Chapter 6

Synthesis and Validation of Cyanine-Based Dyes for DIGE

Michael E. Jung, Wan-Joong Kim, Nuraly K. Avliyakulov, Merve Oztug, and Michael J. Haykinson

Abstract

The application of difference gel electrophoresis (DIGE), in particular its most common "minimal labeling" variety, utilizes *N*-hydroxysuccinimide esters of Cy2, Cy3, and Cy5 dyes, which are commercially available. We describe methods for the efficient synthesis of all three dyes from relatively inexpensive and commercially available precursors in only a few steps and with relatively high yields. In model DIGE experiments, the newly synthesized dyes proved to be indistinguishable from commercially available ones and have been shown to be stable for years while stored under argon as dry solids or after being dissolved in *N*, *N*-dimethylformamide.

Key words: DIGE, Difference gel electrophoresis, Benzoxazolium Cy2, Propyl Cy3, Ethyl Cy5, Cy dye synthesis

1. Introduction

Difference gel electrophoresis (DIGE) is the latest in a series of major advances in the field of two-dimensional (2D) gel electrophoresis, which was originally introduced by O'Farrel (1). The key feature of the DIGE method relies on labeling of protein samples before electrophoresis with fluorescent dyes (originally using two *N*-hydroxysuccinimide (NHS) esters of Cy3 and Cy5) followed by protein separation of the combined samples on the same 2D gel (2). Multiplexed labeled proteins comigrate in the gel ensuing full coregistration of spectrally different gel images, thus minimizing gel-to-gel technical variation commonly associated with regular 2D gels. The introduction of a third Cy2-based dye allowed the implementation of an internal standard for DIGE, which further improved the method. The internal standard concept is based on labeling with Cy2 a pooled sample consisting of equal aliquots of

Rainer Cramer and Reiner Westermeier (eds.), *Difference Gel Electrophoresis (DIGE): Methods and Protocols*, Methods in Molecular Biology, vol. 854, DOI 10.1007/978-1-61779-573-2_6,

all samples. The same amount of the Cy2-labeled standard is then loaded on all the gels in the experiment along with individual samples labeled with Cy3 and Cy5. The ratios of Cy3-to-Cy2 and Cy5-to-Cy2 can then be calculated and normalized against the Cy2-labeled internal standard (3). This approach resulted in further elimination of technical noise in DIGE experiments and enabled the analysis of the relative abundances for each protein spot resolved on gels with high precision and unprecedented statistical power (4, 5).

The DIGE method was originally introduced in 1997 (2); in a few years, it was commercialized by GE Healthcare, which offered a full line of reagents and equipment necessary to successfully implement the technology, including the dyes for labeling. Besides being commercially available, the dyes could be synthesized using known procedures (2, 6, 7). In this chapter, we provide detailed procedures for the efficient synthesis of all three dyes used in DIGE, benzoxazolium dye Cy2, and two indocyanine dyes, propyl Cy3 and ethyl Cy5, and their NHS esters, from commercially available precursors. We also validated the newly synthesized dyes in DIGE experiments by comparing them to commercially available dyes and evaluated the effects of long-term and short-term storage of the synthesized dyes on DIGE output.

2. Materials

2.1. Dye Synthesis

The following reagents were obtained from Sigma-Aldrich: 2-methylbenzoxazole, bromoethane, 1,2-dichlorobenzene, 4-(bromomethyl) phenylacetic acid, *N,N′*-diphenylformamidine, *N,N′*-disuccini-midyl carbonate (DSC), 2,3,3-trimethyl-3*H*-indole, 1-bromopropane, 6-bromohexanoic acid, malondialdehyde bis(dimethyl acetal), 1,3,3-trimethyl-2-methyleneindoline.

Other reagents and common solvents were obtained from Fisher Scientific: anhydrous *N,N*-dimethylformamide (DMF), diethyl ether, acetonitrile, acetic anhydride, dichloromethane, hexane, methanol, absolute ethanol, anhydrous triethylamine, anhydrous pyridine, glacial acetic acid, anhydrous sodium acetate, and HCl. Aniline was obtained from Alfa Aesar.

2.2. DIGE Experiments

The following reagents and materials were supplied by GE Healthcare: urea, thiourea, CHAPS, 2D Quant kit, CyDye fluor minimal dyes Cy2, Cy3, and Cy5, and IPG strips. Other reagents were obtained from Sigma-Aldrich (DMF, SDS, DTT, iodoacetamide, L-lysine, magnesium acetate), and Fisher Scientific (glycerol, isopropanol).

All 2D electrophoresis equipment, the IPGPhor II (first-dimension isoelectric focusing) and the DALT 12 apparatus (second-dimension SDS-PAGE) as well as the DeCyder v. 6.5 software for DIGE analysis were obtained from GE Healthcare.

3. Methods

3.1. Dye Synthesis

General notes

All reactions were carried out under an atmosphere of nitrogen, and all commercial reagents were used as provided by the manufacturers. ^{1}H and ^{13}C NMR data were obtained on a Bruker 400 MHz spectrometer. ^{1}H NMR and ^{13}C NMR data are reported in parts per million (d) downfield from tetramethylsilane. The following abbreviations are used: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), and br (broad). Infrared spectra were recorded on a Thermo Nicolet Avatar 370 FT infrared spectrophotometer as a liquid film or as a thin crystalline film. All IR data are reported in wavenumbers (per cm). Thin-layer chromatography (TLC) was performed using Merck silica gel 60F254 0.2 mm alumina-backed plates. Visualization was accomplished using ultraviolet light or one of the following stains: anisaldehyde, phosphomolybdic acid, and potassium permanganate. Flash chromatography was carried out using ICN Biomedicals silica gel 60 (230–400 mesh). Mass spectrometry analysis was carried out using electrospray ionization (ESI) on an IonSpec FT mass spectrometer.

3.1.1. Cy2 Synthesis

The synthesis of the Cy2, the benzoxazolium dye, and its NHS ester (see Fig. 1) began with alkylation of the commercially available 2-methylbenzoxazole **(1)** with two different alkyl halides: alkylation with ethyl bromide gave the ethyl salt **(2)** (8) in 22% yield (this compound is also commercially available), while alkylation with the commercially available 4-bromomethylphenylacetic acid **(3)** gave an 80% yield of the salt **(4)** (9). Condensation of the ethyl salt **(2)** with diphenylformamidine in the presence of excess acetic anhydride as solvent afforded the acetanilidylvinyl indolium salt **(5)** in 93% yield (10–13). This compound was then reacted with the other benzoxazolium salt **(4)** in ethanol in the presence of triethylamine to give the desired dye **(6)** in 75% yield as a deep yellow powder. The dye was easily converted into the NHS ester by treatment with DSC in the presence of pyridine to give the activated dye **(7)** in 86% yield. The spectroscopic data were in agreement with the structures assigned (see Note 1).

A. *N-Ethyl-2-methylbenzoxazolium bromide* **(2)**. A mixture of 2-methylbenzoxazole **(1)** (Aldrich, 2.24 g, 0.01 mol) and bromoethane (Aldrich, 2.5 mL, 0.03 mol) in 1,2-dichlorobenzene (Aldrich) was heated at 110°C for 24 h. The solution was cooled to room temperature, and the residue obtained was filtered and washed with diethyl ether. The solid obtained was dried under vacuum to give the salt **(2)** as a white powder (0.60 g, 22%) (14, 15). This compound is also commercially available from various suppliers (Aldrich, Alfa Aesar, TCI, etc.). IR (neat): 3,084, 3,047, 2,974, 2,929, 2,859, 2,729, 1,593,

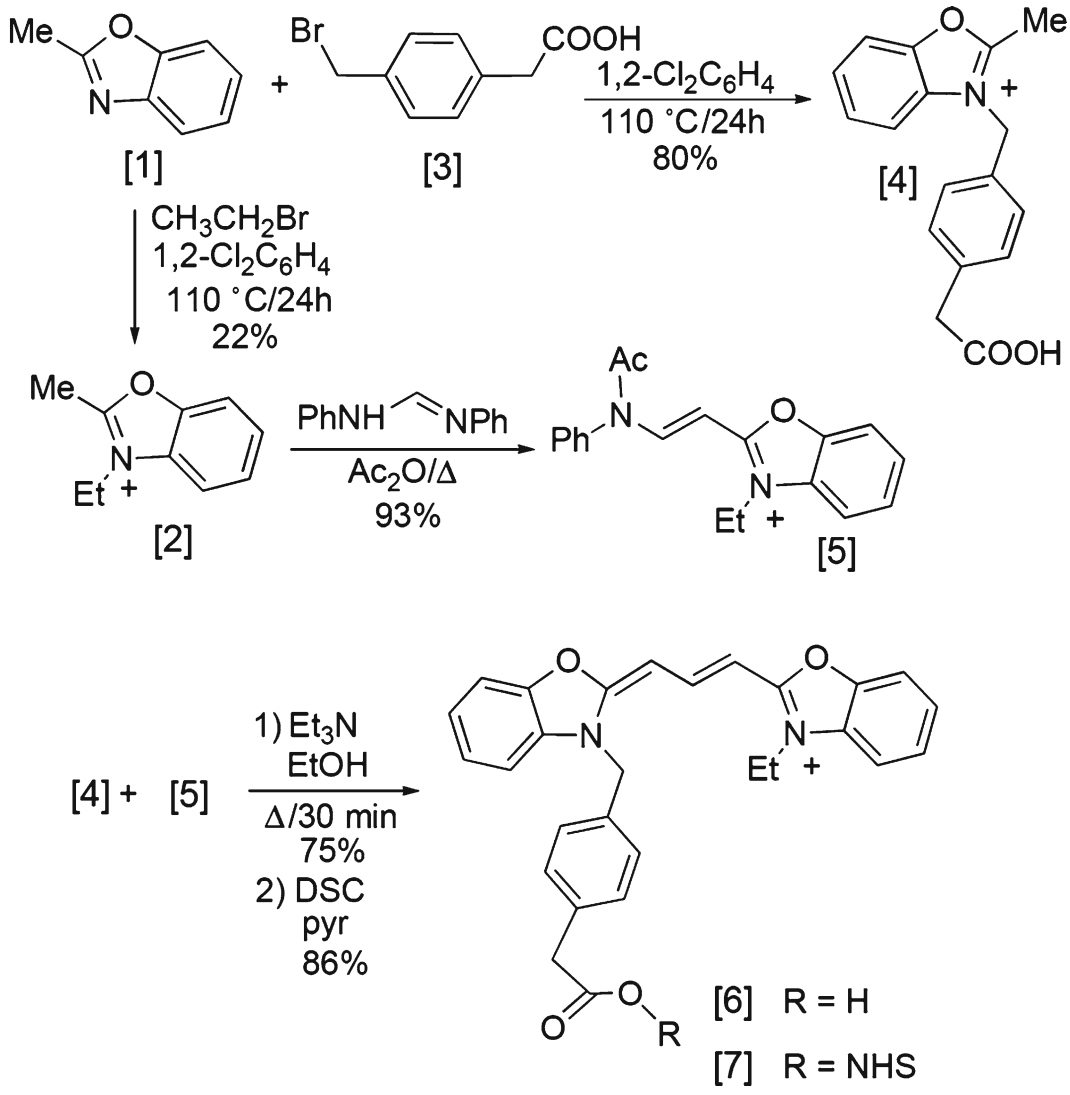

Fig. 1. Synthesis of the benzoxazolium Cy2 dye and its *N*-hydroxysuccinimide (NHS) ester.

1,462, 1,388, 1,188, 1,147, 1,025, 759/cm. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.09–8.23 (2H, m), 7.71–7.80 (2H, m), 4.60 (2H, q, J=7.3 Hz), 3.02 (3H, s), 1.43 (2H, t, J=7.3 Hz). ^{13}C NMR (100 MHz, DMSO-d_6): δ 169.03, 147.91, 129.73, 129.04, 128.13, 115.00, 113.42, 42.38, 13.84, 13.40.

B. *N-((4-Carboxymethyl)phenylmethyl)-2-methylbenzoxazolium bromide* **(4)**. A mixture of 2-methylbenzoxazole **(1)** (Aldrich, 2.24 g, 0.01 mol) and 4-(bromomethyl)phenylacetic acid **(3)** (Aldrich, 3.22 g, 0.01 mol) in 1,2-dichlorobenzene (Aldrich) was heated at 110°C for 12 h. The solution was cooled to room temperature, and the residue obtained was filtered and

washed with acetonitrile. The solid obtained was dried under vacuum to give the salt **(4)** as a light yellow powder (3.20 g, 80%) (9). IR (neat): 3,014, 1,733, 1,579, 1,456, 1,360, 1,226, 1,164, 753/cm. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.13 (4H, s), 7.01 (1H, br t, J=7.8 Hz), 95 (1H, br d, J=8.1 Hz), 85 (1H, br d, J=7.8 Hz), 68 (1H, br t, J=7.6 Hz), 5.20 (1H, d, J=15.0 Hz), 4.19 (1H, d, J=15.0 Hz), 3.48 (2H, s), 3.17 (3H, s). ^{13}C NMR (100 MHz DMSO-d_6): δ 173.18, 170.49, 153.38, 1375, 133.98, 130.15, 130.04, 129.54, 129.46, 128.38, 119.71, 117.15, 50.79, 40.84, 22.21.

C. *2-(2-Phenylacetamido-E-1-ethenyl)-N-ethylbenzoxazolium salt* **(5)**. A mixture of *N*,*N*′-diphenylformamidine (Aldrich, 0.38 g, 1.98 mmol) and the salt **(2)** (0.26 g, 1.65 mmol) in acetic anhydride (10 mL) was refluxed for 30 min. The solution was cooled to room temperature, the solvent was removed under reduced pressure, and the residue was purified by flash chromatography on silica gel (dichloromethane/hexane/methanol = 5:1:1) to give the salt **(5)** as a light yellow powder (0.41 g, 93%) (9). IR (neat): 3,084, 3,064, 2,978, 1,719, 1,646, 1,613, 1,589, 1,491, 1,466, 1,413, 1,372, 1,319, 1,252, 1,151, 1,004, 755, 702/cm. ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.19 (1H, d, J=13.8 Hz), 7.26–7.83 (9H, m), 5.33 (1H, d, J=13.8 Hz), 4.44 (2H, q, J=7.4 Hz), 2.02 (3H, s), 1.31 (3H, t, J=7.4 Hz).^{13}C NMR (100 MHz, $CDCl_3$): δ 173.25, 169.92, 162.86, 147.68, 1488, 138.12, 131.30, 130.86, 129.62, 128.57, 127.85, 113.93, 112.21, 87.14, 42.36, 23.45, 13.79.

D. *2-(3-(3-(4-(2-carboxyethyl)phenylmethyl)-2(3H)-benzoxazolylidene)-1-propenyl)-3-ethylbenzoxazolium, Cy2* **(6)**. A mixture of the enamide **(5)** (0.10 g, 0.32 mmol), the salt **(4)** (0.92 g, 0.32 mmol), and dry triethylamine (0.1 mL) in absolute ethanol (10 mL) was refluxed for 30 min. The solvent was removed under reduced pressure and the crude residue was purified by flash chromatography on silica gel (dichloromethane/hexane/methanol = 5:1:1) to give the dye **(6)** as a deep yellow powder (0.11 g, 75%). IR (neat): 3,408, 2,927, 1,710, 1,609, 1,565, 1,508, 1,461, 1,394, 1,347, 1,280, 1,201, 1,154, 1,116, 1,083, 978, 906, 748/cm. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.38 (1H, dd, J=13.2, 13.3 Hz), 7.16–7.48 (12H, m), 5.90 (1H, d, J=13.3 Hz), 5.88 (1H, d, J=13.2 Hz), 5.22 (2H, s), 4.12 (2H, br q, J=7.2 Hz), 3.51 (2H, s), 1.30 (3H, t, J=7.2 Hz). ^{13}C NMR (100 MHz $CDCl_3$): δ 174.31, 1637, 162.25, 161.74, 148.00, 1488, 1473, 131.23, 130.93, 130.58, 130.38, 127.17, 1233, 1223, 125.59, 125.38, 111.01, 110.86, 110.76, 110.53, 85.95, 85.26, 47.41, 45.52, 39.60, 12.98.

E. *2-(3-(3-((4-(2-((2,5-Dioxo-1-pyrrolidinyl)oxy)-2-oxoethyl)phenyl)methyl)-2(3H)-benzoxazolylidene)-1-propenyl)-3-ethylbenzoxazolium, Cy2-NHS ester* **(7)**. Anhydrous pyridine (0.1 mL)

and DSC (Aldrich, 21 mg, 0.08 mmol) were added to a stirred solution of the dye **(6)** (25 mg, 0.05 mmol) in dry DMF (Fisher, 2 mL) under nitrogen. The reaction mixture was stirred at 60°C for 1.5 h. After evaporation of the solvent, the deep yellow residue was purified by column chromatography on silica gel (dichloromethane/hexane/methanol = 5:1:1) to give the pure Cy2-NHS ester **(7)** (26 mg, 86%) as an orange powder. IR (neat): 2,924, 2,851, 1,736, 1,565, 1,507, 1,461, 1,395, 1,348, 1,280, 1,201, 1,115, 1,082, 747/cm. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.40 (1H, t, J = 13.2 Hz), 7.22–7.49 (12H, m), 52 (1H, d, J = 13.2 Hz), 40 (1H, d, J = 13.2 Hz), 5.49 (2H, s), 4.31 (2H, br q, J = 7.1 Hz), 3.85 (2H, s), 2.78 (4H, s), 1.46 (3H, t, J = 7.1 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): δ 168.99, 1651, 162.18, 161.73, 1495, 1482, 132.87, 132.02, 131.37, 130.82, 130.13, 128.36, 1209, 125.28, 125.08, 110.98, 110.90, 110.76, 110.66, 87.15, 849, 37.19, 25.60, 13.34. MS (EI): *m/z* (%) = 550 (100), 453 (6), 304 (6).

3.1.2. Cy3 Synthesis

The synthesis of the propyl Cy3 dye and its NHS ester (see Fig. 2) began with the alkylation of the commercially available 2,3,3-trimethyl-3*H*-indole **(8)** with propyl bromide to give the 1-propyl-2-methyleneindoline **(9)** (16, 17) in an unoptimized yield of 44%. Condensation with commercially available *N*,*N*′-diphenylformamidine in the presence of excess acetic anhydride as solvent afforded the acetanilidylvinyl indolium salt **(10)** in 87% yield. The second component of the dye was prepared by alkylation of trimethylindole **(8)** with 6-bromohexanoic acid in dichlorobenzene at 110°C for 12 h to give 67% yield of the methylindolium salt **(11)** (2, 18–20). This compound was then reacted with the acetanilidylvinyl indolium salt **(10)** in ethanol in the presence of triethylamine to give the desired dye **(12)** in 85% yield as a red powder (14, 15). The dye was easily converted into the NHS ester by treatment with DSC in the presence of pyridine to give the activated dye **(13)** in 96% yield. Again, all the pertinent spectroscopic data, especially high-field NMR and mass spectrometry, were in agreement with the structures assigned (see Note 1).

A. *3,3-Dimethyl-2-methylene-1-propylindoline* **(9)**. A mixture of 2,3,3-trimethyl-3*H*-indole **(8)** (Aldrich, 0.2 g, 1.25 mmol) and 1-bromopropane (Aldrich, 2.28 mL, 0.025 mol) in 1,2-dichlorobenzene (Aldrich) was heated at 110°C for 24 h. The solution was cooled to room temperature, and the residue obtained was filtered and washed with a mixture of acetonitrile/diethyl ether (1/1). The solid obtained was dried under vacuum to give the 3,3-dimethyl-2-methylene-1-propylindoline **(9)** as a light red powder (0.11 g, 44%) (see Note 1). IR (neat): 2,966, 2,925, 1,617, 1,601, 1,474, 1,454, 1,356, 1,290,

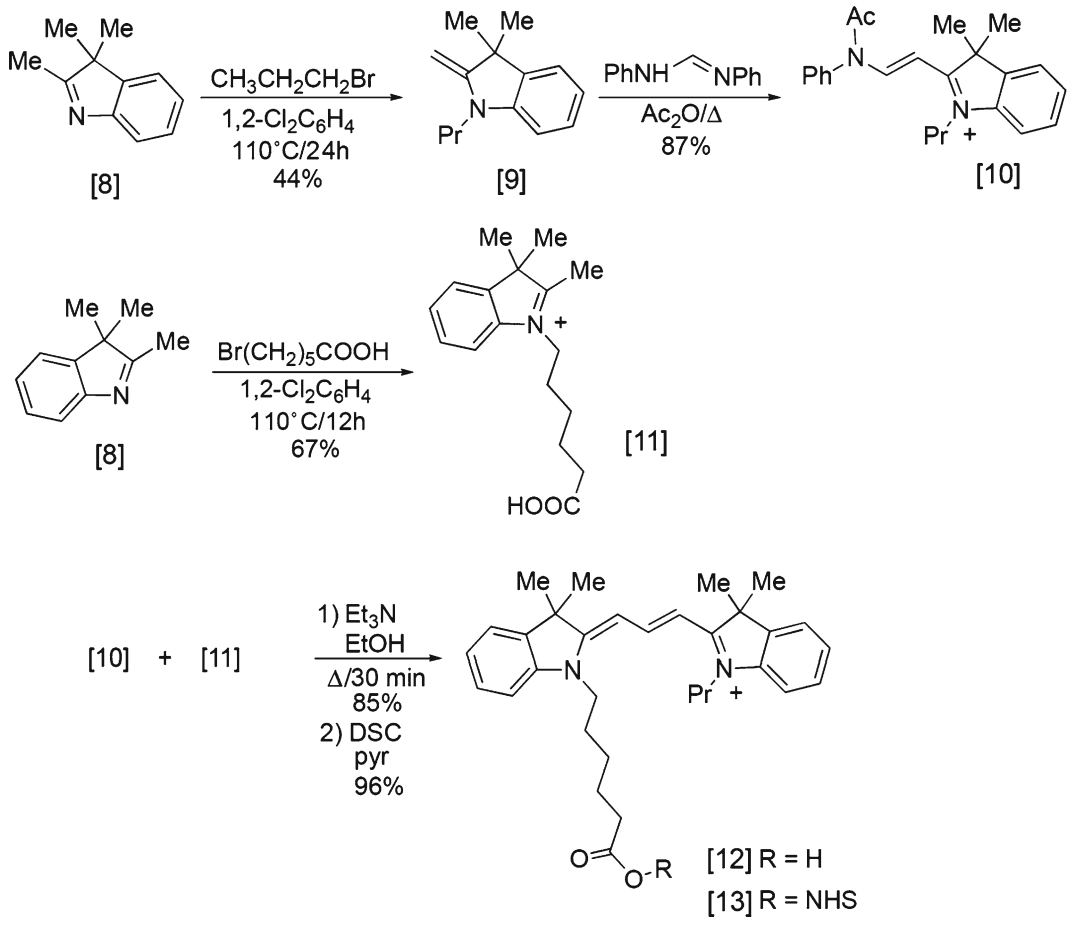

Fig. 2. Synthesis of the propyl Cy3 dye and its NHS ester.

1,119, 931, 767/cm. ^{1}H NMR (400 MHz, D_2O): δ 7.66 (1H, m), 7.60 (1H, m), 7.45–7.50 (2H, m), 4.65 (2H, s), 4.30 (2H, t, *J*=7.4 Hz), 1.86 (2H, m), 1.42 (6H, s), 0.87 (3H, t, *J*=7.4 Hz). ^{13}C NMR (100 MHz, D_2O): δ 141.77, 140.92, 129.75, 128.99, 123.26, 115.15, 54.33, 49.20, 21.72, 20.85, 10.18.

B. *2-(2-Phenylacetamido-E-1-ethenyl)-3,3-dimethyl-1-propylindolium salt* **(10)**. A mixture of *N,N'*-diphenylformamidine (Aldrich, 0.35 g, 1.78 mmol) and 3,3-dimethyl-2-methylene-1-propylindoline **(9)** (0.30 g, 1.48 mmol) in acetic anhydride (10 mL) was refluxed for 30 min. The solution was cooled to room temperature, the solvent was removed under reduced pressure, and the residue was purified by flash chromatography on silica gel (dichloromethane/hexane/methanol=5:1:1) to give the salt **(10)** as a light yellow powder (0.45 g, 87%). IR (neat): 2,965, 2,926, 1,680, 1,638, 1,603, 1,580, 1,553, 1,492,

1,369, 1,311, 1,200, 1,130, 996, 757/cm. ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.10 (1H, d, J=14.2 Hz), 7.05–7.70 (9H, m), 5.34 (1H, d, J=14.2 Hz), 4.06 (2H, t, J=7.1 Hz), 2.05 (3H, s), 1.70 (6H, s), 1.60 (2H, m), 0.67 (3H, t, J=7.4 Hz). ^{13}C NMR (100 MHz $CDCl_3$): δ 162.80, 154.24, 142.85, 139.56, 129.48, 128.41, 125.42, 123.32, 121.96, 119.42, 109.13, 94.35, 47.84, 45.19, 29.26, 27.86, 20.15, 11.45.

C. *1-(5-Carboxypentyl)-2,3,3-trimethyl-3H-indolium bromide* **(11)**. A mixture of 2,3,3-trimethyl-3*H*-indole **(8)** (Aldrich, 0.2 g, 1.25 mmol) and 6-bromohexanoic acid (Aldrich, 0.36 g, 1.80 mmol) in 1,2-dichlorobenzene was heated at 110°C for 12 h. The solution was cooled to room temperature, and the residue obtained was filtered and washed with a mixture of acetonitrile/diethyl ether (1/1). The solid obtained was dried under vacuum to give the product **(11)** as a light red powder (2.3 g, 67%) (2, 18–20). IR (neat): 3,405, 2,936, 1,724, 1,624, 1,460, 1,392, 1,168, 767/cm. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.93–7.97 (1H, m), 7.79–7.87 (1H, m), 7.56–7.64 (2H, m), 4.43 (2H, t, J=7.7 Hz), 2.82 (3H, s), 2.19 (2H, t, J=7.2 Hz), 1.81 (2H, m), 1.52 (2H, m), 1.50 (6H, s), 1.35 (2H, m). ^{13}C NMR (100 MHz, DMSO-d_6): δ 1998, 174.77, 142.33, 141.51, 129.85, 129.40, 123.99, 115.97, 54.62, 47.90, 33.83, 27.41, 25.87, 24.48, 22.47, 14.51.

D. *2-(3-(1-(5-Carboxypentyl)-1,3-dihydro-3,3-dimethyl-2H-indol-2-ylidene)-1-propenyl)-3,3-dimethyl-1-propyl-3H-indolium, propyl Cy3* **(12)**. A mixture of the salt **(11)** (0.08 g, 0.28 mmol), enamide **(10)** (0.10 g, 0.28 mmol), and dry triethylamine (0.1 mL) in absolute ethanol (10 mL) was refluxed for 30 min. The solvent was removed under reduced pressure, and the crude residue was purified by flash chromatography on silica gel (dichloromethane/hexane/methanol = 5:1:1) to give the dye propyl Cy3 **(12)** as a deep red powder (0.12 g, 85%) (16, 17). IR (neat): 3,407, 2,969, 2,934, 2,876, 2,734, 2,673, 1,557, 1,453, 1,427, 1,242, 1,192, 1,130, 1,030, 930, 754/cm. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.31 (1H, dd, J=13.4, 13.5 Hz), 7.61 (2H, d, J=7.4 Hz), 7.36–7.47 (4H, m), 7.26 (2H, t, J=7.1 Hz), 54 (1H, d, J=13.5 Hz), 53 (1H, d, J=13.4 Hz), 4.08 (2H, t, J=7.2 Hz), 3.43 (2H, t, J=7.2 Hz), 2.14 (2H, t, J=7.2 Hz), 1.75 (2H, m), 1.66 (12H, s), 1.52 (2H, m), 1.40 (2H, m), 1.19 (2H, m), 0.84 (3H, t, J=7.4 Hz). ^{13}C NMR (100 MHz DMSO-d_6): δ 174.20, 161.01, 1445, 143.11, 141.37, 137.32, 129.11, 127.92, 1213, 122.98, 122.21, 118.45, 105.73, 102.72, 74.11, 49.36, 44.06, 43.30, 30.26, 27.94, 27.90, 226, 20.89, 19.44, 11.79, 11.4.

E. *2-(3-(1,3-Dihydro-3,3-dimethyl-1-propyl-2H-indol-2-ylidene)-1-propenyl)-1-(6-((2,5-dioxo-1-pyrrolidinyl)oxy)-6-oxohexyl)-3,3-dimethyl-3H-indolium, propyl Cy3-NHS ester* **(13)**. Anhydrous

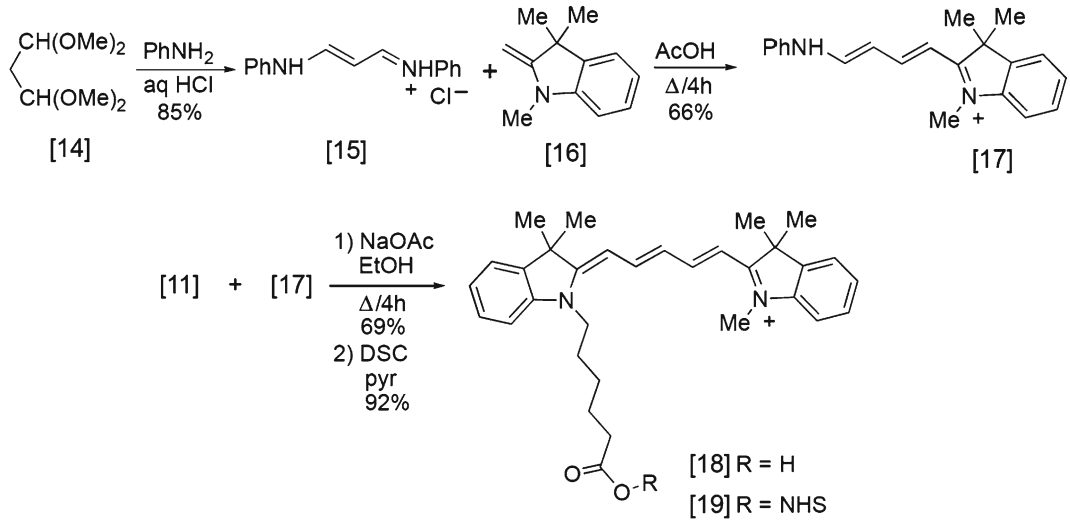

Fig. 3. Synthesis of the methyl Cy5 dye and its NHS ester.

pyridine (0.1 mL) and DSC (21 mg, 0.08 mmol) were added to a stirred solution of the dye **(12)** (27 mg, 0.05 mmol) in dry DMF (2 mL) under nitrogen. The reaction mixture was stirred at 60°C for 1.5 h. After evaporation of the solvent, the deep red residue was purified by column chromatography on silica gel (dichloromethane/hexane/methanol = 5:1:1) to give the pure propyl Cy3-NHS ester **(13)** (31 mg, 96%) as a red powder (2, 6). IR (neat): 2,924, 2,853, 1,737, 1,555, 1,456, 1,428, 1,371, 1,248, 1,196, 1,158, 1,116, 1,019, 930, 796, 579, 680/cm. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.32 (1H, dd, J = 13.4, 13.4 Hz), 7.61 (2H, d, J = 7.6 Hz), 7.38–7.47 (4H, m), 7.26 (2H, t, J = 7.4 Hz), 48 (2H, d, J = 13.5 Hz), 4.09 (4H, m), 2.74 (4H, s), 2.66 (2H, t, J = 7.2 Hz), 1.74 (2H, m), 1.69 (2H, m), 1.66 (12H, s), 1.48 (2H, m), 1.19 (2H, m), 0.95 (3H, t, J = 7.4 Hz). ^{13}C NMR (100 MHz, DMSO-d_6): δ 174.20, 173.40, 169.17, 168.61, 151.50, 142.30, 140.52, 129.11, 128.86, 125.04, 122.03, 111.14, 110.96, 105.17, 48.90, 440, 44.20, 30.70, 29.71, 28.20, 27.21, 25.82, 25.62, 24.53, 21.17, 11.30. MS (EI): m/z (%) = 582 (100), 485 (22).

3.1.3. Cy5 Synthesis

For the synthesis of the methyl Cy5 dye and its NHS ester (see Fig. 3), the three-carbon spacer **(15)** had to be prepared. Condensation of commercially available malondialdehyde bis(dimethyl acetal) **(14)** with aniline under acidic conditions afforded the anilino anilinium salt **(15)** in 85% yield (2, 21). The reaction of **(15)** with the commercially available 1,3,3-trimethyl-2-methyleneindoline **(16)** in refluxing acetic acid afforded the anilinobutadienyl salt **(17)** in 66% yield (2, 22). Finally, the reaction of the activated indolium salt **(17)** with the methylindolium salt **(11)** synthesized

earlier (see Subheading 3.1.2, step C and Fig. 2) in ethanol in the presence of sodium acetate afforded the desired dye **(18)** in 69% yield as a blue powder. The dye was easily converted into the NHS ester by treatment with commercially available DSC in the presence of pyridine to give the activated dye **(19)** in 92% yield. All spectroscopic data, especially high-field NMR and mass spectrometry, were in agreement with the structures assigned (see Note 1).

A. *N-((1E)-3-(Phenylimino)prop-1-enyl)benzenamine hydrochloride* **(15)**. A solution of distilled water (140 mL), HCl (10 mL), and aniline (Alfa Aesar, 7.4 mL, 0.08 mol) was added dropwise to a solution of distilled water (171 mL), HCl (8.5 mL), and malondialdehyde bis(dimethyl acetal) **(14)** (Aldrich, 10.5 mL, 0.06 mol) with stirring at 50°C. The precipitate was isolated by filtration to give malondialdehyde dianil hydrochloride **(15)** (12 g, 85%) as an orange powder (2, 21). IR (neat): 3,425, 1,642, 1,620, 1,580, 1,492, 1,343, 1,273, 1,194, 749, 683/cm. ^{1}H NMR (400 MHz, DMSO-*d6*): δ 12.72 (2H, d, *J* = 13.2 Hz), 8.92 (2H, t, *J* = 12.4 Hz), 7.40 (8H, m), 7.20 (2H, m), 50 (1H, t, *J* = 11.5 Hz). ^{13}C NMR (100 MHz, DMSO-*d6*): δ 158.89, 139.18, 130.33, 1231, 117.86, 99.09.

B. *2-(4-Phenylamino-1E,3E-butadien-1-yl)-1,3,3-trimethy-lindolium chloride* **(17)**. A mixture of malondialdehyde dianil hydrochloride **(15)** (1 g, 4.49 mmol) and 1,3,3-trimethyl-2-methyleneindoline **(16)** (Aldrich, 0.93 mL, 4.49 mmol) in glacial acetic acid (10 mL) was refluxed for 4 h. The solution was cooled to room temperature, the acetic acid was removed under reduced pressure, and the residue was purified by flash chromatography on silica gel (dichloromethane/hexane/methanol = 5:1:1) to give the product **(16)** as a red powder (0.9 g, 66%) (22). ^{1}H NMR (400 MHz, DMSO-*d6*): δ 8.18 (1H, d, *J* = 9.8 Hz), 7.52 (1H, t, *J* = 13.3 Hz), 74–7.36 (9H, m), 48 (1H, dd, *J* = 14.1, 9.9 Hz), 5.55 (1H, d, *J* = 12.5 Hz), 3.24 (3H, s), 1.55 (6H, s). ^{13}C NMR (100 MHz, DMSO-*d6*): δ 129.41, 129.17, 128.04, 125.24, 121.71, 121.36, 120.41, 118.18, 107.27, 468, 28.38.

C. *2-(5-(1-(5-Carboxypentyl)-1,3-dihydro-3,3-dimethyl-2H-indol-2-ylidene]-1,3-pentadienyl]-1,3,3-trimethyl-3H-indolium, methyl Cy5* **(18)**. A solution of the anil **(16)** (0.20 g, 0.65 mmol), the acid **(11)** synthesized earlier in Subheading 3.1.2, step C (0.18 g, 0.65 mmol), and anhydrous sodium acetate (0.11 g, 0.79 mmol) in absolute ethanol (50 mL) under nitrogen was refluxed for 4 h. The solid was purified by flash chromatography on silica gel (dichloromethane/methanol = 5:1) to give the methyl Cy5 dye **(18)** (0.22 g, 69%) as a blue powder (2). IR (neat): 3,406, 2,925, 1,716, 1,575, 1,470, 1,425, 1,371, 1,335, 1,217, 1,146, 1,016, 1,040, 923, 796, 750, 708/cm. ^{1}H NMR (400 MHz, DMSO-*d6*): δ 8.30

(2H, t, J=13.1 Hz), 7.58 (2H, d, J=7.4 Hz), 7.34 (4H, m), 7.21 (2H, m), 53 (1H, t, J=12.3 Hz), 25 (2H, dd, J=13.8, 13.9 Hz), 4.06 (2H, t, J=7.2 Hz), 3.56 (3H, s), 2.15 (2H, t, J=7.2 Hz), 1.69 (2H, m), 1.64 (12H, s), 1.52 (2H, m), 1.32 (2H, m). ^{13}C NMR (100 MHz DMSO-*d6*): δ 175.03, 173.69, 172.95, 154.47, 143.21, 142.47, 141.54, 141.47, 128.80, 125.83, 125.16, 125.05, 122.89, 122.76, 111.49, 103.77, 103.51, 55.39, 49.30, 43.67, 34.16, 31.56, 27.61, 27.44, 27.15, 212, 24.73.

D. *2-(5-(1,3-Dihydro-1,3,3-trimethyl-2H-indol-2-ylidene)-1,3-pentadienyl]-1-(6-((2,5-dioxo-1-pyrrolidi-nyl)oxy]-6-oxohexyl]-3,3-dimethyl-3H-indolium, methyl Cy5-NHS ester* **(19)**. Anhydrous pyridine (0.1 mL) and DSC (23.6 mg, 0.09 mmol) were added to a stirred solution of the acid **(18)** (0.36 g, 0.06 mmol) in dry DMF (2 mL) under nitrogen. The reaction mixture was stirred at 60°C for 1.5 h. After evaporation of the solvent, the deep blue residue was purified by column chromatography on silica gel (dichloromethane/hexane/methanol=5:1:1) to give the pure NHS ester of the dye **(19)** (0.33 g, 92%) as a blue powder (2, 6). IR (neat): 2,926, 1,733, 1,496, 1,481, 1,456, 1,372, 1,336, 1,217, 1,182, 1,149, 1,097, 1,040, 1,016, 924, 796, 756, 708, 668/cm. ^{1}H NMR (400 MHz, DMSO-*d6*): δ 8.30 (2H, t, J=13.1 Hz), 7.58 (2H, d, J=7.4 Hz), 7.36 (4H, m), 7.20 (2H, m), 53 (1H, t, J=12.4 Hz), 25 (2H, t, J=14.2 Hz), 4.05 (2H, t, J=7.2 Hz), 3.56 (3H, s), 2.77 (4H, s), 2.64 (2H, t, J=7.2 Hz), 1.68 (2H, m), 1.65 (2H, m), 1.64 (12H, s), 1.44 (2H, m). ^{13}C NMR (100 MHz, DMSO-*d6*): δ 173.74, 173.26, 172.93, 170.71, 169.35, 154.49, 151.10, 143.21, 142.44, 141.54, 141.47, 129.11, 128.81, 125.83, 125.19, 125.05, 122.87, 122.76, 111.50, 103.44, 49.31, 49.03, 43.63, 31.54, 30.44, 27.61, 27.43, 284, 25.90, 25.66, 24.37. MS (EI): *m/z* (%)=580 (100).

3.2. Validation of Newly Synthesized Dyes in DIGE Experiments

To validate the newly synthesized dyes, which will be called LAB dyes further, we applied them in a regular DIGE experiment and compared them to commercially available dyes from GE Healthcare, which further will be described as COM dyes.

For the experimental model, we chose a comparison between two strains of *Escherichia coli* K-12 MG1655: a wild-type (WT) and a Fis^{-} mutant strain (both strains were kindly provided by Daniel Yoo and Reid Johnson, Department of Biological Chemistry, UCLA School of Medicine). Fis is a small nucleoid-associated protein, the expression levels of which undergo dramatic changes during cell growth: it has been shown that if stationary phase cells are transferred into a fresh rich medium such as LB (1% Tryptone, 0.5% yeast extract, 1% NaCl, pH 7.3), in about 1 h Fis levels increase from about 100 copies per cell to tens of thousands of

copies (23). Fis has been implicated in transcriptional regulation of multiple genes (24); hence, differential expression of many different proteins may also be expected when WT *E. coli* and Fis⁻ mutant cells are analyzed using the DIGE method. This study is designed to compare individual protein abundances calculated by the DIGE method after using either LAB or COM dyes for protein labeling.

3.2.1. Sample Preparation

Five independently grown *E. coli* WT and five Fis⁻ mutant cultures were grown in LB for 15 h. A stationary overnight culture was diluted 1/50 in prewarmed LB medium and incubated for 45 min at 37°C as Fis protein level is known to reach its maximum at this time (23). Immediately after removing the cell cultures from the incubator, they were quickly chilled on ice and harvested by centrifugation at 10,000 × *g* for 10 min at 4°C. The pellets were washed twice with 20 mM Tris–HCl, pH 8.0, 5 mM magnesium acetate. Cell pellets (from approximately 10^{10} cells) were resuspended in the lysis buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 20 mM Tris–HCl, pH 8.8, and sonicated on ice for six cycles of 30 s each at high-power setting with 1 min of interruption between the cycles (see Note 2). The samples were centrifuged at 14,000 × *g* for 15 min at 4°C, and the protein concentration was determined using 2D Quant kit.

3.2.2. Labeling with Cy Dyes

A total of 50 μg of each protein sample was labeled with 400 pmol of either Cy3 or Cy5 dyes, standard conditions for a so-called minimal labeling. A pool of 25-μg aliquots collected from each sample was labeled with 400 pmol Cy2 dye/every 50 μg of protein and used as an internal standard for each gel. The labeling reaction was stopped after 30-min incubation on ice by adding 1 μL of 10 mM L-lysine/400 pmol dye and incubated on ice for additional 10 min. The rehydration solution containing 7 M urea, 2 M thiourea, 4% CHAPS, 5% glycerol, 10% isopropanol, 1% DTT, and 0.5% IPG 4–7 buffer was added to the labeled protein samples to a final volume of 450 μL. The samples were incubated for 20–30 min at room temperature (RT) and then centrifuged at 12,000 × *g* for 5 min at RT.

3.2.3. IEF, SDS-PAGE, and Scanning

Protein samples (450 μL) were loaded overnight onto a 24-cm pH 4–7 IPG strips (GE Healthcare) using an IPGPhor II apparatus (GE Healthcare). Isoelectric focusing (IEF) was performed at 20°C at 50 μA for a total of 80,000 Vh. After the IEF, IPG strips were incubated at RT in equilibration buffer containing 50 mM Tris–HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, and 10 mg/mL DTT for 15 min with gentle shaking, and then in the buffer containing 50 mM Tris–HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, and 40 mg/mL iodoacetamide for another 15 min. IPG strips were then rinsed in the 1× SDS running buffer and placed on top of the 12.5% SDS-PAGE gels and run at 25 V for 1 h, 50 V for

another hour, and then at 2 W/gel overnight at 24°C in an Ettan DALT 12 apparatus (GE Healthcare). Next morning, gels were scanned, and fluorescently labeled proteins were visualized by the Typhoon Trio Variable Mode Imager (GE Healthcare) using the following parameters: Cy2 dye-labeled proteins using 488 nm excitation wavelength and 520BP40 emission filter (520 nm center, 40 nm bandpass), Cy3 dye-labeled proteins using 532 nm excitation wavelength and 580BP30 emission filter, and Cy5-labeled proteins using 633 nm excitation and 670BP30 emission filter. All gels were scanned at 100-μm resolution, the PMT (photomultiplier tube) voltage was adjusted to keep all the recognized grayscale image values below 80,000, which are within the linear range of the scanner. Images were cropped using ImageQuant v.5.2 software (GE Healthcare) to remove areas outside of the gel image.

3.2.4. Image Analysis

Image analysis was performed using the DeCyder software v. 6.5 (GE Healthcare). The same software parameters were applied for the analysis of all images in the automatic batch mode. The estimated number of spots was set in the DeCyder DIA module at 2,500 in all cases. Gel matching was performed within the DeCyder BVA module automatically without land-marking or manual matching to minimize the operator impact on the analysis. The following filtering criteria were applied for further protein-of-interest (POI) selection: spot presence in all spot maps, average ratios of ≥1.4 and ≤−1.4, *t* test ≤0.01, and spot volume of ≥1.0e+05 and ≤1.0e+07.

3.2.5. Validation Results

In order to determine how similarly the newly synthesized (LAB) and commercially available dyes (COM) behave in DIGE, two identical DIGE experiments were conducted using LAB dyes and COM dyes. For each experiment, ten protein samples comprising five WT and five Fis$^-$ samples were used for labeling ($N=5$). The dyes were "swapped" to minimize the potential effects of individual dyes: three samples in the WT group were labeled with Cy3, and two with Cy5, while three Fis$^-$ group samples were labeled with Cy5, and two with Cy3. In both cases (LAB and COM), the Cy2 dye was used to label the common internal standard consisting of equal aliquots from all ten samples. Cy3 and Cy5 samples were paired randomly in order to avoid bias related to loading, and the Cy2-labeled internal standard was loaded onto each gel along with a Cy3/Cy5 sample pair. Although experiments with LAB and COM dyes were run independently and conducted on two separate days, spot patterns were found to be remarkably similar (a portion of two gels representing each experiment is shown in Fig. 4). A nonsupervised spot detection was performed on both sets of gels representing five WT and five Fis$^-$ samples labeled with two different dyes. As shown in Table 1, the number of detected spots for both LAB- and COM-labeled samples is very similar across all gels representing both LAB and COM experiments with the differences of 4.5 and 6% in CV values.

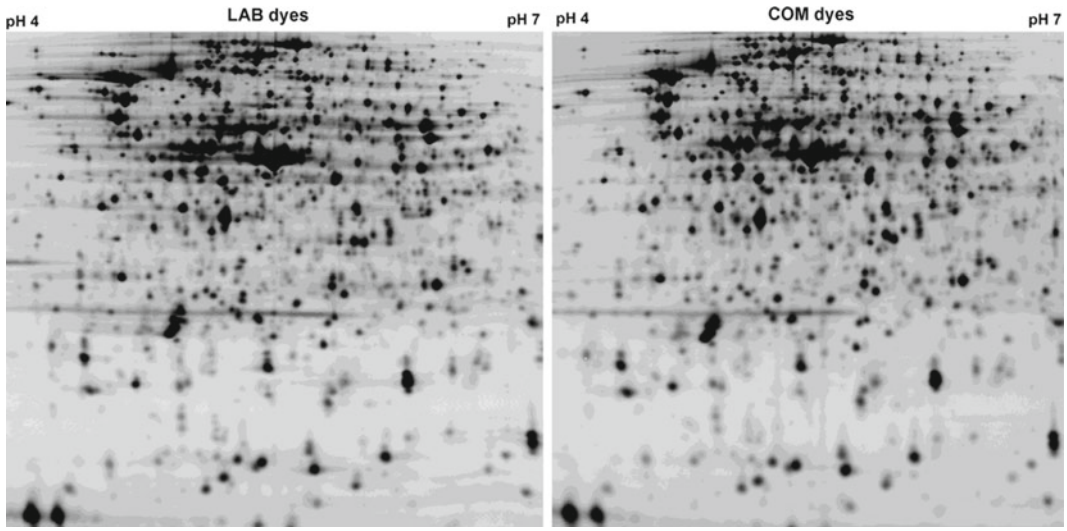

Fig. 4. Gel images of *Escherichia coli* protein samples labeled with newly synthesized (LAB) and commercial (COM) dyes. Spot patterns produced by using the two different dyes for labeling are remarkably similar.

Table 1
Comparison of spot maps for samples labeled with LAB and COM dyes

Sample	Number of detected spots (per gel, 1 through 5)	Mean	Standard deviation	CV (%)
LAB	2,172 (1), 2,143 (2), 2,243 (3), 2,090 (4) 1,990 (5)	2,128	95	4.5
COM	2,000 (1), 2,071 (2), 2,166 (3), 2,376 (4), 2,204 (5)	2,163	143	6

To check for similarities, individual spots average ratio measurements and an automated POI selection filter was then applied, and 37 protein spots were selected that satisfied the selection criteria, as described in Subheading 3.2.4 above. Out of 37 spots passing the filter, 27 were found to be upregulated in Fis$^-$ cells, and 10 protein spots were downregulated in Fis$^-$ cells, as compared to WT (see Note 3).

Comparison of average ratios in protein abundance for all 37 spots plotted in Fig. 5 shows that selected spots in both LAB- and COM-labeled samples are very similar. As shown in Table 2, the difference in average ratios for the majority of protein spots in both LAB and COM samples is small, as 29 spots (80% of total) differ less than 5% from each other, while the rest of the spots differ by no more than 15%. Paired Student's *t*-test analysis performed on

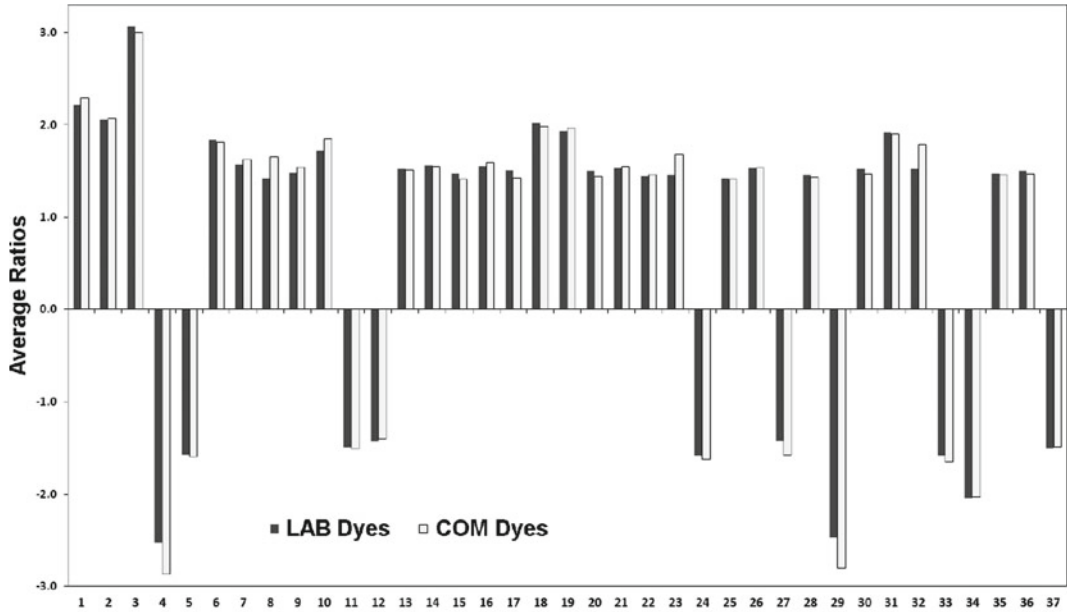

Fig. 5. Comparison of average ratios of individual protein measurements in samples labeled with newly synthesized (LAB) and commercial (COM) dyes. Spot numbers are shown at the *bottom* of the graph, while standardized log abundances for proteins selected are shown on the *y*-axis.

Table 2
Comparison of average ratios for individual spots in samples labeled with LAB and COM dyes

Deviation from mean average ratio (%)	≤1	1–5	5–10	10–15	≥15
Number of spots (total = 37)	8	21	2	6	0

this set has also confirmed that there is no statistical difference between the corresponding average ratios for LAB- and COM-labeled protein spots.

3.3. Dye Storage and Stability

3.3.1. Aliquoting the Dyes for a Long-Term Storage

Newly synthesized NHS dyes were weighed into regular 1.5-mL Eppendorf tubes (0.5 mg dye/tube) and stored as dry solids. The tubes were filled with argon gas, closed tightly, and stored at –20°C. The use of argon presumably reduces the amount of oxygen and water vapors in contact with the dye and therefore extends the dyes' storage life (see Subheading 3.3.3 below and Note 4).

3.3.2. Dyes Dilution and Storage

For everyday work, dyes were dissolved in anhydrous DMF at a final concentration of 2 nmol/μL. This solution was distributed into smaller aliquots (usually 25–30 μL) and stored in 0.5-mL Eppendorf tubes at −80°C. Before closing, the tubes were filled with argon gas. These aliquots were taken out of the −80°C freezer as necessary for creating working dye solutions for protein labeling. Before returning the remaining aliquots to the freezer, the tubes were filled with argon gas again.

3.3.3. Long-Term Storage and Dye Stability

To evaluate the effects of long-term storage of dry dyes on their performance in DIGE, as well as the effect of dilution in DMF on dye stability, we compared two groups of dye samples. The first group of newly synthesized dyes (Cy2, Cy3, and Cy5) was placed in storage immediately after synthesis and stored at −20°C as dry solids for 5 years under argon atmosphere, as described in Subheading 3.3.1. These tubes were taken out of the freezer and dissolved in fresh anhydrous DMF right before being used in a DIGE experiment. This dilution is further called NEW dilution, and it was the same dilution used to compare newly synthesized dyes with commercially available ones, as described in Subheading 3.2 above. The second group of new dyes was diluted in DMF at 2 nmol/μL at the time of dye synthesis and then stored in DMF for 5 years, as described in Subheading 3.3.2. This sample is further called OLD dilution and was used to compare to the freshly made NEW dilution in DIGE experiments.

OLD and NEW dyes dilutions were used for protein labeling in the same type of experiment, as described in Subheading 3.2 above, except that it was done on a slightly smaller scale: instead of five pairs of samples, only three WT and three Fis^- protein samples were used ($N=3$). As previously, after labeling, IEF and SDS-PAGE separation, protein spots were analyzed using DeCyder software in an automated fashion. Spot detection and automatic matching was performed without manual intervention, and a protein filter was applied as described in Subheading 3.2.4. Out of 25 protein spots that satisfied the selection criteria, 16 are upregulated, and 9 are downregulated in Fis^- cells, as compared to WT cells (see Fig. 6).

Average ratios in protein abundance for all 25 spots plotted in Fig. 6 shows that selected spots in both OLD and NEW dilution-labeled samples are very similar. As presented in Table 3, the majority of protein spots (21 spots, 84% of total) in both OLD and NEW dilution samples differ by less than 10% from each other.

In summary, three dyes used in DIGE, benzoxazolium dye Cy2, propyl Cy3, and ethyl Cy5, and their NHS esters were synthesized from commercially available precursors. Newly synthesized dyes were tested in DIGE experiments and were shown to be indistinguishable from commercially available dyes at the level of individual protein abundance measurements. Synthesized dyes

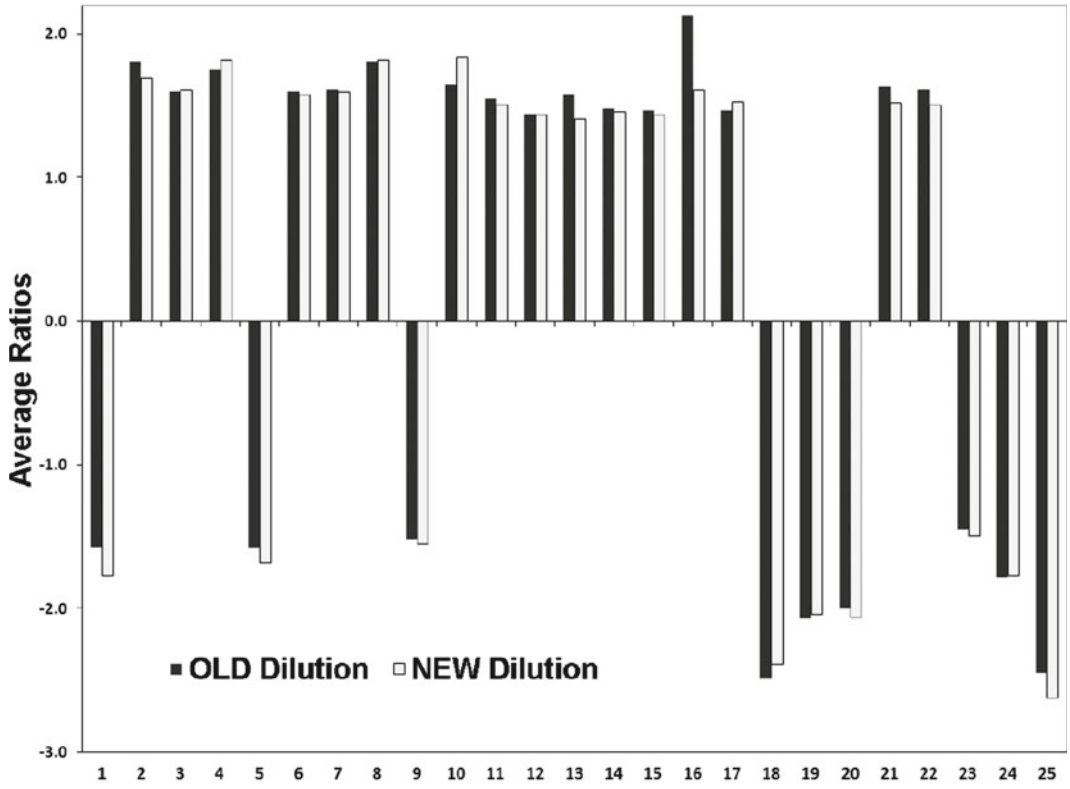

Fig. 6. Comparison of average ratios of individual protein measurements in samples labeled with NEW and OLD dyes dilutions (see text for details). Spot numbers are shown at the *bottom* of the graph, while standardized log abundances for proteins selected are shown on the *y*-axis.

Table 3
Comparison of average ratios for individual spots in samples labeled with OLD and NEW dye dilutions

Deviation from mean average ratio (%)	≤1	1–5	5–10	10–15	≥15
Number of spots (total = 25)	5	11	5	3	1

were shown to be stable for years when stored as dry solids under argon, the dyes are equally stable when stored in anhydrous DMF in the argon atmosphere.

4. Notes

1. We have no information on the counterions of these final quaternary ammonium salt dyes, but they are almost certainly not acetate since no peak for an acetate group appears in the proton NMR of the final compounds.
2. Charged urea/thiourea degradation products may cause protein carbamylation, and therefore it is advisable to deionize urea and thiourea before use. To prepare 25 mL of the deionized buffer, add 10.5 g of urea and 3.8 g thiourea to 11 mL of milli-Q water. Fill a small column with 7–9 g of ion-exchange resin AG501X8 (Bio-Rad) and wash it with milli-Q water. After water removal, pass the urea/thiourea solution through the column 3–4 times until the solution conductivity is around 0.2–0.4 μS. Add 1 g of CHAPS, 0.33 mL of 1.5 M Tris–HCl, pH 8.8, and water to make a 25-mL solution and then filter it through a 0.45-μm filter. For longer-term storage, distribute the lysis buffer into 1.5-mL Eppendorf tubes and store at –80°C.
3. The identifications of these and other proteins affected by Fis at the early logarithmic phase of *E. coli* cell growth will be published elsewhere.
4. Dye precursors, such as **(6)**, **(12)**, and **(18)** (see Figs. 1–3), could be successfully stored as dry solids at –20°C under argon, so when more NHS dyes are needed, one last reaction could be performed to convert the acid form of the dye into its NHS ester.

Acknowledgments

Wan-Joong Kim and Nuraly K. Avliyakulov contributed equally to this study. We thank the Dean's Office of the David Geffen School of Medicine at UCLA and Senior Associate Dean Leonard Rome for the generous and continuing support of this work.

References

1. O'Farrel PH (1975) High resolution two-dimensional electrophoresis of proteins. J Biol Chem, 250: 4007–4021.
2. Unlu M, Morgan ME, Minden JS (1997) Difference gel electrophoresis. A single gel method for detecting changes in protein extracts. Electrophoresis 18: 2071–2077.
3. Alban A, David SO, Bjorkesten L, Andersson C, Sloge E, Lewis S, Currie I (2003) A novel experimental design for comparative two-dimensional gel analysis: two-dimensional difference gel electrophoresis incorporating a pooled internal standard. Proteomics 3: 36–44.
4. Lilley KS, Friedman DB (2004) All about DIGE: quantification technology for differential-display 2D-gel proteomics. Expert Rev Proteomics 1: 401–409.

5. Karp NA, Lilley KS (2005) Maximising sensitivity for detecting changes in protein expression: experimental design using minimal CyDyes. Proteomics 5: 3105–3115.
6. Tonge R, Shaw J, Middleton B, Rowlinson R, Rayner S, Young J, Pognan F, Hawkins E, Currie I, Davison M (2001) Validation and development of fluorescence two-dimensional differential gel electrophoresis proteomics technology. Proteomics 1: 377–39.
7. Jung ME, Kim W-J (2006) Practical syntheses of dyes for difference gel electrophoresis. Bioorg Med Chem 14: 92–97.
8. Jedrzejewska B, Kabatc J, Pietrzak M, Paczkowski J (2003) Hemicyanine dyes: synthesis, structure and photophysical properties. Dyes Pigments 58: 47–58.
9. Hamilton AL, Birch MN, Hatcher MJ, Bosworth N, Scott B (1999) Energy transfer assay method and reagent. PCT Int Appl WO/1999/064519.
10. Schouten JA, Ladame S, Mason SJ, Cooper MA, Balasubramanian SG (2003) Quadruplex-specific peptide–hemicyanine ligands by partial combinatorial selection. J Am Chem Soc 125: 5594–5595.
11. Gorb LT, Romanov NN, Fedotov KV, Tolmachev AI (1981) Meso-ionic compounds with a nitrogen bridging atom. Polymethine dyes of the thiazolo[3,2-a]quinolinium 1-oxide series. Khim Geterotsikl Soedin, 481–484.
12. Abramenko PI, Zhiryakov VG (1975) Polymethine dyes, derivatives of 6-furo[2,3-b] pyridine. Khim Geterotsikl Soedin, 475–479.
13. Bailey J, Elvidge JA (1973) Synthesis and properties of dyes containing the pyrano[2,3-d] pyrimidine nucleus. J Chem Soc, Perkin Trans, 1: 823–828.
14. Cummins WJ, West RM, Smith JA (1999) Cyanine dyes. PCT Int Appl WO/1999/005221.
15. Jackson P, Cummins WJ, West R, Smith JA, Briggs MSJ (1998) Analysis of carbohydrates. PCT Int Appl WO/1998/015829.
16. Wurthner F (1999) DMF in acetic anhydride: A useful reagent for multiple-component syntheses of merocyanine dyes. Synthesis, 2103–2113.
17. Durr H, Ma Y, Cortellaro G (1995) Preparation of photochromic molecules with polymerizable organic functionalities. Synthesis: 294–298.
18. Lee LG, Woo SL, Head DF, Dubrow RS, Baer TM (1995) Near-IR dyes in three-color volumetric capillary cytometry: Cell analysis with 633- and 785-nm laser excitation. Cytometry 21: 120–128.
19. Mader O, Reiner K, Egelhaaf H-J, Fischer R, Brock R (2004) Structure-property analysis of pentamethine indocyanine dyes identification of a new dye for life-science applications. Bioconjugate Chem 15: 70–78.
20. Reichardt C, Engel HD (1988) An improved method for the synthesis of 1,3,3-trialkyl-2-alkylideneindolines. Chem Ber 121: 1009–1011.
21. Shiobasa Y, Ishida S (1960) Malonaldehyde dianil. Yamanouchi Pharmaceuticals Patent, Japan JP35 017020.
22. Kiprianov AI, Buryak VYu (1972) Cyanine dyes with two conjugated chromophores. XVIII. Effect of steric hindrance on the absorption spectra of bis(hemicyanines) from isomeric phenylenediamines with methyl groups on the benzene rings. Zh Org Khim 8: 1707–1712.
23. Ball CA, Osuna R, Ferguson KC, Johnson RC (1992) Dramatic changes in Fis levels upon nutrient upshift in *Escherichia coli*. J Bacteriol 174: 8043–805.
24. Bradley MD, Beach MB, de Koning AP, Pratt TS, Osuna R (2007) Effects of Fis on *Escherichia coli* gene expression during different growth stages. Microbiology 153: 2922–2940.