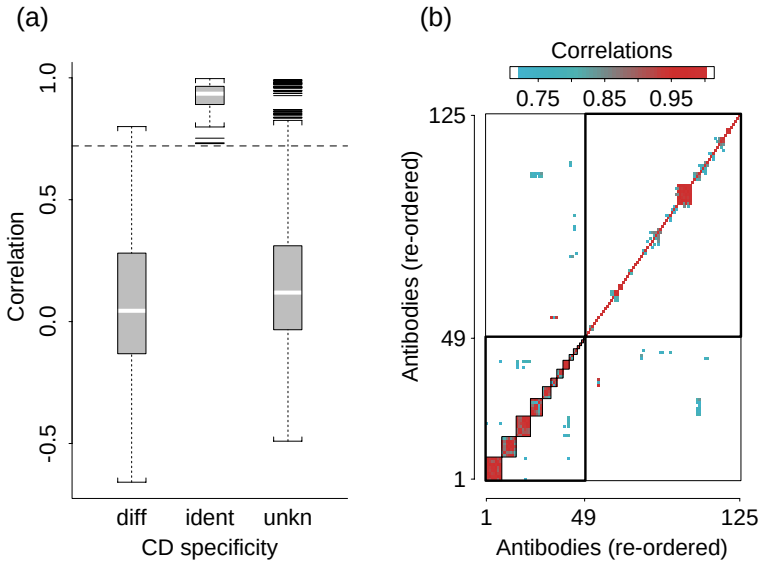

Fig. 2. (a) Boxplots of the correlation score distributions for the 102 pairs of identical, 1074 pairs of different and 2850 pairs of unknown CD specificity. The horizontal lines show the threshold value r_T=0.721. (b) A correlation between the baseline-adjusted mean values of log-fluorescence. The image shows only correlation values exceeding the threshold r_T=0.721. The antibodies were re-ordered as described in Section 2. The thick black lines bound the subsets of antibodies with known (left lower corner) and unknown (right upper corner) CD specificity. The smaller rectangles bounded by the thin black lines mark the groups of antibodies of known identical specificity.

with a cell population shows no staining with antibody then the corresponding experiments with the subpopulations of this cell type may be unnecessary. Two different approaches were considered to calculating correlation coefficients. In the first approach, the calculation of a correlation coefficient was based on the non-missing values of AMLF. In the second approach we imputed AMLF=0 values as an outcome of the intentionally omitted experiments and treated these as if they were observed. Resulting values of correlation coefficients obtained by using these approaches were very similar and we used the values of correlation coefficients based on the imputation method.

3. Results

In a set of 2850 pairs formed by antibodies of unknown CD specificity we found 60 pairs (2.1%, formed by 49 antibodies) with correlation coefficients exceeding the threshold 0.721 value. Visual inspection of the densities suggested that biochemical investigation of the similarity of CD specificity for 28 of these pairs (formed by 25 antibodies) was warranted. Fig. 3 shows the correlation scores for these antibodies, which were re-ordered using the correlation-based hierarchical clustering described in Section 2. The minimum correlation score for these pairs was equal to 0.722. For convenience we organized the antibodies in 8 groups such that each of the antibodies within the groups had high correlation of the baseline adjusted log-fluorescence (AMLF) values with the AMLF values for at least one of the antibodies within the same group and low correlation with the AMLF values for antibodies in other groups. We predict that some of the antibodies within these groups may have identical CD specificity. This hypothesis will be investigated by molecular methods.

The correlation scores for 22 pairs formed by antibodies of unknown CD specificity with the antibodies of known CD specificity exceeded the 0.721 threshold. We visually inspected the fluorescence staining profiles for these pairs and found that reactivity patterns associated with antibodies 402, 413 and 636

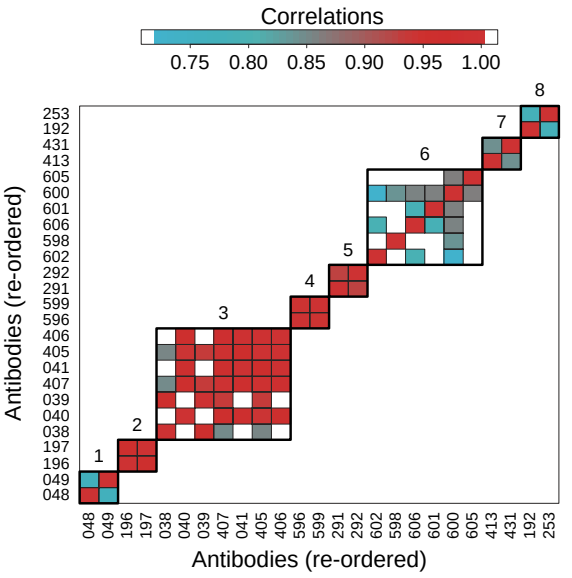

Fig. 3. The correlation between the baseline-adjusted mean values of log-fluorescence for the subset of 25 antibodies described in Section 3. The image shows only the correlations that exceeded the $r_T = 0.721$ threshold for pairs of antibodies with the similarity of antibody reactivities confirmed by visual inspection of the densities and SiZer maps. The numbers above each block of antibodies identify a group of antibodies.

were similar to those of antibodies from the CD15, PAI and CD14 clusters.

4. Discussion

We used an approach for detecting antibodies with similar reactivities suggested by Salganik et al. (in press). We started from a preliminary automatic detection of a small subset of the pairs formed by the antibodies of unknown CD specificity that had "unusually high" correlations between their baseline-adjusted log-fluorescence values. The similarity in the reactivities of these antibodies was re-evaluated by visual inspection of the fluorescence distributions of log-fluorescence. We found several groups of antibodies with highly similar reactivity patterns.

An empirically selected 99% quantile threshold correctly detected all of the pairs of the identical CD specificity in the training dataset and reduced by a factor of approximately 50 the number of pairs requiring detailed visual comparison of their densities. Our limited experience with the analysis of several blind panel datasets (see Salganik et al., in press for some examples) illustrates the potential value of this novel approach.

While the initial results of the data analysis look encouraging we emphasize that a detailed comparison of the existing approaches and the subsequent analysis of multiple blind panel datasets is required before any of the methods may be recommended as a standard. This work will require a non-trivial additional effort because the collection of the blind panel data requires a substantial effort of multiple laboratories (e.g. Swart et al., 2005) and publicly available datasets are scarce. In spite of these technical difficulties we anticipate that the current methodology as well as many other different approaches to the analysis of blind panel experiments will be further developed and tested in the future.

Acknowledgments

We thank Donald Halstead, Inge Koch and Jim Ware for their useful comments and suggestions. This work was partially supported by a grant from the Flow Cytometry Facility, Renal Division, Brigham and Women's Hospital, and a grant from the University of New South Wales Faculty Research Grants Program.

Appendix A. Estimation and comparison of densities of log-fluorescence distributions

Each of the density estimates displayed in Fig. 1 was obtained by an application of the kernel density estimation procedure described by e.g. Wand and Jones (1995) to the raw log-fluorescence values. This estimate may be viewed as a smoothed alternative to the histogram that is more familiar to biomedical researchers.

Let x denote a value of log-fluorescence. A histogram estimate of the density of log-fluorescence distribution may be obtained by dividing the range $(x_{\min}, x_{\max})$ of the observed log-fluorescence values into B histogram bins of equal width $\Delta = (x_{\min} - x_{\max})/B$

and estimating the density at an arbitrary point x as

$$\hat{f}_{\mathrm{H}}(x;\Delta) = \frac{\text{number of observations in bin containing } x}{n\Delta},$$

where n is the number of observations in the sample. Fig. 4 shows two histogram density estimates obtained from the sample of $n=207$ log-fluorescence measurements observed in the cell population ACT.PBL.56 after staining the cells with antibody 038. This relatively small sample of observations was obtained by a selection of the data for a relevant cell population (i.e. by gating) from a much larger dataset of a multi-color flow cytometry measurements.

A comparison of the estimates based on the different values of a "binwidth" Δ illustrates the well known dependence of the density estimate from the choice of the smoothing parameter's values. A stronger smoothing of the original data achieved by an increase of a "binwidth" Δ leads to the desirable decrease of variability of a density estimate and the undesirable decrease in the spatial resolution of the resulting curve.

In our work we estimated densities of log-fluorescence using a kernel density estimator with a Gaussian kernel and selected a "bandwidth" parameter h by the normal reference rule (see Wand and Jones, 1995 among others). The resulting density estimate is shown in Fig. 4. We also used the "SiZer" statistical methodology developed by Chaudhuri and Marron (1999) and the software developed by J.S. Marron (http://www.stat.unc.edu/faculty/marron.html) for evaluating the statistical significance and sensitivity of the observed multi-modality in the log-fluorescence distributions to the choice of the smoothing parameter.

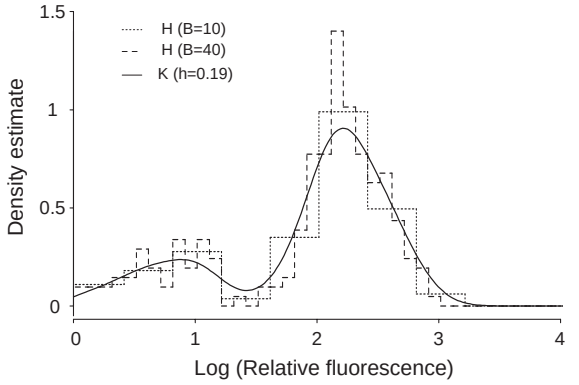

Fig. 4. Histogram-based (dotted and dashed lines) and kernel density (solid line) estimates for log-fluorescence distribution observed in the cell population ACT.PBL.56 after staining the cells with the antibody 038. The numbers in the legend show the number of bins B used for the histogram density estimates and the bandwidth parameter h used for the kernel density estimate.

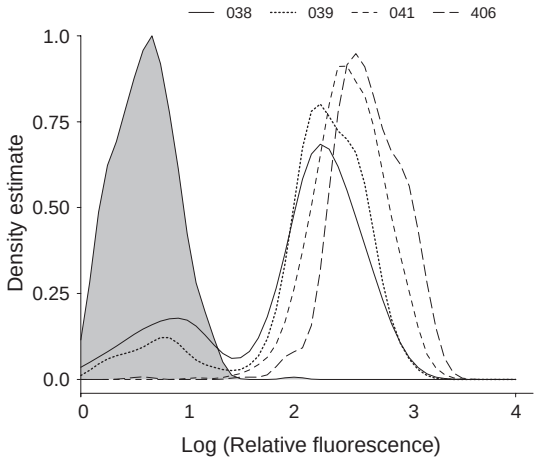

Fig. 5. Density estimates for the log-fluorescence distributions of cell populations ACT.PBL.56, ACT.PBL.456, ACT.PBL.4, ACT.PBL.19 stained by the antibodies 038, 039, 041, and 406. Density estimates of log-fluorescence intensity distributions for unstained cell populations are shown as gray polygons, and distributions for the cell populations stained by antibodies are denoted by solid and dashed lines. The density curves are scaled so that the maximum value of the density estimates is equal to one.

Fig. 5 illustrates the importance of the visual comparison of densities of log-fluorescence for the evaluation of the similarity of specificities of antibodies. It shows the density estimates for the fluorescence distributions observed in the cell populations ACT.PBL.56 stained by the antibodies 038, 039, 041 and 406. These curves demonstrate an important difference in the distributions of fluorescence: antibodies 038 and 039 have bimodal distributions of fluorescence while the fluorescence distributions for antibodies 041 and 406 are unimodal. An application of the methodology of Chaudhuri and Marron (1999) showed that the presence of a subpopulation with low values of log-fluorescence in the populations of cells stained by the antibodies 038 and 039 was statistically significant and may be detected at multiple levels of spatial resolution, corresponding to multiple values of

the bandwidth h. The qualitative difference in the features of fluorescence distributions suggests that the antibodies 038 and 039 are likely to bind to different antigens than antibodies 041 and 406.

References

Bernard, A., Boumsell, L., Dausset, J., Milstein, C., Schlossman, S.F. (Eds.), 1984. Leukocyte TypingSpringer Verlag, Berlin.

Chaudhuri, P., Marron, J.S., 1999. SiZer for exploration of structures in curves. Journal of the American Statistical Association 94, 807.

Gilks, W.R., Shaw, S., 1995. Statistical analysis. In: Schlossman, S., et al., (Eds.), Leucocyte Typing V. Oxford University Press, Oxford, p. 8.

Gilks, W.R., Oldfield, L., Rutherford, A., 1989. Statistical analysis. In: Knapp, W., et al., (Eds.), Leucocyte Typing IV. Oxford University Press, Oxford, p. 6.

Hallam, J., Ying, Z., Gail, H.M., Shaw, S., 1997. T-cell blind panel: evolving strategy for finding similarities between monoclonal antibodies on the basis of flow cytometric analysis of multiple cell subsets in peripheral blood. In: Kishimoto, T., et al., (Eds.), Leucocyte Typing VI. Garland Publishing, Inc., New York, p. 82.

Hastie, T., Tibshirani, R., Friedman, J., 2001. The Elements of Statistical Learning. Data Mining, Inference, and Prediction. Chapman and Hall, London.

Kishimoto, T., Kikutani, H., von dem Borne, A.E.G.Kr., Goyert, S.M., Mason, D.Y., Miyasaka, M., Moretta, L., Okumura, K., Shaw, S., Springer, T.A., Sugamura, K., Zola, H. (Eds.), 1997. Leucocyte Typing VI: White Cell Differentiation Antigens. Garland Publishing, Inc., New York.

Knapp, W., Dorken, B., Gilks, W., Rieber, E.P., Schmidt, R.E., Stein, H., von dem Borne, A.E.G.Kr. (Eds.), 1989. Leucocyte Typing IV. Oxford University Press, Oxford.

Mason, D., Andre, P., Benussian, A., Buckley, C., Civin, C., Clark, E., de Haas, M., Goyert, S., Hadam, M., Hart, D., Horesi, V., Jones, Y., Meuer, S., Morissey, J., Schwartz-Albiez, R., Shaw, S., Simmons, D., Turni, L., Uguccioni, M., van der Schoot, Vivier, E., Zola, H. (Eds.), 2002. Leucocyte Typing VII. Oxford University Press, Oxford.

McMichael, A.J., Beverley, P.C.L., Cobbold, S., Crumpton, M.J., Gilks, W., Gotch, F.M., Hogg, N., Horton, M., Ling, N., MacLennan, I.C.M., Mason, D.Y., Milstein, C., Spiegelhalter, D., Waldmann, H. (Eds.), 1987. Leucocyte Typing III: White Cell Differentiation Antigens. Oxford University Press, Oxford.

Reinherz, E.L., Haynes, B.F., Nadler, L.M., Bernstein, I.D. (Eds.), 1986. Leucocyte Typing IISpringer-Verlag, New York.

Salganik, M.P., Milford E.L., Hardie D.L., Shaw, S., Wand, M.P., in press. Classifying antibodies using flow cytometry data: class prediction and class discovery. Biometrical Journal.

Schlossman, S.F., Boumsell, L., Gilks, W., Harlan, J.M., Kishimoto, T., Morimoto, C., Ritz, J., Shaw, S., Silverstein, R., Springer, T., Tedder, T.F., Todd, R.F. (Eds.), 1995. Leucocyte Typing V. Oxford University Press, Oxford.

Shaw, S., Luce, G.G., Gilks, W.R., Anderson, K., Ault, K., Bochner, B., Boumsell, L., Denning, S.M., Engleman, E.G., Fleisher, T., Freedman, A.S., Fox, D.A., Gailit, J., Guttieres-Ramos, J.C., Hurtubise, P.E., Lansdorp, P., Lotze, M.T., Mawhorter, S., Marti, G., Matsuo, Y., Minowada, J., Michelson, A., Picker, L., Ritz, J., Roos, E., van der Schoot, C.E., Springer, T.A., Tedder, T.F., Telen, M.J., Thompson, J.S., Valent, P., 1995. Leucocyte differentiation antigen database. In: Schlossman, S., et al., (Eds.), Leucocyte Typing V. Oxford University Press, Oxford, p. 8.

Spiegelhalter, D.J., Gilks, W.R., 1987. Statistical analysis. In: McMichael, A.J., et al., (Eds.), Leucocyte Typing III. Oxford University Press, Oxford, p. 14.

Swart, B., Salganik, M.P., Dagderviren, A., Hodge, G., Hollemweguer, E., Horesi, V., Macardle, P., Milford, E., Millard, D., Narin, J., Tinckam, K., Wand, M.P., Dandie, G.W., Zola, H., 2005. The HLDA8 blind panel: Findings and conclusions. Journal of Immunological Methods 305, 75.

Wand, M.P., Jones, M.C., 1995. Kernel Smoothing. Chapman and Hill, London.