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Technical note

Reconstructing a 3-dimensional image of the results of antinuclear antibody testing by indirect immunofluorescence

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ABSTRACT

Indirect immunofluorescence anti-nuclear antibody testing (IIF-ANAT) is an essential screening tool in the diagnosis of various autoimmune disorders. ANA titer quantification and interpretation of immunofluorescence patterns are determined subjectively, which is problematic.
First, we determined the examination conditions under which IIF-ANAT fluorescence intensities are quantified. Next, IIF-ANAT was performed using homogeneous, discrete speckled, and mixed serum samples. Images were obtained using Bio Zero BZ-8000, and 3-dimensional images were reconstructed using the BZ analyzer software. In the 2-dimensional analysis, homogeneous ANAs hid the discrete speckled pattern, resulting in a diagnosis of homogeneous immunofluorescence. However, 3-dimensional analysis of the same sample showed discrete speckled-type ANA in the homogeneous background.
This study strengthened the current IIF-ANAT method by providing a new approach to quantify the fluorescence intensity and enhance the resolution of IIF-ANAT fluorescence patterns. Reconstructed 3-dimensional imaging of IIF-ANAT can be a powerful tool for routine laboratory examination.

1. Introduction

Indirect immunofluorescence antinuclear antibody testing (IIF-ANAT) is essential for diagnosing autoimmune diseases. According to the guidelines from the National Committee for Clinical Laboratory Standards, ANA testing should provide ANA titers and fluorescence patterns (Kavanaugh et al., 2000). IIF-ANAT has improved since its introduction into the clinical laboratory, but ANA titration and discrimination of ANA fluorescence patterns remain problematic.

ANA titer is clinically important because high ANA titers are commonly observed in patients with autoimmune diseases. In addition, some of ANAs, including anti-dsDNA and anti-centromere antibodies, are reported to correlate with disease activity (Satoh et al., 2009). ANA titer is determined by repeating a positive test with serial dilution until the test becomes negative; the maximum dilution that tests positive is the reported titer. Because positivity is determined by laboratory technicians, the assay is dependent on subjective interpretation, resulting in inter-laboratory, inter-observer, and inter-examination variability (Satoh et al., 2007).

Interpretation of immunofluorescence patterns presents another problem. Fluorescence patterns develop because of ANA recognition of specific nuclear antigens; for example, a speckled pattern is associated with anti-Smith and anti-snRNP antibodies and a homogeneous pattern is associated with

Abbreviations: 2D, 2-dimensional; 3D, 3-dimensional; IIF-ANAT, indirect immunofluorescence antinuclear antibody testing; MBL, Medical & Biological Laboratories; PBS, phosphate-buffered saline.

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antibodies to DNA, histones, and nucleosomes. Discriminating ANA immunofluorescence patterns is important because each pattern is correlated with a different autoimmune disease, sometimes in terms of pathogenesis and sometimes in association with a disease-specific immune abnormality (Rokeach and Hoch, 1992; Gonzalez et al., 2004; Migliorini et al., 2005). Discrimination of immunofluorescence patterns is often difficult because serum samples frequently contain more than one ANA type (Sanchez-Guerrero et al., 1996).

To make IIF-ANAT more objective, we addressed these issues by (i) quantifying ANA immunofluorescence intensities and by (ii) reconstructing 3-dimensional (3D) images of IIF-ANAT results to distinguish between ANA immunofluorescence patterns derived from more than one ANA.

2. Materials and methods

2.1. Reagents and serum samples

ANA reference sera containing homogeneous or discrete speckled ANAs (HEPASERA-1) and Hep-2 slides were purchased from Medical & Biological Laboratories (MBL; Nagoya, Japan). The other serum samples were collected from patients at our hospital who provided their written informed consent.

2.2. Quantification of fluorescence intensity of indirect immunofluorescence ANA testing

IIF-ANAT was performed according to a previously described method (Dellavance et al., 2009). Briefly, Hep-2 slides were incubated with HEPASERA-1 diluted 1:640 with phosphate-buffered saline (PBS) for 30 min at room temperature (RT). The slides were washed with PBS for 15 min, incubated with fluorescein isothiocyanate-conjugated anti-human IgG antibody (MBL) for 1 h at 37 °C, washed with PBS for 15 min, and covered with Fluoromount/Plus mounting reagent (Japan Tanner Corporation, Osaka, Japan). Images were obtained with Bio Zero BZ-8000 (Keyence, Osaka, Japan). The nuclear fluorescence intensity (130 μm^2/nucleus) was measured using BZ analyzer software (Keyence) according to the manufacturer's instructions.

2.3. 3D reconstruction of the results of indirect immunofluorescence ANA testing

For 3D reconstruction, IIF-ANAT was performed as described above, except that HEp-2 slides were created using the cells obtained from RIKEN BioResource Center (Tsukuba, Japan). The HEp-2 cells were cultured on 2-chamber polystyrene vessel tissue culture-treated glass slides (Becton Dickinson, Franklin Lakes, NJ), washed with PBS, and fixed with methanol/acetone (1:1) for 3 min at −20 °C. The slides were subjected to IIF-ANAT, and 3-dimensional images were reconstructed using BZ Analyzer software (Keyence) according to the manufacturer's instructions. Briefly, 30–40 2-dimensional images were captured every 0.2 μm in the vertical direction using ×40 oil immersion objective lens. The images were reconstructed to a 3D image using real time 3D module of BZ Analyzer software. Fluorescence patterns were analyzed by rotating the reconstructed 3D image.

2.4. Statistical analysis

Statistical analysis was performed in Microsoft Excel®. Statistical significance was evaluated with the Student's *t*-test.

3. Results

3.1. Determination of assay condition for measuring fluorescence intensity

We first examined the cell-to-cell variability in fluorescence intensity. We measured the fluorescence intensities of 20 cells from 4 different fields and compared the values. As shown in Table 1, the average intensity of a single cell in 4 different fields did not differ significantly when evaluated by Student's *t*-test, indicating that differences in nuclear protein expression do not affect the examination, and random fields can be used for immunofluorescence quantification. Thus, we measured 20 cells per slide to determine the fluorescence intensity.

Next, we optimized the exposure time. First, we determined whether exposure time affects the quantification limit of IIF-ANAT by testing ANA-negative and -positive samples; a sample containing homogeneous ANA titer of 1:40 was used as ANA-positive control. Six exposure times between 0.5 and 3 s were examined. In all conditions, the ANA-positive sample yielded greater fluorescence; the shorter the exposure time, the greater the signal-to-noise ratio (Table 2). Next, we sought exposure time that can quantify samples containing different ANA titers. Discrimination of high and low ANA titer samples was difficult with longer (1.5, 2, and 3 s) and shorter (0.5 s) exposure times, respectively (Fig. 1). We concluded that exposure times between 1 and 2/3 s are appropriate to quantify the fluorescence intensity of IIF-ANAT.

3.2. Three-dimensional reconstruction of the results of indirect immunofluorescence antinuclear antibody testing using ANA reference sera

We tried to identify a discrete speckled pattern in a homogeneous background, since this is often difficult in routine examination. First, ANA-reference sera were used in the assay. Two-dimensional (2D) analysis helped discriminate homogeneous (Fig. 2A) from discrete speckled (Fig. 2B) patterns created by control serum samples, each containing a single ANA type; however, when the homogeneous and discrete speckled sera were combined, homogeneous ANAs hid the discrete speckled pattern in 2D analysis (Fig. 2C). To resolve the image in Fig. 2C, the same nucleus in each sample was subjected to 3D reconstruction. For the control, 3D images of a single ANA type were analyzed; the homogeneous pattern was maintained in the 3D analysis (Fig. 2D) and discrete speckled serum yielded a comet-like appearance

Table 1
Variability of ANA fluorescence intensities in 4 different fields.

Field	1	2	3	4
Number of cells measured	20	20	20	20
Average intensity	89.45	95.40	90.15	91.65
Standard deviation	18.60	10.34	9.77	11.80