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Technical Note

Use of Double Labeling and Photo CD for Morphometric Analysis of Injured Skeletal Muscle[1]

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We used computer-assisted analysis of myofiber cross-sectional areas to measure skeletal muscle responses to injury and disease. We developed a simple, inexpensive method for measuring myofiber size in human muscle samples using Kodak photo compact discs (CDs) as the image source. The photo CD serves as a permanent image storage medium and provides a high-resolution image that can be used to detect small myofibers. The use of double labeling for dystrophin and desmin allowed positive identification of both degenerating and regenerating fibers in a single biopsy specimen. (*J Histochem Cytochem 43:1179–1184, 1995*)

KEY WORDS: Skeletal muscle; Image analysis; Eccentric exercise; Dystrophin; Desmin; Myofiber area; Pathology; Regeneration.

Introduction

Myofiber damage in human muscle has been reported for a variety of exercise training protocols. Muscle damage is most commonly seen after eccentric muscle actions, such as repeated lowering of heavy weights. The damage results from the high tension experienced by human muscle fibers, which can result in extracellular matrix damage (12), focal disturbances of sarcomeres (4), and necrotic fibers (1). Immunohistochemical methods can be quite useful in describing such injury but often require long exposure times if repeated measurements are to be made. Long exposure to light causes deterioration of the fluorescence.

Recently, we described a technique for using photographed images and computer-assisted analysis for myofiber size measurements in rat soleus muscles (10). The technique used a 35-mm slide as the permanent record but required a special camera to enter the image into the analysis system. In the present work, human muscle biopsies taken after resistance exercise protocols were photographed and the 35-mm slide was sent to a commercial service to produce a photo compact disc (CD). The photo CD was used for direct entry into the image analysis program and for permanent storage.

Using the photo CD images, we developed a relatively simple procedure of analysis employing methods readily available to most investigators and clinical pathologists. Photographs of muscle tissue stained for both dystrophin and desmin were obtained from cross-sections of skeletal muscle from one subject with obvious tissue damage. Photo CD representations from this sample were used to validate the technique, to make morphometric measurements of muscle fibers, and to describe the morphology of the muscle, which included necrotic cells, damaged myofibers, and regenerating myofibers.

Materials and Methods

Muscle biopsy samples were obtained from the biceps brachii of volunteers taking part in a resistance exercise protocol described elsewhere (5). The exercise protocol to produce muscle damage consisted of eight bouts of eight repetitions with a resistance of 80% of maximum (i.e., 1 RM). The subjects in this study performed only eccentric muscle actions (i.e., the load was lowered using the elbow flexor muscles to resist the movement). The muscle samples were removed 48 hr after the protocol. At this time the isometric peak torque was 40% of pre-exercise values, indicative of material fatigue or structural damage (see 5 for details). The biopsy samples were cleaned of fat and connective tissue, mounted in embedding medium, and immediately frozen in isopentane cooled by liquid nitrogen. The tissue was sectioned at −20°C at a thickness of 8 μm and the sections were collected on glass slides and stored at −20°C until use. The samples were shipped from Hamilton, Ontario to Morgantown, West Virginia on dry ice and stored until use.

Localization of dystrophin and desmin was performed on the biopsy samples taken 48 hr after the exercise bout. A modification of a double labeling technique using two monoclonal antibodies (8) was used. Briefly, monoclonal antibodies (MAbs) to dystrophin (MAb 1645; Chemicon International, Temecula, CA) were diluted 20-fold in PBS, pH 7.4. A 50-μl aliquot was applied to the slides and the slides were incubated for 30 min at room temperature. After a brief wash with PBS, a 50-μl aliquot of

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rhodamine-labeled goat anti-mouse IgG (1:20 dilution) was applied and incubated again for 30 min. The slides were washed. A blocking step consisted of applying 50 µl of goat anti-mouse IgG F(ab)$_2$ fragments (1:20 dilution) (ICN Biomedicals; Irvine, CA) for 30 min. The blocking step was used to mask any antigenic regions remaining on the first MAb (anti-dystrophin IgG) and to prevent any fluorescein-labeled anti-mouse IgG from binding to the dystrophin antibody.

After a brief wash, a second MAb to desmin (M760; Dako, Carpinteria, CA) was applied. After a brief wash with PBS, a 50-µl aliquot of fluorescein-labeled goat anti-mouse IgG (1:20 dilution) (Chemicon International; Temecula, CA) was applied and incubated again for 30 min. After a final wash in PBS, coverslips were applied to the slides.

Areas of interest were photographed with a Leica fluorescence microscope for both dystrophin and desmin at final magnifications of ×50 and ×100 of the same field. A 35-mm slide (Ektachrome 400) was made and a copy was sent to a commercial service for Kodak photo CD processing. The 35-mm slide was scanned at a Kodak processing center using a Kodak PCD film scanner producing an 18 megabyte data file. The scanned image was compressed (Kodak PCD Data Manager), resulting in a 4.5 megabyte file. The compressed file was sent to the Kodak photo CD disc writer to store the image on a write-once compact disc. The photo CD was used for subsequent image analysis.

To calculate myofiber cross-sectional areas by computer-assisted analysis, two different methods of image capture were used. Method 1 has been published previously (10). It utilizes a video camera to enter the image recorded on the 35-mm slide into the Optimas image analysis program (V 4.0; Bioscan, Edmonds, WA). Method 2 uses a CD ROM drive and the Kodak photo CD image. Because the Optimas program does not read Kodak photo CD files, the photo CD images were displayed using Adobe Photoshop (V 3.0; Adobe Systems, Mountain View, CA), converted to "gray-scale" images, and saved for analysis by the Optimas program. This file conversion step adds about 5 min of additional processing time per image.

Before making myofiber cross-sectional area measurements the image analysis system was calibrated in both the horizontal and vertical direction using a photograph of a calibration slide. Use of the two methods resulted in different images. The photo CD version was of higher resolution (374,496 pixels^2) than the camera vesion (202,095 pixels^2) of the calibration slide. The photo CD used a square pixel, whereas the camera version used a rectangular pixel. Therefore, corrections had to be made so that display, processing, and measurements were the same. To compensate for horizontal and vertical differences in the photo CD and camera images, Optimas was calibrated separately for both types of images. Corrections were made based on a standard calibration grid photographed under identical conditions.

Each small fiber was measured (Tables 1–3) three times and the data are presented as mean ± SD for the three trials. There was no statistical significance between the two methods of measurement.

Results

A muscle biopsy taken 48 hr post exercise provided 12 whole uninjured fibers within the field of view (Figures 1A and 1B). The camera and photo CD images of the dystrophin-stained sample (Figure 1A) were processed with Optimas using the same procedure; only whole myofibers were used for fiber measurements (Table 4). Fiber cross-sectional areas for both methods were compared by a least-squares linear regression analysis (Figure 2). The correlation coefficient for the data was 0.9969 ($p<0.0001$). Therefore, the data were identical for the two methods. As expected, a Student's t-test confirmed that there was no difference between the two sets of data ($p<0.97$).

Small myofibers that stained for both dystrophin and desmin were present (Figure 3). These myofibers were also assessed using the two methods. The small myofiber containing desmin and dystrophin in Figures 3C and 3D was almost 400 µm^2 (Table 1). With detailed analysis using higher-resolution images, two very small myofibers could be resolved in Figure 3A (see Figure 4); both fibers were less than 50 µm^2 in cross-sectional area (Tables 2 and 3).

Table 1. *Comparison of morphometric measurements for the small myofiber in Figure 3C*

	Cross-sectional area (µm^2)	Major diameter (µm)	Perimeter (µm)
Method 1 (camera)	373 ± 13	31.0 ± 0.4	78.0 ± 1.1
Method 2 (photo CD)	385 ± 15	32.0 ± 0.8	81.0 ± 2.3

Table 2. *Comparison of morphometric measurements for the upper small myofiber in Figures 3A and 4*

	Cross-sectional area (µm^2)	Major diameter (µm)	Perimeter (µm)
Method 1 (camera)	48.0 ± 3.0	11.0 ± 0.6	28.0 ± 1.2
Method 2 (photo CD)	45.0 ± 0.6	10.0 ± 0.1	27.0 ± 0.3

Table 3. *Comparison of morphometric measurements for the lower small myofiber in Figures 3A and 4*

	Cross-sectional area (µm^2)	Major diameter (µm)	Perimeter (µm)
Method 1 (camera)	25.0 ± 2.4	8.0 ± 0.7	21.0 ± 0.9
Method 2 (photo CD)	22.0 ± 1.0	8.0 ± 0.2	19.0 ± 0.3

In addition to morphometric analysis, dual localization of antibodies to dystrophin and desmin were used to assess myofiber damage and regeneration after repeated eccentric muscle actions (Figures 1 and 4). Cell damage was defined as a disruption of a cellular structure.

Dystrophin antibodies can reveal disruptions in the sarcolemma, as shown in Figure 1C, in which fragments of dystrophin-positive material still remain on the surface of desmin-positive material (Figure 1D). Desmin staining can also reveal areas of sarcomere damage (3). In addition, the bright rim of desmin seen around uninjured fibers (Figure 1B) was lost in injured fibers (Figure 1D). The use of two MAbs (dystrophin and desmin) on a single biopsy sample has simplified the identification of degenerating and regenerating myofibers.

Discussion

Methodology

A simple and inexpensive method for image analysis using a Kodak photo CD as an image source was developed and tested for its

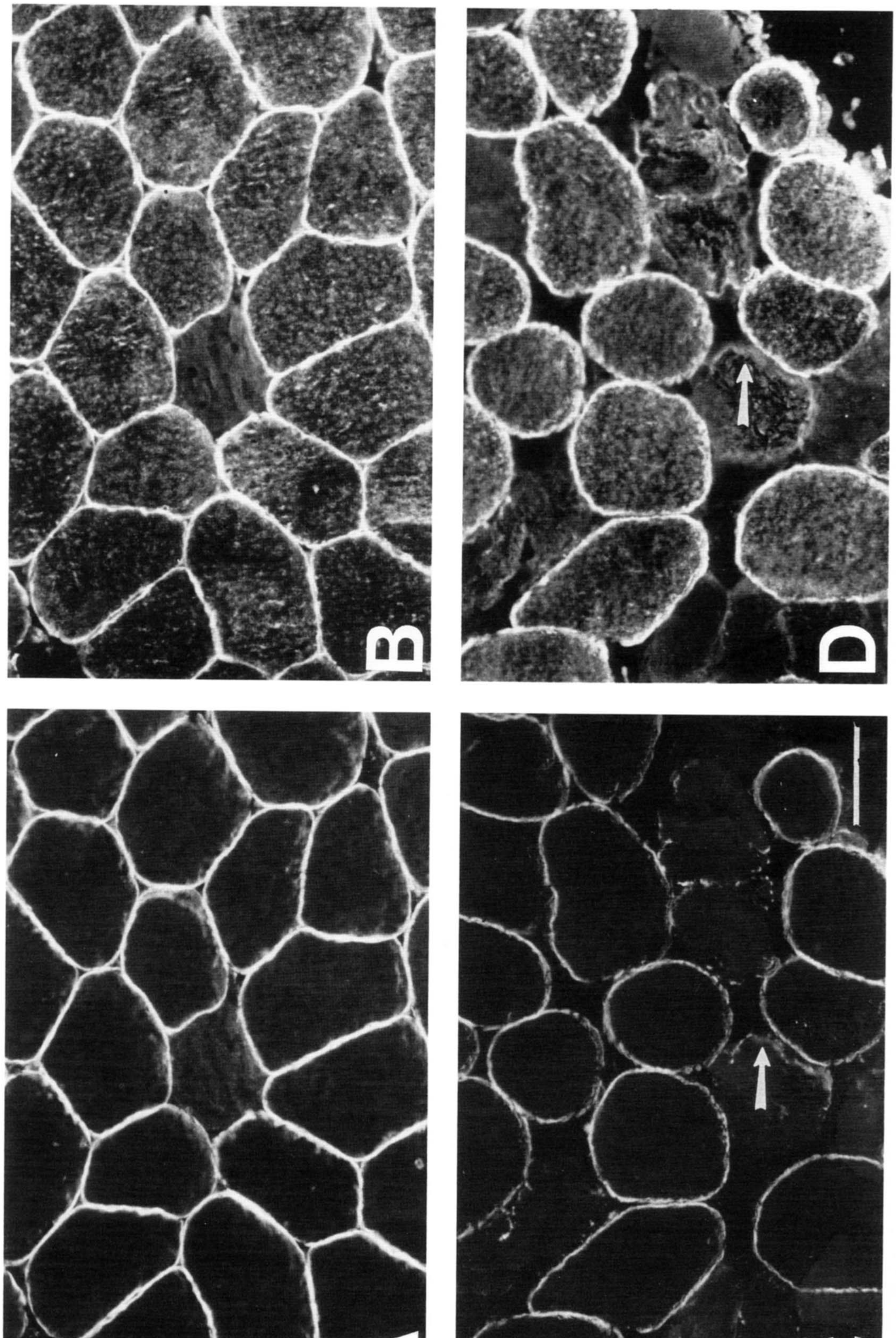

Figure 1. Micrographs of human skeletal muscles stained for both **(A,C)** dystrophin and **(B,D)** desmin. Original magnification × 50. Bar = 100 μm.

Table 4. *Comparison of morphometric measurements for the myofibers in Figure 1A*

	Cross-sectional area (μm^2)	Major diameter (μm)	Perimeter (μm)
Method 1 (camera)	12,820 ± 1995	162 ± 16	450 ± 38
Method 2 (photo CD)	12,851 ± 1949	161 ± 14	450 ± 36

accuracy compared to a video camera method of image capture (10). Because the method uses 35-mm negatives or slides for photo CD replication, image analysis can be performed on material collected long before the advent of computer-assisted image analysis. There are two additional advantages of using the commercial service provided by Kodak: (a) the photo digitization produces a higher-resolution image than one obtained by video cameras, and (b) the use of a photo CD eliminates the need for a video camera, frame grabber hardware, and other special attachments. In this study, the base resolution (768 × 512 pixels) of the photo CD image was used, but higher-resolution images (3072 × 2048 pixels) are also available from the same CD.

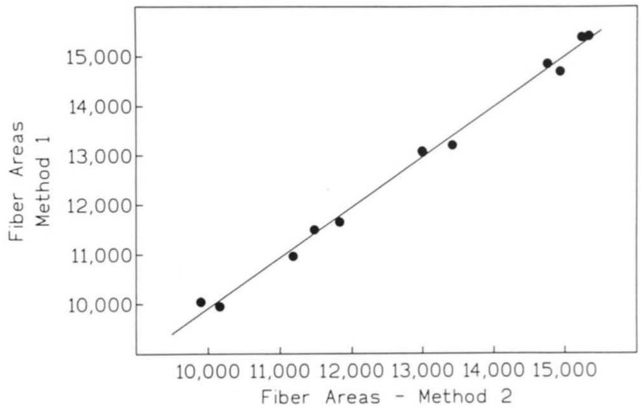

Figure 2. Fiber area using Method 1 as a function of fiber area using Method 2.

Although software limitations exist with Optimas in working with high-resolution images, these can be offset by using Adobe Photoshop to locate a portion of the original image, to display a portion of it at the highest resolution (e.g., Figure 4), and to save

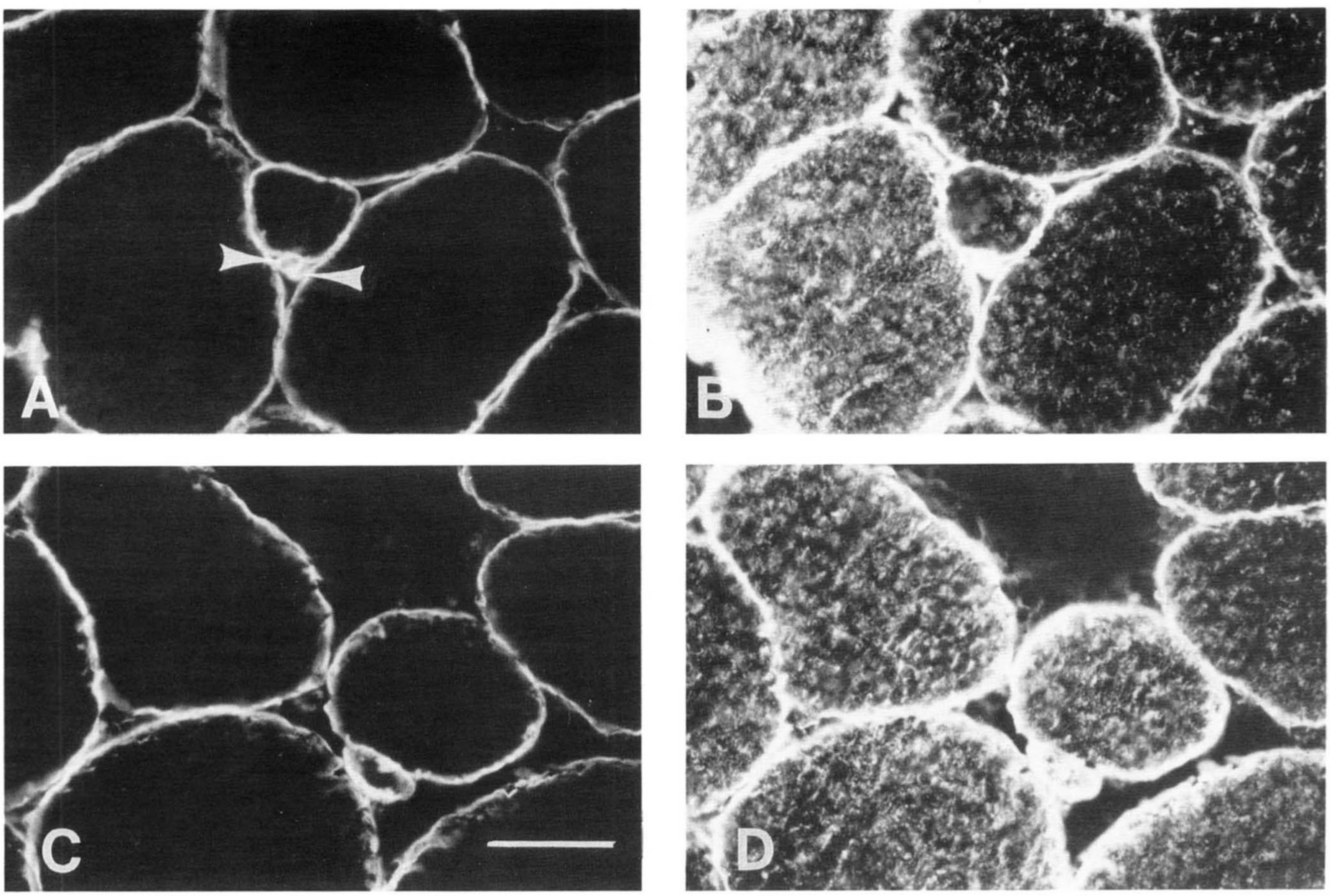

Figure 3. Micrographs of human skeletal muscles stained for both (**A,C**) dystrophin and (**B,D**) desmin. Original magnification × 100. Bar = 50 µm.

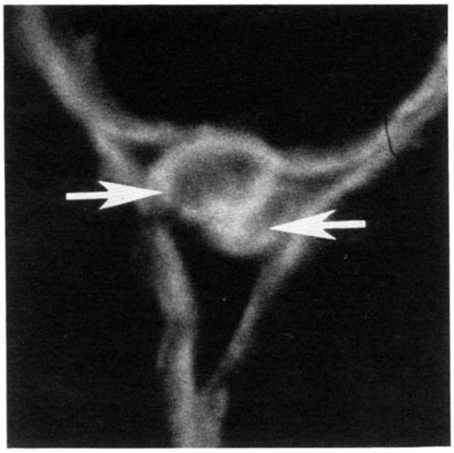

Figure 4. Image enhancement of two small fibers (arrows) from Figure 3A stained for dystrophin.

the high-resolution image as a separate file for analysis using Optimas. This process can be viewed as taking a higher-magnification picture of a sample with a reduced field of view. Therefore, it is possible to extract information from lower-magnification images that would otherwise require higher-magnification original photographs, if the material is well defined in the original low-magnification photograph.

The photo CD method has the additional advantage of allowing repeated measures to be performed on the same sample (Tables 1–3). Because long exposures are necessary to repeat the morphometry, the fluorescence of the biopsy specimen would typically fade and require new camera settings for each measurement. Using photo CD, only one photo is taken and the image can be tested repeatedly over a period of days.

Muscle Morphology

Many of the characteristics seen in human myopathies were observed in the biopsy sample from the exercise-injured biceps brachii. Myofiber degeneration was observed by the loss of desmin staining at the periphery of the cell (6). Myofiber necrosis could be identified by a loss of dystrophin and a decrease in the intensity of the desmin stain. These necrotic myofibers corresponded to the fibers with a ghost-like appearance described for gastrocnemius muscles after an ultra-marathon race (1). In addition, sarcomere damage was verified by electron microscopy (5).

Evidence of muscle regeneration was observed at 48 hr after exercise-induced injury. First, small fibers, which were the size of myoblasts (<50 μm^2), stained brightly for the muscle proteins desmin and dystrophin. The desmin stain was very intense, which is characteristic of regenerating fibers (6,11). Second, small myofibers the size of newborn muscle fibers (<500 μm^2) (2) were identified on the basis of their positive staining for both desmin and dystrophin. Although the intensity of the stain for desmin and dystrophin was consistent with small regenerating myofibers, it is possible that the muscle damage produced tapered ends of some myofibers that appear as myoblasts.

The unexpectedly large cross-sectional area of the undamaged myofibers requires further comment. MacDougall et al. (9) reported that the cross-sectional areas of myofibers from biceps brachii muscles of untrained men and elite body builders were 6700 μm^2 and 10,560 μm^2, respectively. Therefore, the myofibers in the present study were twice the cross-sectional area of similar untrained men. Because the image analysis system was calibrated and other myofiber measurements from rat soleus (10) and gastrocnemius (unpublished observations) muscles were identical to published reports, the myofibers in this sample must have been swollen. Muscle swelling was observed after repeated eccentric muscle actions (7), but myofiber measurements were not made. It is reasonable to expect that extracellular matrix disruption and myofiber protein degradation would lead to additional osmotically active molecules both within and around myofibers. The round shape of many of the fibers supports the hypothesis that the unexpected large size of the myofibers was due to swelling.

In summary, a computer-assisted analysis of myofiber cross-sectional areas from injured human muscle samples was developed using a photo CD as the image source. The photo CD serves as a permanent image storage medium and provides a high-resolution image that can be used to detect small myoblasts. The use of double labeling for dystrophin and desmin allowed positive identification of both degenerating and regenerating fibers in a single biopsy specimen.

Acknowledgments

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