Journal of
Proteomics & Bioinformatics

Fu et al., J Proteomics Bioinform 2016, 9:1
DOI: 10.4172/jpb.1000382

Research Article Open Access

Protein Subcellular localization profiling of Prostate Cells by Dissociable Antibody MicroArray (DAMA) Staining Technology

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Abstract

Background: Dissociable Antibody MicroArray (DAMA) staining technology is a new approach for global analysis of protein expression and subcellular localization profiles in fixed cells. We have developed the technology and demonstrated its application on identifying differentially expressed proteins in breast cancer cells. We have determined the subcellular localization profiles of 360 proteins in breast cells and identified a protein with unique subcellular localization in breast cancer cells, by combining data from four 96-antibody format arrays. In this report, we have further developed the technology to determine the subcellular localization profiles of 400 proteins simultaneously from single chip, in a 400-antibody format.

Method: We have determined the subcellular localization profiles of 400 arrayed antibodies in two normal prostate cell lines (PWR-1E and PZ-HPV-7), and three cancer cell lines (Du145, LNCap and VCap). The subcellular localization profiles were compared and analyzed by Chipview, the program for image database management and analysis.

Results: A protein, GRK2 was identified to have unique localization in some of the cancer cells: localized in the membrane of two cancer cell lines, and in the cytosol of normal cell lines and one cancer cell line. The identified subcellular localization difference was confirmed by individual immunostaining.

Conclusion: DAMA staining technology could be a powerful method for global subcellular localization profiling in high throughput format.

Keywords: Protein microarray; Protein subcellular localization profile; Prostate cancer; Proteomics; DAMA staining

Introduction

Prostate carcinoma is the most common form of cancer in men and the second leading cause of death in the United States [1,2]. Efforts to improve the early detection of cancer by use of prostate-specific antigen (PSA) and transrectal ultrasound has result in increasing numbers of prostate biopsies submitted for examination to exclude early prostate cancer [3,4]. Diagnosis of prostate carcinomas can be difficult in needle biopsies or in minimal residual cancer of radical prostatectomies [5]. In addition, it's known that the development of prostate cancer is co-regulated by many kinds of oncogenic and tumor suppressor gene. Therefore, developing a new approach in the global analysis of protein biomarkers in prostate cancer cells is very important to the diagnosis and treatment for prostate cancer.

Protein microarrays have recently been attracting great attention for their potential use in high-throughput studies of protein function [6-11] and have been utilized to study protein expression profiles [12-14], protein-protein interactions [15,16], and drug discovery [17]. Most of the microarrays use the captured microarray platform [8-10] that determines the protein expression and modification profiles using lysate of the target samples. We have developed a novel platform called Dissociable Antibody MicroArray (DAMA) staining technology [18-20] that combines the power of immuno-histochemical staining with the high throughput characteristics of protein microarrays. This technology provides a new approach in the global analysis of protein expression and subcellular localization profiles in fixed cells and tissues.

Differently from the captured microarray platform, the DAMA staining technology uses fixed cells on coverslip. Those cells were permeabilized by the standard protocols for immunohistochemical staining. A set of selected antibodies immobilized on the proprietary chip membrane in a matrix format was delivered to the sample by the DAMA staining technology. The corresponding spots of staining would be identified according to the antibody matrix design. The amount of antibodies bound to the antigens in a specific spot could be detected with the chromogenic reporter-conjugated secondary antibodies. This way, the set of protein expression profile could be determined in a high throughput format. By using this approach, we have previously determined the expression profiles of 312 proteins in three normal breast cell lines and seven breast cancer cell lines [19]. We have identified and validated five proteins with higher expression level in breast cancer cells than in normal breast cells [19].

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Received October 28, 2015; **Accepted** January 11, 2016; **Published** January 15, 2016

Citation: Fu G, Song XC, Yang X, Mo Y, Zhou GW (2016) Protein Subcellular localization profiling of Prostate Cells by Dissociable Antibody MicroArray (DAMA) Staining Technology. J Proteomics Bioinform 9: 001-008. doi:10.4172/jpb.1000382

Combining this matrix-format antibody delivering and staining technology with fluorescent-conjugated secondary antibody detection, we can determine the subcellular localization profile of hundreds of proteins simultaneously. We have utilized the automatic image-taking and developed software for image database management and data analysis. We have determined the subcellular localization profiles of 360 proteins in nine different breast cell lines [20]. We have identified and validated that one protein, Cyclin B1, with distinctively different subcellular localization between normal and cancer cell lines [20].

In this report, we have further developed the DAMA staining technology to accommodate the high throughput format. Instead of the previous 96-Antibody per chip, we improved the protocol to profile the subcellular localization of 400 proteins per chip simultaneously. This further development makes it possible to accommodate a much broader coverage of protein of interest; or in some specific cases, to detect a certain set of protein targets with their different secondary modification forms, which could be of useful and confirmative information in biomedical research. We have demonstrated the usage of this technology by determining the subcellular localization profiles of 400 proteins in two normal prostate cell lines and three prostate cancer cell lines.

Materials and Method

Preparation of antibody microarray Array-400

Antibody microarray Array-400 obtained from Hypromatrix (Worcester, MA) was used. Array-400 contained 400 antibody spots, in a 16 × 25 format. The antibodies were spotted by a robotic piezoelectric non-contact microarrayer on a proprietary membrane chip. Array-400 has an overall size of 15 mm (height) × 24 mm (width), with 1 mm distance between neighboring spots. Each antibody spot contained 50 ng antibodies and had a diameter of ~ 200 μm (generated a corresponding staining spot of ~ 200 μm in diameter on the sample slide). The antibody lists for Array-400 are shown in Figure 1. All antibodies were selected from Hypromatrix's collection, which all passed their internal quality controls.

Cell culture and sample preparation

Five prostate cell lines, including two normal lines (PWR-1E and PZ-HPV-7) and three cancer cell lines (Du145, LNCap and VCap), were purchased from American Type Culture Collection (ATCC). Cells were maintained and propagated as recommended by the ATCC and were grown on 10 cm culture dish until ~ 90% confluency and fixed with pre-cooled methanol/acetone (1:1) solution in -20°C for 10 minutes.

Dissociable antibody microarray (DAMA) staining and traditional immunostaining

Fixed cells on a coverslip were permeabilized with 0.5% Triton X-100 and blocked with 10% goat serum in PBS. Array-400 array membrane was placed over the fixed cells, with antibody side of the membrane facing the fixed cells. The membrane was then incubated at room temperature, with 0.2 kg of weight added, and was removed from the fixed cells in two hours. The antibodies were delivered into the fixed cell in a matrix pattern. The unbounded antibodies were washed away with PBS buffer. The bound antibodies were stained with the FITC-conjugated secondary antibodies (both goat-anti- rabbit and goat-anti-mouse antibodies) for 30 minutes. Cells were then stained with DAPI for 5 minutes before mounted on a slide for fluorescent microscopy [20].

For the traditional immunostaining, individual primary antibodies were diluted 500 - 2000 times in PBS buffer and were delivered to the surface of the coverslip with fixed cells, followed by incubation at room temperature for 2 hours. The unbounded antibodies were washed away with PBS buffer. The bound antibodies were stained by FITC-conjugated secondary antibodies for 30 minutes. Cells were then stained with DAPI for 5 minutes and mounted on a slide for fluorescent microscopy.

Obtain the DAMA staining images by a de-convolution microscope

The molecular images of 400 proteins were obtained from a single slide with cultured cells by using Leica imaging system. The system includes a Leica deconvolution microscope (Leica Microsystem, Bannockburn, IL), a 12-bit digital CCD camera (The Cooke Corporation, Romulus, MI), a motorized stage with the X-Y movement and a controlling program (SlideBook) with the multi-well capture package. The anti-Histone H1 antibodies spotted at the four corner of the array were used as reference spots to determine the orientation of the slide and the positions of all spotted antibodies by the program. The molecular images of all 400 proteins were obtained automatically from a single coverslip with cultured cells using the multi-well capture-package of SlideBook. The captured images were stored into database managed by Chipview for further analysis. Protein molecular images from individual traditional staining were obtained by using the standard image-capturing package.

Database setup and image analysis by ChipView

ChipView, a user-friendly GUI program, was developed to manage and analyze large amount of images obtained from the DAMA staining experiments [20]. The program establishes and maintains an image database to store the images of the 400 proteins in different samples together with the information of experimental conditions and outputs analysis results from the database. It also provides a platform to extract the subcellular localization information by comparing these images in various manners.

Results

We have previously developed the DAMA staining technology as a new approach for the global analysis of protein expression profiles in fixed cells [19]. We have also developed and optimized the technology for protein subcellular localization profiling, established ChipView, a program for management and analysis of molecular image database [20]. We have utilized the technology to identify proteins with unique subcellular localization in breast cancer cell lines [20]. The goal of this work is to further expand the DAMA staining technology to profiling the subcellular localization in a high throughput format. For this purpose, we have optimized the protocols, and have extended the testing capability from profiling the subcellular localization of 96 proteins [20] to profiling that of 400 proteins simultaneously. We have also utilized it in identifying the proteins with differential subcellular localization in prostate cancer cells. The technology includes the following steps: determination of protein subcellular localization profiles by DAMA staining, data analysis and data mining by CHIPVIEW program and evaluation by individual immunostaining analysis.

Determination of the subcellular localization profiles of 400 proteins in 5 different prostate cell lines by the DAMA staining technology

Protein subcellular localization profiles of 400 proteins in five

Array-400 Antibody list by position

	1	2	3	4	5	6	7	8	9	10	11	12	13
A	Histone	Histone	14-3-3	Abl (c-Abl/Bcr Abl)	Akt 1/2	Bin 1	Ankyrin	Bad	Bak	Bax	BOK	Bag-1	Bcl-2
B	γ-catenin	Histone	Cbl (c-Cbl)	CBP	Cdk1/Cdc2	Cdk2	Cdk4	Cdk6	Chk	C-IAP1	C-IAP2	Clathrin	Cyclin A
C	Egr-2	Egr-3	Histone	EphA1	EphA4	EphB1 (EIK)	FAK	Fas/CD95/APO-1	Fas Ligand	FGFR1 (Flg)	FGFR2 (Bek)	FGFR3	FGFR4
D	BAP1	BRCA1	HCAM	VCAM-1	Caspase1	Caspase2	Caspase3	Caspase4	CD 3 epsilon	CD27	CD28	CD40	CD45
E	DcR2	DR5	Dynamin	eps8	erb2 (Neu, Her 2)	erbB3	erbB4	ERK1	ERK2	Estrogen R a	Ets-1/2	FADD	FLIPs/l
F	GRB2	GRB7	GRK 2	IFN-a R a	IFN-g R a	IL 1 R	IL 2 R alpha	IL 2 R, beta	IL2/IL4/IL7/IL9/IL13	IL3/IL5/GMCSF	IL4R a	ISGF3 gamma	Jak1
G	Met	MGMT	MMp-9	Mos proto-oncogene	Ikappa B-α	Ikappa B-α (phospho)	Ikappa B-β	Ikappa B-ρ	Ikappa B-ε	IKKa	IKKb	Nibrin	NIK
H	Phospholipase D	PI3kinase P85	PKC alpha, beta, gamma	PP1,2A,2B,PPX	PSD-95	RACK1	Rad51	Rad52	Raf-1 (c-Raf-1)	raf (c-raf, p: ser 338)	RAIDD	RalA	Rap1
I	Integrin b 3	IRAK	IRF1	IRF2	LIFR	Mad-1	Max	MDM2	mdr	Myc (c-Myc)	MyoD	NCK	NF-1
J	p16	P63 (KET)	P73	p130Cas	p300	PAR-4	PARP	Patched	Pax-5	Paxillin	PCNA	PTEN	PTP1 (SH)
K	Rel (c-Rel)	Rel B	Ret	Rho A	RIP	Ron alpha	Rsk-1	Sam68	Selectin (E-selectin)	Selectin (L-Selectin)	sp1	sp2	Blk
L	TCR beta	TGFb R1	TGFb R2	Thyroid R a1(b1)	TRAF5	TRAF6	TRAIL	TrkA, B, C	TSG101	Tuberin	Tyk2	Vav	Selectin (P-Selectin)
M	Stat5a	Stat5b	Stat6	TNFR1	TNFR2	TOSO	TRADD	TRAF1	TRAF2	TRAF3	VDR	VEGFR1	VEGFR2
N	Homer-3A	HS1(Lck BP-1)	HSP27	HSP60	HSP-70	MAD4	MGMT	NEUROD1	NMI	ING1-P33	PACT	PCNA	PITPNB
O	rhoG	SLM-2	USF1	ABIN-1	ABIN-1	Amphiphysin	Amphiphysin	ARF6	ARF6	BID	DMC1	Bin1	Bin1
P	Histone	Cyclin D3	Cyclin D3	DFF45/ICAD	DFF45/ICAD	Esterase D	Esterase D	FAF-1	FAF-1	FHIT	FHIT	Gankyrin	Gankyrin

	14	15	16	17	18	19	20	21	22	23	24	25
A	Bcl-xS/L	A1	Brk	Btk	C/EBP beta	E-Cadherin	N-Cadherin	Pan-Cadherin	BLCAM	α-Catenin	β-Catenin	Histone
B	Cyclin B	Cyclin D3	Cyclin E	Cyclin H	cytochrome C	DAXX	Desmoglein	DFF45/ICAD	E2F1	EGFR	EGFR (p-EGFR: tyr 1173)	Egr-1
C	Annexin VI	Apaf1	APC	ARC	ATF-2	ATF-2 (p-ATF-2)	B7-1	B7-2	Mcl-1	Bcl-3	Bcl-6	Bim
D	Cdc6	Cdc25A	CDC42GAP	Clusterin	Connexin-43	Cortactin	CPAN	CREB	CREM-1	Crk	Csk	CUL-1
E	Flt-3/2	Flt-4	Fos (c-Fos)	Frizzled	GADD34	GAK	GATA-1	GATA-2	GATA-3	G-CSF R	gp130	Granzyme B
F	Jak2	Jak3	JNK1,2,3	JNK1,2,3(phosphorylated)	Jun B (c-Jun,Jun-D)	Jun (p-c-Jun:)	KAP	c-Kit	MEF2	MEK1	MEKK1	MEKK2
G	p19Skp1	p21WAF1/CIP1	p27	p35	p38 MAPK	p38 MAP kinase (p: tyr 182)	p45 skp2	p53	p55 CDC	PDGF Receptor a	PDGF Receptor b	Phospholipase Cγ
H	Rap2	GSK-3 alpha	HDAC1	Lck BP-1 (HS1)	HSP-70	ICSBP	Id1	Insulin R b	Integrin a 1	Integrin a 5	Integrin a V	Integrin b 1 (CD29)
I	NF1GRP	NF2	NFATC	NF-kappa B 50	NF-kappa B 52	NF-kappa B p65	NOS (eNOS)	NOS (I)	NOS (n)	Ntk	Nurr 1(Nur 77)	Ob Receptor
J	PTP2 (SH)	Rab3	Rab5	Rab11	Rac1	RAR r (a,b,r)	RXR a,b,r	Ras	Ras-GAP	Rb p107	Rb (p110)	RbP130 (Rb2)
K	c-Fgr	fyn	Lck	Lyn	Src	Yes	SRF	SURVIVIN	Syk	Syntaxin	TANK	TCR apha
L	SHC	Sik	Smad1 (1/2/3)	Smad4	SOCS-1	Sos1/2	Stat1	Stat1(P-Stat1)	Stat2	Stat3	Stat3 (p-Stat3)	Stat4
M	WT	XRCC4	YY1	ZAP70 Kinase	ABIN	Calbindin	Clusterin	CNTFR	Cyclin D3	DFF45/ICAD	Gankyrin	HLF
N	Prohibitin	RAB1A	RAB2	RAB3A	RAB5A	RAB6	RAB7	RAB11A	N-ras	REF-1	RGS10	RHEB
O	Calbindin	Calbindin	Cathepsin	Cathepsin	CDC34	CDC34	CDC42	CDC42	Clusterin	Clusterin	CNTFR	CNTFR
P	GRB2	GRB2	HDAC1	HDAC1	HLF	HLF	Homer-3A	Homer-3A	HSP27	HSP27	HSP60	Histone

Figure 1: List of 400 antibodies and their positions in the Array-400.

different human prostate cell lines were obtained by using the DAMA staining technology. The five prostate cell lines include two normal lines (PWR-1E and PZ-HPV-7) and three carcinoma cell lines (Du145, LNCaP and VCaP). The cells were grown on 4 cm × 2 cm coverslips upto 80-90% confluence, and fixed and permeabilized by the optimized protocol. The 400 known primary antibodies, spotted on a membrane, were delivered to the fixed and permeabilized cells in 16 × 25 format. The bound antibodies were then stained with FITC-conjugated secondary antibodies. Five images for each spot for all the 400 proteins, with FITC stained protein in green channel, DAPI stained nucleus in blue channel, were taken by an automatic image-taking system in the microscope (equipped with a computer-controlled motorized stage). A total of approximately 10,000 molecular images was obtained and stored in ChipView, a program for database management and analysis.

ChipView was developed to manage and analyze the large amount of molecular images obtained by DAMA staining technology [20]. The program organizes imported images according to both the sample ID (i.e., cell lines in this work) and the spot positions (i.e., the corresponding antibody ID). Automatic data processing could also be carried out to modify the image quality and to assign a custom-defined SCL code for each protein by the procedure described below. Image data can be either pulled out based on sample names to show profiling outlines, or based on specific antibody of interest to provide cross-sample comparisons. Selected images can be conveniently customized into ChipView pictures. The composite images of those 400 proteins in five prostate cells, PZ- HPV-7, PWR-1E, DU145, LNCaP, VCaP, are shown in Figures 2a–2e, respectively. Each image corresponds to the molecular image of a specific protein, as listed in Figure 1, in that cell line.

Assignment of the protein subcellular localization codes for each of the protein by using the ChipView program

The subcellular localization of each of the 400 proteins in different prostate cell lines was assigned with a SCL code by analyzing each molecular image with the ChipView program. The coding method is the similar to the published procedure on breast cancer cells [20]. In a brief summary, Code N is assigned to nuclear distribution, defined by overlaying with DAPI staining with exclusion from cytosolic region; code C is for cytosolic distribution with exclusion from nucleus; code D is assigned to the protein with both nuclear and cytosolic distributions; code M is assigned to a cell membrane pattern and code S is assigned if multiple spots are shown in the cell images. For the protein with unclear images due to various reasons, a "U" was assigned as the subcellular localization code.

The assigned subcellular localization codes of the 400 proteins in the five different prostate cell lines are summarized in Figure 3. Each colored bar represents the subcellular localization of a specific protein in the cell line. Blue, green, dark green, red, and light blue colored bars represent protein localization to the nucleus (N), cytoplasm (C), plasma membrane (M), spots (S) or diffusion (D), respectively. The summary of different SCL assignments in five different cell lines is shown in Table 1.

GRK2 was confirmed to have differentially subcellular locations in some prostate cancer cell lines by individual immunostaining

From the comparison of SCL profiles of the 400 proteins in five prostate cell lines, we intend to identify any proteins with one subcellular localization code in the three cancer cells, and with another subcellular localization code in the two normal cells. We did not find any proteins fit the criteria. However, one protein, GRK2 (localized in F3 position of the array), was identified as having unique subcellular localization in some cancer cell lines (Figure 3). GRK2 was identified as localizing on the membrane in two cancer cell lines (Du145, VCap), and localizing at cytosol in the third cancer cell line (LNCap) and in other two normal cell lines (PWR-1E and PZ-HPV-7).

The observed difference in GRK2 subcellular localization was further validated by the individual immunostaining, for the five tested cell lines, and for an additional normal prostate cell line, RWPE-1 (Figure 4). The images of GRK2 in those six cells were determined by using confocal microscopy, overlapped with the staining of the Alex-555 linked agglutinin, a membrane marker. GRK2 was confirmed to localize on the plasma membrane in two prostate cancer cells, Du145, VCaP (Figures 4e and 4f), and in the cytosol of other prostate cancer cell, LNCap (Figure 4d) and of other normal prostate cells PWR-1E, PZ-HPV-7 and RWPE-1 (Figures 4a–4c).

Discussion

Dissociable antibody microarray (DAMA) staining is a novel protein microarray platform that combines the high throughput property of microarray technology with the high sensitivity and high specificity of classic immunostaining technology. It could be used to study both protein expression and subcellular localization profiles in high throughput fashion. In this report, we have modified the technology and protocol so that the molecular images of 400 proteins could be obtained from one experiment. We have moved the technology one step forward for profiling the subcellular localization of proteins in high throughput format. We have determined the molecular images of 400 proteins in five prostate cell lines and analyzed those images with ChipView, a program for database management and analysis. In addition, we have assigned the subcellular localization profiles of those proteins in the five cell lines from those images, and have examined and compared their SCL patterns between prostate normal and cancer cell lines. As a validation of the 400-antibody array, we have identified and validated that one protein, GRK2, as being localized on the membrane of two cancer cells, and localized mainly to the cytoplasm in three tested normal cell lines and one cancer cell line.

To develop the application of DAMA staining technology on protein expression profiling, we have first developed the 320-antibody array, in 16 × 20 format, on a 5cm × 5cm membrane with 2 mm between different antibody spots. The cells were grown and fixed on 10 cm-cell culture dishes. The alkaline phosphatase-conjugated antibodies with the substrate were used to determine the protein expression profiles [20]. To further develop the application on protein subcellular localization profiling, we have developed the 96-antibody array, in 8 × 12 format, on a 1 cm × 1cm membrane with 1mm between different antibody spots. The cells were grown on standard coverslips instead of cell culture dishes [20]. In this report, we have further developed the 400-antibody array, in 16 × 25 format, on a 2 cm × 3 cm membrane with 1 mm between different antibody spots. The cells were grown on standard coverslips. In addition, the bound antibodies were detected by using fluorescent microscope, which required the software modification for the microscope. We shall further develop the 1000-antibody array on a 2 cm × 4 cm membrane, with 1mm between different antibody spots, and to start the application of the technology on protein subcellular localization profiling in high-throughput format in the future.

In our previous report, we have compared the subcellular localization profiles of 360 proteins in nine different breast cell lines,

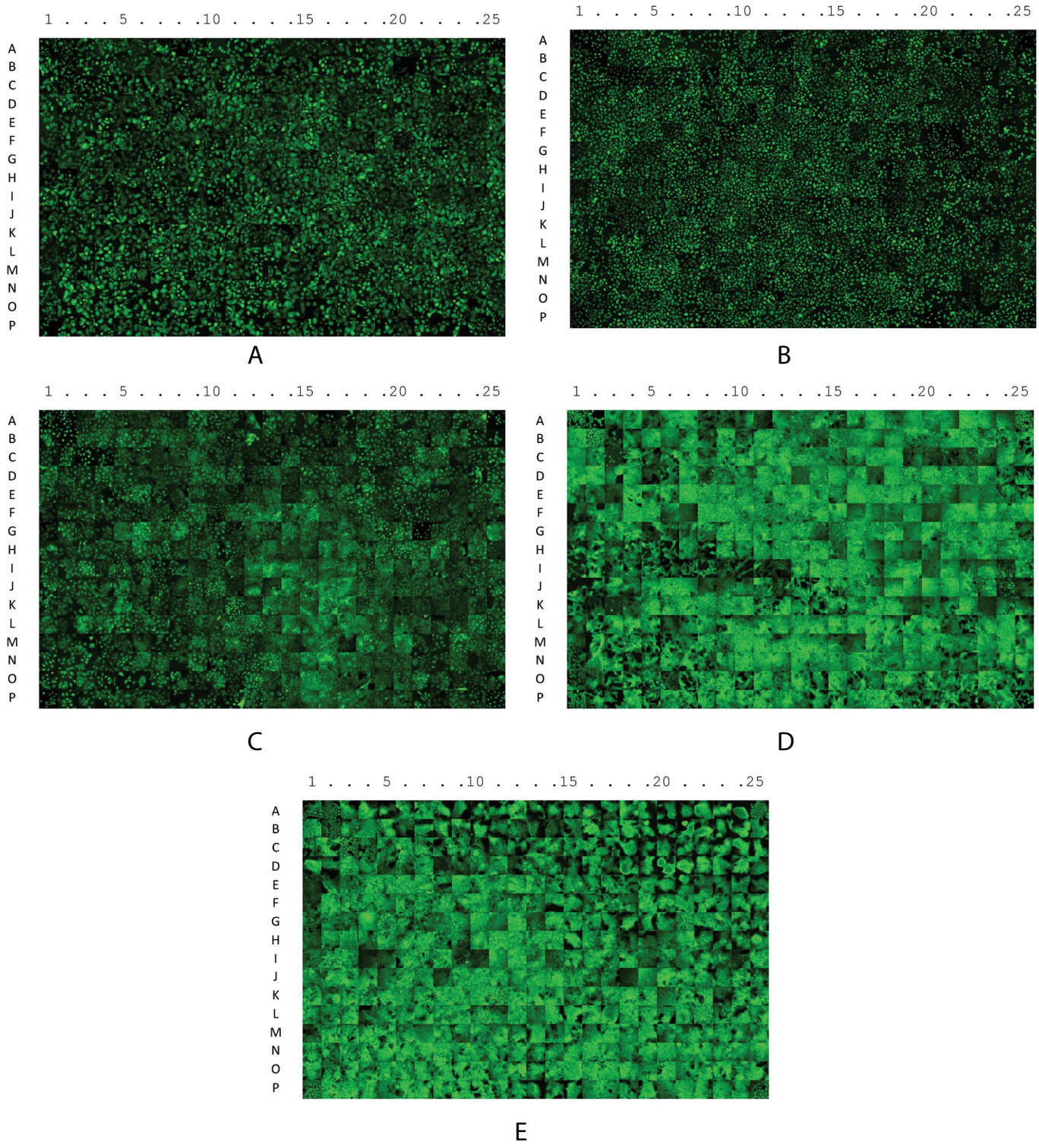

Figure 2: Summary of the molecular images of the 400 proteins in five different prostate cells, as determined by the DAMA staining technology. a) PZ-HPV-7; b) PWR-1E; c) DU145; d) LNCaP; e) VCaP.

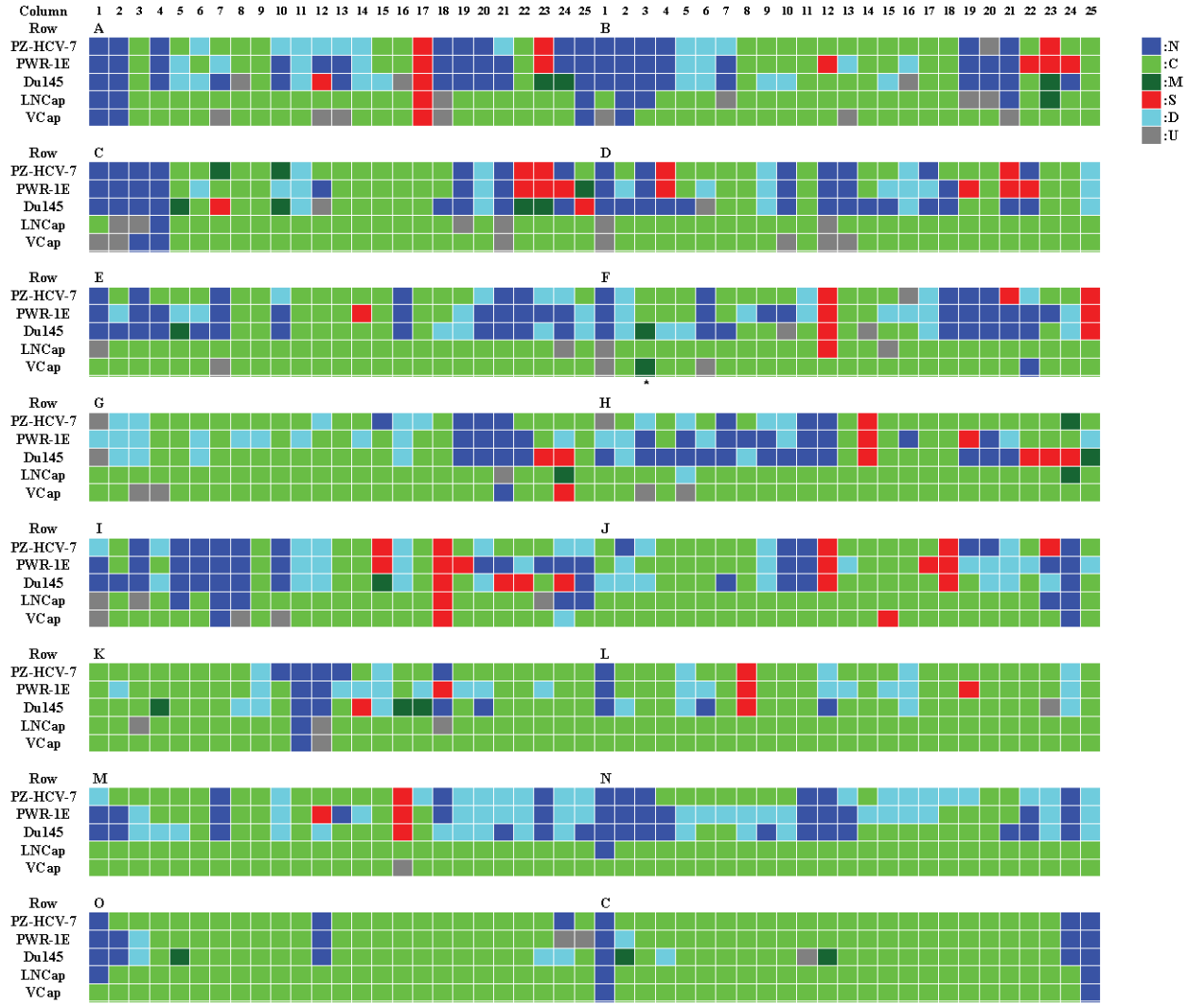

Figure 3: The subcellular localization profiles of the 400 proteins in five different prostate cells. Positions in the arrays are labeled on the top. N1 and N2 represent the two normal cell lines (PWR-1E and PZ-HPV-7) and C1 to C3 represent three prostate cancer cell lines (Du145, LNCaP, VCaP), respectively. Blue, orange, green, dark green, red, and light blue colored bars represent that the subcellular localization of the protein is in the nucleus (N), cytoplasm (C), plasma membrane (M), spots (S) or diffusion (D), respectively, as assigned from their corresponding DAMA staining images.

# of SCL code	PZ-HCV-7	PWR-1E	Du145	LNCaC	VCaC
N	79	107	122	19	13
C	229	163	162	353	355
M	3	1	17	3	1
S	18	29	21	3	4
D	67	98	68	1	1
U	4	2	10	21	26

Table 1: Summary of SCL patterns of 400 proteins in 5 different prostate cell lines.

and we have identified at least one protein with unique localization between 7 cancer cell lines and 2 normal cell lines. On the other hand, in current report, we have not identified any proteins with unique subcellular localization in cancer cells, among three prostate cancer cell lines and two normal prostate cell lines. One possible reason is that only three subcellular localization codes (N, C, D) were assigned to all the images in our previous publication. In current report, in addition to N, C, D assignment, two more subcellular localizations, M and S (representing membrane and spot distribution), have been identified and assigned to the subcellular localization profiles. Therefore, more

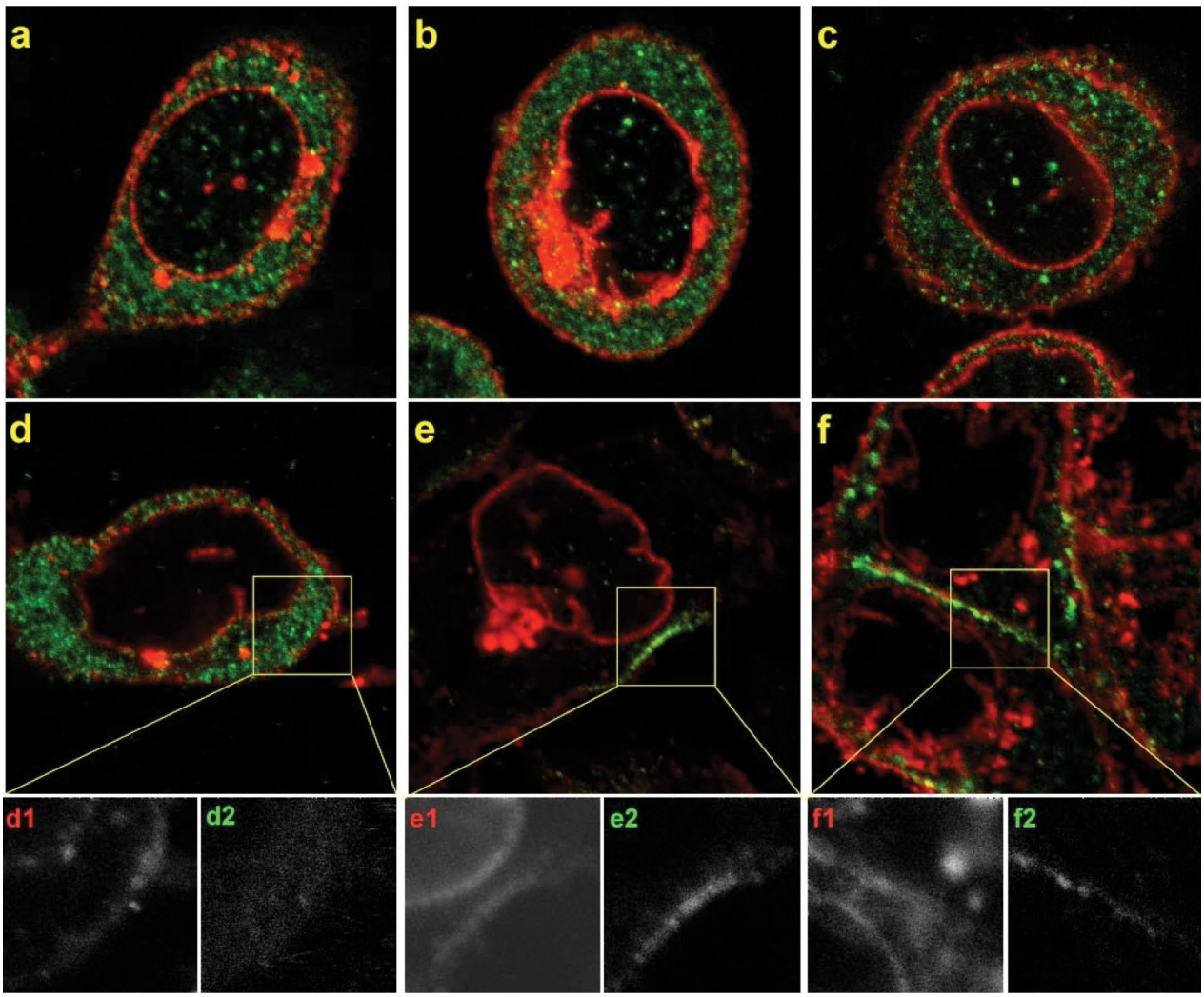

Figure 4: Classic individual immunostaining to confirm the unique subcellular localization pattern of GRK2 in cancer and normal cells. The images of GRK2 in six prostate cell lines (green) were overlapped with the staining of the Alex-555 linked agglutinin (red). Images were taken by using the confocal microscopy with 40x lens. A) PWR-1E. b) PZ- HPV7. C) RWPE-1. D) LNCaP. E) VCaP. F) DU145. The selected regions in the images of prostate cancer cell lines are enlarged in separate channels, to show the relative localization of GRK2 to the plasma membranes, (d1, e1 and f1 for red and d2, e2 and f2 for green).

cell line samples and tissue samples may be needed in order to identify a subcellular localization pattern that could be the characteristic of a certain subtype of prostate cancer.

Acknowledgement

This work was supported by NIH grant CA110047 and a grant from Susan G Komon Breast Cancer Foundation.

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