Effects on immunorecognition of transfer of cells from 10% NBF to 70% ethanol

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Abstract

Because of schedule limitations, cells and tissues initially fixed in 10% neutral buffered formalin (NBF) may sometimes be transferred and held in 70% ethanol until immunostaining can be performed in order to reduce the decrease in immunorecognition that might occur due to antigens being masked through the formation of methylene bridges on prolonged fixation in formaldehyde. The specific parameters as to time of fixation in 10% NBF prior to transfer to 70% ethanol as well as the effects on immunorecognition of transfer to ethanol have not been documented adequately in the literature.

Two cell lines, DU145 (prostate cancer) and SKOV-3 (ovarian cancer) were used as models of fixation in which aliquots of each of these cell lines were fixed in 10% NBF for 5 minutes and 12, 15, 18, 36, 108 and 180 hours. Concomitantly, after 12 hours of fixation in 10% NBF, aliquots of the exact same cells were transferred to 70% ethanol for 3, 6, 24, 96 and 168 hours. For the cells to be contrasted all experiments were designed to perform immunostaining concurrently. They were stained with PCNA, cytokeratins AE1/AE3, and EGFr (membrane and cytoplasmic).

Methods

Duration of fixation in 10% NBF (hrs)	Duration of fixation in 10% NBF fixation for 12 hrs + duration in (hrs) in 70% ethanol
0.08333	_
12	_
15	12+3
18	12+6
36	12+24
108	12+96
180	12+168

Table 1. Experimental design

The two cell lines, DU145 (prostate cancer) and SKOV3 (ovarian cancer) obtained from American Type Culture Collection (ATCC) were maintained in RPMI 1640 and DMEM respectively with 10% fetal calf serum plus supplements (MEM vitamin solution (Invitrogen), l- glutamine (Invitrogen) and antibiotic-antimycotic (Invitrogen) reagents and maintained in an incubator at 5% CO_2 at 37˚C.

The DU145 and SKOV3 cell lines were grown on coverslips. When confluency was about 70% (usually after two days of cell growth), the cells on coverslips were quickly rinsed twice in PBS then either fixed in 10% NBF alone or fixed in 10% NBF for 12hrs at the end the 10% NBF was replaced with 70% ethanol (AAPER Alcohol and Chemical Co. Shelbyville, KY) for comparable duration as shown in (Table 1) above.

Each of these cell lines were fixed in 10% NBF for 5 minutes and 12, 15, 18, 36, 108 and 180 hours. Concomitantly, after 12 hours of fixation in 10% NBF, aliquots of the exact same cells were transferred to 70% ethanol for 3, 6, 24, 96 and 168 hours.

The plating was done over several days in a decreasing time schedule so that all fixation times were synchronized to enable immunostaining to be done at the same time.

When the designated time point for all cultures had been reached, the fixed cells on coverslips were rinsed in Tris buffer for 10 min, dehydrated through graded concentrations of ethanol i.e., 70%; 95%; and absolutes; at 2 min at each concentration, then permeabilized in acetone (Fisher Scientific, Fairlawn, NJ) for 15 sec then rehydrated via the graded concentrations of ethanols i.e., absolute; 95%; and 70%, before washing in Tris buffer for 2 min. Endogenous peroxidase was quenched by exposure to 3% of aqueous H_2O_2 for 5 min and rinsed in Tris buffer. To reduce nonspecific staining 3% goat serum was added to cells on coverslips for 1 hr at room temperature.
The fixed cells on coverslips were stained with three monoclonal antibodies each at a fixed concentration. The three antibodies were:

•PCNA (Santa Cruz Biotechnology, Santa Cruz, CA) dilution 1:18,000.

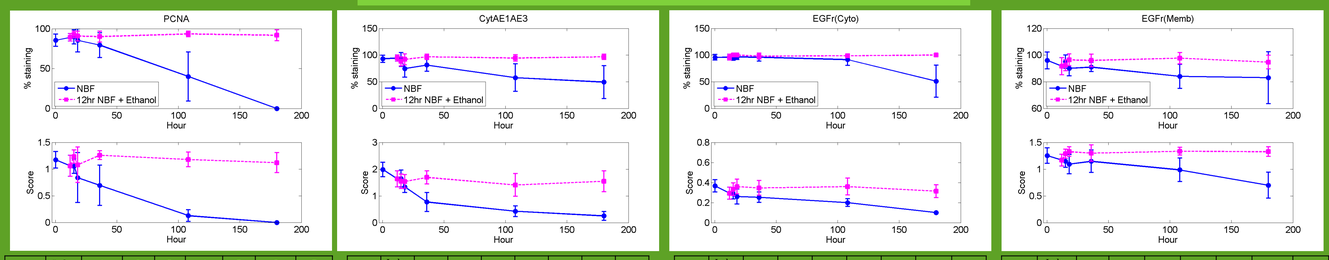
•Anti keratin AE1/AE3 (Boehringer Mannheim Corp, Indianapolis, IN) 5µg/ml dilution 1:40.

•Anti EGFr (Zymed, San Francisco, CA) 3µg/ml dilution 1:5.

All primary antibodies were diluted in PBE pH 7.6. For each antibody and cell line, a delete was included for which the primary antibody was replaced with 3% goat serum. Next the cells on coverslips were rinsed with Tris buffer pH 7.6; then the cells were incubated with biotinylated goat anti-mouse polyclonal rabbit detection antibody for 10 min (Signet, Dedham, MA) and 5 min with HRP conjugated streptavidin (Signet, Dedham, MA). Color was developed with diaminobenzedine (DAB) for 7 min (Biogenex, San Ramon, CA) producing an insoluble chromogen. The cells on coverslips were rinsed with DI water, counter stained in Mayer's hematoxylin (Sigma-Aldrich, St. Louis, MO) for 1 min 15 sec, blued in tap water, dehydrated through graded concentrations of ethanols: 70%; 95%; and absolute ethanol, before clearing in three changes in xylene (Fisher Scientific, Fairlawn, NJ). The cells on coverslips were mounted on the microscope slides using permount (Fisher Scientific, Fairlawn, NJ).

Each experiment was independently repeated three times and five random fields were evaluated at each point of evaluation using the methods we have reported previously (Otali et al. 2009).

DU145 Results



Index (n)	1	2	3	4	5	6	7
Time (hrs) in 10% NBF	0.083	12	15	18	36	108	180
1	0.083	-	0.05	0.02	0.007	4.51 E-05	3.10 E-19	5.51 E-23
2	12	-	-	0.46	0.05	0.001	7.77 E-16	9.32 E-19
3	15	-	-	-	0.05	0.001	9.84 E-19	3.62 E-23
4	18	-	-	-	-	0.18	1.68 E-06	6.74 E-08
5	36	-	-	-	-	-	2.78 E-06	4.72 E-08
6	108	-	-	-	-	-	-	6.05 E-05

Table 2A shows complete p-values of t-tests between any sample pairs for immunostaining score of DU145 cells staining with PCNA. For example p14=0.007 is the p-value of the t-test comparing sample 1 i.e., 5 min in 10% NBF with sample 4 i.e., 18 hrs in 10% NBF.

Index (n)	1	2	3	4	5	6	7
Time (hrs) in 10% NBF	0.083	12	15	18	36	108	180
1	0.083	-	0.001	0.002	8.38 E-07	1.39 E-11	2.89 E-17	4.01 E-19
2	12	-	-	0.55	0.006	3.72 E-08	9.21 E-14	9.46 E-16
3	15	-	-	-	0.006	4.33 E-08	1.80 E-13	2.18 E-15
4	18	-	-	-	-	7.39 E-06	6.27 E-11	2.53 E-13
5	36	-	-	-	-	-	0.001	8.78 E-06
6	108	-	-	-	-	-	-	0.007

Table 3A shows complete p-values of t-tests between any sample pairs for immunostaining score of DU145 cells staining with cytokeratin AE1/AE3: For example p14=8.38E-07 is the p-value of the t-test comparing sample 1 i.e., 5 min in 10% NBF with sample 4 i.e., 18 hrs in 10% NBF.

Index (n)	1	2	3	4	5	6	7
Time (hrs) in 10% NBF	0.083	12	15	18	36	108	180
1	0.083	-	0.001	0.0003	9.40 E-05	4.01 E-06	5.65 E-10	9.23 E-13
2	12	-	-	0.37	0.09	0.03	9.55 E06	2.51 E-10
3	15	-	-	-	0.13	0.04	7.10 E-06	3.34 E-11
4	18	-	-	-	-	0.39	0.005	2.97 E-07
5	36	-	-	-	-	-	0.002	1.41 E-09
6	108	-	-	-	-	-	-	1.12 E-08

Table 4A shows complete p-values of t-tests between any sample pairs for cytoplasmic immunostaining score of DU145 cells staining with EGFr. For example p14=9.40E-05 is the p-value of the t-test comparing sample 1 i.e., 5 min in 10% NBF with sample 4 i.e., 18 hrs in 10% NBF.

Index (n)	1	2	3	4	5	6	7
Time (hrs) in 10% NBF	0.083	12	15	18	36	108	180
1	0.083	-	0.04	0.02	0.006	0.06	0.0003	1.55 E-07
2	12	-	-	0.31	0.09	0.37	0.004	6.34 E-07
3	15	-	-	-	0.17	0.5	0.009	1.27 E-06
4	18	-	-	-	-	0.77	0.08	5.63 E-05
5	36	-	-	-	-	-	0.03	3.16 E-05
6	108	-	-	-	-	-	-	0.003

Table 5A shows complete p-values of t-tests between any sample pairs for immunostaining score of DU145 cells membrane staining with EGFr. For example p14=0.006 is the p-value of the t-test comparing sample 1 i.e., 5 min in 10% NBF with sample 4 i.e., 18 hrs in 10% NBF).

Index (n)	1	2	3	4	5	6
T= 12 (hrs) in 10% NBF+ EtOH	12+0	12+3	12+6	12+24	12+96	12+168
1	12+0	-	1.0	0.58	1.0	0.97	0.8
2	12+3	-	-	0.05	0.75	0.14	0.03
3	12+6	-	-	-	0.97	0.85	0.66
4	12+24	-	-	-	-	0.03	0.006
5	12+96	-	-	-	-	-	0.16

Table 2B shows complete p-values of t-tests between any sample pairs for immunostaining score of DU145 cells staining with PCNA. For example p14=0.68 is the p-value of the t-test comparing sample 1 i.e., 12 hrs in 10% NBF with sample 4 i.e., 12 hrs in 10% NBF + 24 hrs in 70% ethanol.

Index (n)	1	2	3	4	5	6
T= 12 (hrs) in 10% NBF+ EtOH	12+0	12+3	12+6	12+24	12+96	12+168
1	12+0	-	0.2	0.18	0.72	0.07	0.25
2	12+3	-	-	0.47	0.93	0.17	0.5
3	12+6	-	-	-	0.95	0.18	0.52
4	12+24	-	-	-	-	0.02	0.11
5	12+96	-	-	-	-	-	0.79

Table 3B shows complete p-values of t-tests between any sample pairs for immunostaining score of DU145 cells staining with cytokeratin AE1/AE3. For example p14=0.72 is the p-value of the t-test comparing sample 1 i.e., 12 hrs in 10% NBF with sample 4 i.e., 12 hrs in 10% NBF + 24 hrs in 70% ethanol.

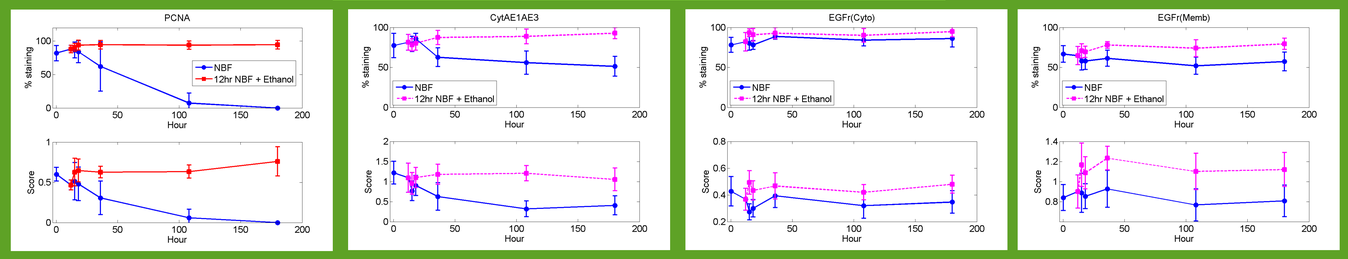
Index (n)	1	2	3	4	5	6
T= 12 (hrs) in 10% NBF+ EtOH	12+0	12+3	12+6	12+24	12+96	12+168
1	12+0	-	0.96	0.99	0.98	0.99	0.81
2	12+3	-	-	0.85	0.7	0.84	0.2
3	12+6	-	-	-	0.31	0.5	0.04
4	12+24	-	-	-	-	0.68	0.10
5	12+96	-	-	-	-	-	0.05

Table 4B shows complete p-values of t-tests between any sample pairs for immunostaining score of DU145 cells cytoplasmic staining with EGFr. For example p14=0.98 is the p-value of the t-test comparing sample 1 i.e., 12 hrs in 10% NBF with sample 4 i.e., 12 hrs in 10% NBF + 24 hrs in 70% ethanol.

Index (n)	1	2	3	4	5	6
T= 12 (hrs) in 10% NBF+ EtOH	12+0	12+3	12+6	12+24	12+96	12+168
1	12+0	-	1.00	1.00	1.0	1.0	1.0
2	12+3	-	-	0.89	0.62	0.94	0.89
3	12+6	-	-	-	0.28	0.59	0.5
4	12+24	-	-	-	-	0.78	0.72
5	12+96	-	-	-	-	-	0.41

Table 5B shows complete p-values of t-tests between any sample pairs for immunostaining score of DU145 cells membrane staining with EGFr. For example p14=1.0 is the p-value of the t-test comparing sample 1 i.e., 12 hrs in 10% NBF with sample 4 i.e., 12 hrs in 10% NBF + 24 hrs in 70% ethanol.

SKOV3 Results



Index (n)	1	2	3	4	5	6	7
Time (hrs) in 10% NBF	0.083	12	15	18	36	108	180
1	0.083	-	1.66 E-05	0.09	0.02	1.22 E-05	1.51 E-15	4.17 E-22
2	12	-	-	0.771	0.59	0.004	1.38 E-13	7.58 E-23
3	15	-	-	-	0.34	0.008	9.02 E-08	1.36 E-09
4	18	-	-	-	-	0.02	6.82 E-08	5.25 E-10
5	36	-	-	-	-	-	0.002	2.10 E-06
6	108	-	-	-	-	-	-	0.02

Table 6A shows complete p-values of t-tests between any sample pairs for immunostaining score of SKOV3 cells staining with PCNA. For example p14=0.02 is the p-value of the t-test comparing sample 1 i.e., 5 min in 10% NBF with sample 4 i.e., 18 hrs in 10% NBF.

Index (n)	1	2	3	4	5	6	7
Time (hrs) in 10% NBF	0.083	12	15	18	36	108	180
1	0.083	-	0.15	3.40 E-05	0.001	8.88 E-06	3.87 E-11	1.62 E-09
2	12	-	-	0.004	0.05	0.006	2.96 E-08	6.13 E-07
3	15	-	-	-	0.93	0.1	3.04 E-06	0.0002
4	18	-	-	-	-	0.009	5.11 E-08	3.24 E-06
5	36	-	-	-	-	-	0.003	0.02
6	108	-	-	-	-	-	-	0.86

Table 7A shows complete p-values of t-tests between any sample pairs for immunostaining score of SKOV3 cells staining with cytokeratin AE1/AE3. For example p14=0.001 is the p-value of the t-test comparing sample 1 i.e., 5 min in 10% NBF with sample 4 i.e., 18 hrs in 10% NBF.

Index (n)	1	2	3	4	5	6	7
Time (hrs) in 10% NBF	0.083	12	15	18	36	108	180
1	0.083	-	0.05	2.73 E-05	0.0003	0.18	0.004	0.02
2	12	-	-	0.0006	0.01	0.8	0.08	0.26
3	15	-	-	-	0.87	1.0	0.94	1.0
4	18	-	-	-	-	1.0	0.75	0.95
5	36	-	-	-	-	-	0.02	0.07
6	108	-	-	-	-	-	-	0.79

Table 8A shows complete p-values of t-tests between any sample pairs for immunostaining score of SKOV3 cells cytoplasmic staining with EGFr. For example p14=0.0003 is the p-value of the t-test comparing sample 1 i.e., 5 min in 10% NBF with sample 4 i.e., 18 hrs in 10% NBF.

Index (n)	1	2	3	4	5	6	7
Time (hrs) in 10% NBF	0.083	12	15	18	36	108	180
1	0.083	-	0.86	0.78	0.61	0.93	0.09	0.27
2	12	-	-	0.42	0.19	0.66	0.02	0.06
3	15	-	-	-	0.29	0.72	0.04	0.11
4	18	-	-	-	-	0.9	0.05	0.19
5	36	-	-	-	-	-	0.008	0.03
6	108	-	-	-	-	-	-	0.75

Table 9A shows complete p-values of t-tests between any sample pairs for immunostaining score of SKOV3 cells membrane staining with EGFr. For example p14=0.61 is the p-value of the t-test comparing sample 1 i.e., 5 min in 10% NBF with sample 4 i.e., 18 hrs in 10% NBF.

Index (n)	1	2	3	4	5	6
T= 12 (hrs) in 10% NBF+ EtOH	12+0	12+3	12+6	12+24	12+96	12+168
1	12+0	-	1.0	1.0	1.0	1.0	1.0
2	12+3	-	-	0.64	0.5	0.55	0.98
3	12+6	-	-	-	0.31	0.38	0.97
4	12+24	-	-	-	-	0.59	0.99
5	12+96	-	-	-	-	-	0.99

Table 6B shows complete p-values of t-tests between any sample pairs for immunostaining score of SKOV3 cells staining with PCNA. For example p14=1.0 (is p-value comparing sample 1 i.e., 12 hrs in 10% NBF with sample 4 i.e., 12 hrs in 10% NBF + 24 hrs in 70% ethanol.

Index (n)	1	2	3	4	5	6
T= 12 (hrs) in 10% NBF+ EtOH	12+0	12+3	12+6	12+24	12+96	12+168
1	12+0	-	1.0	0.99	1.0	0.98	1.0
2	12+3	-	-	0.03	0.22	0.006	0.32
3	12+6	-	-	-	0.85	0.29	0.96
4	12+24	-	-	-	-	0.06	0.67
5	12+96	-	-	-	-	-	0.99

Table 7B shows complete p-values of t-tests between any sample pairs for immunostaining score of SKOV3 cells staining with cytokeratin AE1/AE3. For example p14=0.77 is the p-value of the t-test comparing sample 1 i.e., 12 hrs in 10% NBF with sample 4 i.e., 12 hrs in 10% NBF + 24 hrs in 70% ethanol.

Index (n)	1	2	3	4	5	6
T= 12 (hrs) in 10% NBF+ EtOH	12+0	12+3	12+6	12+24	12+96	12+168
1	12+0	-	1.0	1.0	1.0	1.0	1.0
2	12+3	-	-	0.13	0.85	0.18	0.26
3	12+6	-	-	-	1.0	0.58	0.71
4	12+24	-	-	-	-	0.01	0.02
5	12+96	-	-	-	-	-	0.62

Table 8B shows complete p-values of t-tests between any sample pairs for immunostaining score of SKOV3 cells cytoplasmic staining with EGFr. For example p14=1.0 is the p-value of the t-test comparing sample 1 i.e., 12 hrs in 10% NBF with sample 4 i.e., 12 hrs in 10% NBF + 24 hrs in 70% ethanol.

Index (n)	1	2	3	4	5	6
T= 12 (hrs) in 10% NBF+ EtOH	12+0	12+3	12+6	12+24	12+96	12+168
1	12+0	-	1.0	1.0	1.0	1.0	1.0
2	12+3	-	-	0.12	0.52	0.77	0.86	0.37
3	12+6	-	-	-	0.93	0.99	1.0	0.84
4	12+24	-	-	-	-	0.81	0.9	0.32
5	12+96	-	-	-	-	-	0.63	0.13

Table 9B shows complete p-values of t-tests between any sample pairs of immunostaining score for SKOV3 cells membrane staining with EGFr. For example p14=1.0 is the p-value of the t-test comparing sample 1 i.e., 12 hrs in 10% NBF with sample 4 i.e., 12 hrs in 10% NBF + 24 hrs in 70% ethanol.

DU145 & SKOV3 ± AR

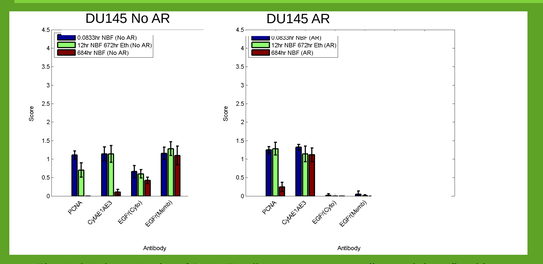


Figure 9A show results of DU145 cells grown on coverslips and then fixed in 10% NBF for 684 hrs compared with 12 hrs fixation in 10% NBF followed by 672 hrs in 70% ethanol immunostained with PCNA, cytokeratin AE1/AE3 and EGFr. Figure 9B shows results of DU145 cells fixed for 684 hrs compared with 12 hrs 10% NBF fixation followed by 672hrs in 70% ethanol then the coverslips were put in 0.01M EDTA pH8.0 pressure cooker immunostained with PCNA, cytokeratin AE1/ AE3 and EGFr.

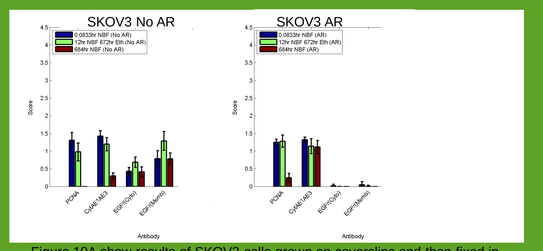


Figure 10A show results of SKOV3 cells grown on coverslips and then fixed in 10% NBF for 684 hrs compared with 12 hrs fixation in 10% NBF followed by 672 hrs in 70% ethanol immunostained with PCNA, cytokeratin AE1/AE3 and EGFr. Figure 10B shows results of DU145 cells fixed for 684 hrs compared with 12 hrs 10% NBF fixation followed by 672hrs in 70% ethanol then the coverslips were put in AR solution was 0.01M EDTA pH8.0 pressure cooker operates two then immunostained with PCNA, cytokeratin AE1/ AE3 and EGFr.

Analysis

Evaluation was performed by a board certified diagnostic pathologist (W.E.G.). Each antibody for a specific cell line at each condition and for each of the three experiments was evaluated during the same session. Two parameters were evaluated, the proportion of cells staining (% staining) and an immunostaining score based on the sum of the intensity from 0 to 4 and the proportion of cells staining at each intensity. Multiplying the proportion of cells at each intensity resulted in an immunostaining score ranging from 0 to 4. Intracellular localization was evaluated separately e.g., for EGFr cell membrane staining was evaluated separately from cytoplasmic staining. Data were analyzed using t-tests with the assumption that both percent of cells staining and staining intensity are normally distributed.

Conclusion

In order to preserve immunorecognition of many antigens in cells, the specimens should be transferred from 10% NBF to 70% ethanol after about 18 hours of fixation.
Immunorecognition will then remain stable for at least one week. Results vary with the antigen-antibody pair being evaluated.
At 108 to 180 hours of fixation in 10% NBF, there was almost complete loss of immunorecognition of PCNA, and cytokeratin AE1/AE3.
The transfer of cells to 70% ethanol after 12 hours of fixation in 10% NBF preserved immunorecognition at all points of evaluation (up to 180 hours i.e. a total time of 12 hrs 10% NBF plus 168 hrs being held in 70% ethanol).

The effects of duration of fixation in 10% NBF on EGFr immunorecognition were less, but transfer to 70% ethanol maintained both cytoplasmic and membranous staining of EGFr.
As shown in figures 9A and 10A, antigen retrieval (AR) at pH8.0 does recover most immunorecogniton of PCNA following fixation for 684 hrs in 10% NBF. However, 12hrs of fixation in 10% NBF followed by 672 hrs in 70% EtOH plus AR recovers most of the staining for PCNA and nearly all of the staining for cytokeratins. Of note, AR at ph8.0 caused loss of immunorecognition for EGFr.

Acknowledgements

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