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Performance Comparison of Image Normalisation Method for DNA Microarray Data

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ABSTRACT

Normalisation is a process of removing systematic variation that affects measured gene expression levels in microarray experiment. The purpose is to get a more accurate DNA microarray result by deleting the systematic errors that may have occurred when making the DNA microarray slid. In this paper, four normalisation methods of Global, Lowess, Quantile and Print-tip are discussed, tested and their final results compared in the form of Matrixes and graphs. Ideal and real microarray slides have been used for this project. It was found that the Print-tip normalisation method showed the closest results to the real result for an ideal microarray slide and it has a straight median line final graph. The Print-tip normalisation method uses more than one normalization factor that is divided among intervals which are dependent on the values of the addition of red and green logarithm.

Keywords: DNA, Microarray, Normalisation, Global, Lowess, Quantile, Print-tip, Background correction, M-A plot

INTRODUCTION

Gene expression measurements provide clues on the regulatory mechanism, biochemical pathways and broader cellular function. By gene expression is the transformation process of gene's information into proteins. The formal transformational pathway of protein begins with the DNA (deoxyribonucleic acid) which is copied to the mRNA (messenger ribonucleic acid) and, finally this molecule passes from nucleus to cytoplasm carrying the information to build proteins (Belean et al., 2011).

There are many microarray analysis software packages in the market. Each software program is concerned with three main tasks: 1) gridding or addressing, which is

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the process of specifying coordinate to every spot on the slide 2) segmentation which decides the classification of each pixel either as foreground which corresponds to be an interest spot or as background which acts as an error or noise 3) Intensity Extraction which is the step to calculate green and red for foreground fluorescence intensity for each spot on the array (Borda et al., 2011; Rao et al., 2008).

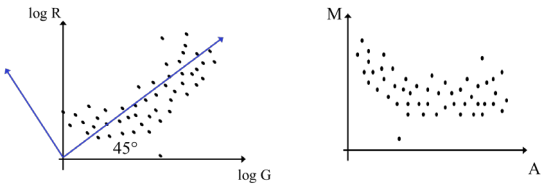
Processes to inspect the results and correct the errors are: 1) background correction method obtained by subtracting the value of the background intensity from the value of foreground intensity or any other suitable method to neglect the effect of background intensity 2) normalisation method which is the objective of this research (Yang et al., 2001).

Normalisation is the process of removing systematic variations that affect measured gene expression levels in microarray experiments. The purpose of normalisation is to adjust for effects which arise from variations in the microarray technology rather than from biological differences between the RNA samples or between the printed probes. Imbalances between the red and green dyes may arise from differences between the labelling efficiencies or scanning properties of the two flours complications perhaps by the use of different scanner settings (Geeleher et al., 2009). The aim of this paper is to review various methods that discuss and compare DNA microarray normalization.

In section II several normalization algorithms are elaborated, while section IV discusses the comparison of these varies methods. Section V and VI presents the methodology and results of analysis of the different methods. The conclusion follows in section VII.

LITERATURE REVIEW

Discussion on the normalisation of DNA microarray is currently well developed. Before we review some of them, we will explain the two types of graphs than can show normalisation quality. First, (log M vs. log R) as shown in Figure 1(a). Second, M-A plot is 45° rotation of standard scatter plot as shown in Figure 1(b). Write R and G for the background-corrected red and green intensities for each spot. Normalisation is usually applied to the log-ratios of expression, which will be written (M = log R – log G). The log-intensity of each spot will be written (A = (log R + log G)/2), a measure of the overall brightness of the spot. (The letter M is a mnemonic for minus while A is a mnemonic for addition) (Dudoit et al., 2002).



(a) (b)

Figure 1. (a) Log R vs. Log G; (b) M-A Plot

This section will discuss and elaborate the methods of DNA microarray normalisation and identify the most suitable for further microarray analysis. The first method is Global normalisation: the underlying assumption of this approach is that the total of mRNA labelled with either R value (sum of red intensities) or G value (sum of green intensities is equal. While the intensity for any one spot may be higher in one channel than the other, when averaged over thousands of spots in the array, these fluctuations should average out. In this method, the value of c out of log (R/G). The c value is equal to the main assumption that equal to log of the total R over total G which can be expressed by the variable K (Yang et al., 2002). The intensity-dependent lowess normalisation runs a line through the middle of the MA plot, shifting the M value of the pair (A,M) by c=c(A), as shown in Equation 3. One estimate of c(A) is made using the loess function: Locally Weighted Scatterplot Smoothing (Berger et al., 2004; Bilban et al., 2002).

$$log_2 R/G \rightarrow log_2 R/G - c = log_2 \ R/(kg) \tag{1}$$

$$\mathrm{K} = \sum R/G \tag{2}$$

$$log_2 R/G \rightarrow log_2 R/G - c(A) = log_2 \ R/(k(A)G) \tag{3}$$

In the Print-tip normalisation, each M-value (Log R – Log G) is normalised by subtracting from it the corresponding value of the tip group loess curve that is dependent on A value ([Log R + Log G]/2) while its value should be fixed. The normalised log-ratios (N) are the residuals from the tip group loess regressions. A simpler form of Print-tip is shown in Equation 4 where loess (A) is the global loess curve plotted in Figure 2. Refer to Figure 3 for the final figure of the Print-tip normalisation (Smyth et al., 2003). The Quantile normalisation method is undertaken by rearranging the genes in each column as in second table in Figure 4. Following which the mean in each row is replaced the whole raw by the mean value as shown in the third table in Figure 4. Finally, reorder each gene in its original place with its new value.

$$N = M - loess(A) \tag{4}$$

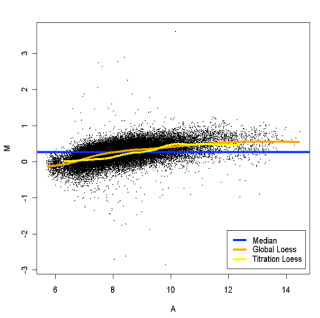


Figure 2. Global normalisation

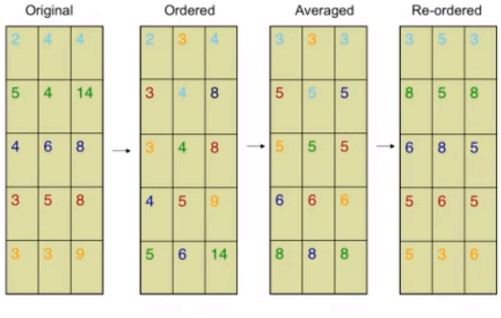


Figure 3. Print-tip normalisation; *Figure 4.*Quantile normalisation

COMPARISON OF DIFFERENT NORMALISATION APPROACHES

In this section, the existing system algorithm as discussed in section III will be analysed and discussed to find out the similarities and variations among the different normalisation methods. Table 1 summarized the comparison of these algorithms.

From Table 1, it can be seen that, all the methods are using mainly the value of M which equal to log of red intensity minus log of green intensity. However, three methods have different value to subtract from M. To illustrate, Global normalisation use the log of addition of each of red and green intensity while the other two methods are using median and global median.

In term of the final shape of the normalisation on M-A graph, there are similarities between Lowess and Print-tip methods because both have a straight median line in the value of (M=0) due to their similarities on subtracting the mean or median from M. However, in Global normalisation, there is a curve around the value of (M=0) due to the subtraction of the total R and G. Quantile normalisation method does not use M-A plot, consequently its final graphs do not always take a straight line of the mean on the (M=0). According to this review, we suggest Print-tip normalisation method to be used because when comparing to the global normalisation its final figure is simpler and easier to read and can also easily be compared to various plots. Straight line on (M=0) is easier to read than the Global and lowess normalisation curve.

Table 1
Comparison between different system algorithms

No.	[1]	[2]	[3]	[4]
Method	Global	Lowess	Print Tip	Quantile
Function	Log (R/ KG)	Log (R/ G) – c(A)	N= M- loess (A)	Mean of rows after reorder
Variable	$K = \sum R/G$	LOWESS function	Global Loess	NA
Shape on M-A graph	Curve	Straight line on (M=0)	Straight line on (M=0) but has some variation	It does not meet M-A plot.

METHODOLOGY

Using Matlab, we developed a code that can extract the intensity for 100 spots. Using 100 spots instead of the whole microarray slide make the process easier and simpler especially to compare the many algorithms used. In order to examine the suitable method which would be more accurate and suitable for this project, an ideal microarray image spots in Figure 5(a), and a real microarray slide in Figure 5(b) were used. Matlab usually reads the image intensity as matrix by pixel, for example our image after cropping is 220*227 pixels while it has only 100 spots. Thus, each spot has around 20 pixel diameters. Next, it calculates the foreground and background then subtract the background value from foreground, and using threshold equal to zero will not allow negative values to appear. In the ideal image the value of background is fixed (Rb = Gb =3) while foreground value is a variant from 0 to 225 as shown in Matrix 1. Then, according to the normalisation method, the formula codes were applied.

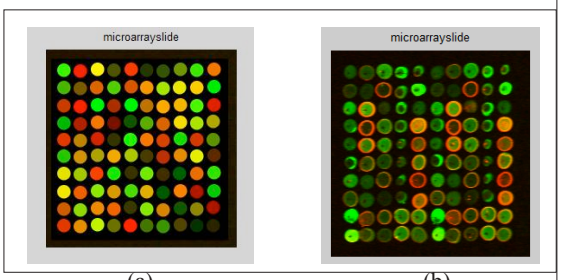


Figure 5. (a) Ideal microarray slide with 100 spot; (b) Real microarray slide with 100 spot;

RedIntensity =

51	243	250	57	242	25	36	91	51	233
57	99	207	67	201	239	148	227	246	1
184	251	1	149	1	172	247	238	65	210
10	155	234	95	14	73	203	239	154	157
214	170	194	36	36	238	201	28	197	73
35	193	155	233	154	54	169	218	219	71
219	147	238	32	39	96	46	1	222	44
236	228	121	203	63	212	1	244	156	15
190	124	230	228	42	117	201	14	121	150
214	220	180	86	249	51	46	60	5	28

GreenIntensity =

244	3	241	66	32	40	66	132	231	96
170	69	76	199	61	129	226	225	192	237
36	1	251	27	251	92	152	166	248	3
172	14	94	6	66	157	77	212	224	150
31	112	12	38	245	96	43	216	33	128
190	91	144	151	188	42	27	118	235	27
220	191	40	230	36	232	33	78	85	224
224	70	230	133	213	157	69	106	1	193
68	210	115	74	40	230	111	68	114	1
32	121	243	94	4	97	130	50	35	25

Matrix 1. Original Intensity of the ideal spots

For Global Normalisation, loops were used to find the total of red and green intensities values for all 100 spots. Then taking the logarithm of the total value and subtracts it from the value of M according to Equation number 1 and 2. Similarly, in the Lowess method, mean of m values was calculated then subtracted, to be on the centre (M=0) according to equation number 3. However, Quantile normalisation is much different than the previous two methods, because it does not require calculation of A and M values. But it requires sorting the matrix in each column. Then taking the average in each raw and finally put each new value in its original location as shown in Figure 5. Finally, Print-tip normalisation, A values (addition of logarithm) has been divided into four groups (<5, <6, <7 and else) and according to each group, mean value of M was taken and defined into variable call PT. After that, the PT value was subtracted from M according to its group. Next section will discuss the results of the various methods tested.

RESULT AND DISSCUSSION

First of all, there is a different in the last result for all the four methods in terms of last intensities values and M-A displaying graphs. Global normalisation and Lowess share a similarity especially when we compare the difference between the green and the red intensity for the same spots. Similarly, Print-tip normalisation which has a similar graph but there is a different according to the interval groups. However, the results for quantile normalisation are fluctuating and the different is larger than all of the other normalisation methods. Normalisation results for the ideal and normal microarray slide are shown in matrix 1 and 2, and M-A graphs in Figure 6 and 7 respectively. As we saw in Matrix 1 above, there are red and green intensities for 100 spots as well as in Matrix 2 bellow. Thus, we have 4 matrixes with the size of (10*10). The first and second for the red and green intensities of ideal image in Matrix 2 while the third and fourth for the red and green intensities for the slide image. Figure 6 and 7 depict M-A plots for ideal and slide image before any method of normalisation was performed. Thus, the illustrations will help us compare them with the next results of various normalisation methods.

RedIntensity =

5	15	22	11	11	4	16	9	12	14
11	6	36	19	30	8	4	41	20	22
10	87	13	12	5	8	88	31	16	3
5	56	23	15	156	8	75	19	11	163
4	63	21	8	67	3	89	33	11	76
8	32	11	6	47	13	26	20	11	73
13	6	20	7	68	15	5	24	8	84
15	9	6	10	109	32	14	8	9	115
10	53	25	32	44	10	83	38	48	45
70	34	13	23	30	51	30	22	38	26

GreenIntensity =

30	50	99	39	16	31	42	102	46	9
31	30	31	28	110	27	22	29	27	43
66	126	26	34	30	68	120	19	39	37
59	33	85	35	108	61	32	83	30	108
32	96	100	17	51	35	91	85	16	52
29	51	47	14	88	36	56	46	14	86
78	44	35	21	23	96	34	32	16	23
53	50	17	13	74	49	49	18	29	74
152	37	45	96	100	133	37	43	93	91
185	52	34	102	69	126	47	28	108	73

Matrix 2. Red and Green Intensity before norm of the slide image;

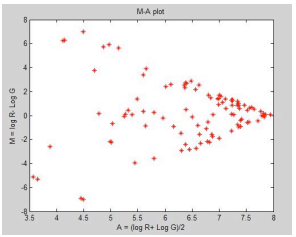


Figure 6. M-A plot before normalisation of the ideal image;

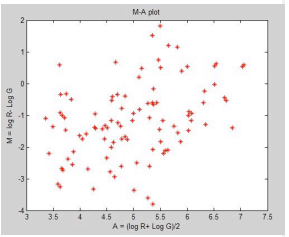


Figure 7. M-A plot before normalisation of the slide image

Matrixes 3 and Matrix 4 show the results of global normalisation for ideal and real DNA microarray slide. Firstly, they show k and c values, c is the logarithm of the total of red intensities over the total of green intensities (k) which is equal to 0.0274 in ideal image and – 0.2358, and that explains to us why the normalisation is important and how the variety of c increased for the real microarray slide image. Thus, the difference between the last and original results in the real microarray slide is larger.

m =

0.1756

RedlowsNorm =

49	230	237	55	229	25	35	87	49	221
55	95	196	64	191	226	141	215	233	2
175	238	2	142	2	163	234	225	63	199
11	147	222	91	15	70	192	226	146	149
203	161	184	35	35	225	191	28	187	70
34	183	147	221	146	52	160	207	208	68
208	140	225	32	38	92	45	2	210	43
224	216	115	192	61	201	2	231	148	16
180	118	218	216	41	112	191	15	115	143
203	208	171	82	236	49	45	58	6	28

GrenLowsNorm =

261	5	258	72	36	44	72	142	247	104
182	75	82	213	66	139	242	241	206	253
40	3	268	30	268	99	163	178	265	5
184	16	101	8	72	168	83	227	240	161
35	121	14	42	262	104	47	231	37	138
203	98	155	162	201	46	30	127	251	30
235	205	44	246	40	248	37	84	92	240
240	76	246	143	228	168	75	114	3	207
74	225	124	80	44	246	120	74	123	3
36	130	260	101	6	105	140	55	39	28

Matrix 3. Red and Green Intensity for global norm of the ideal image

m =

-1.1662

RedlowsNorm =

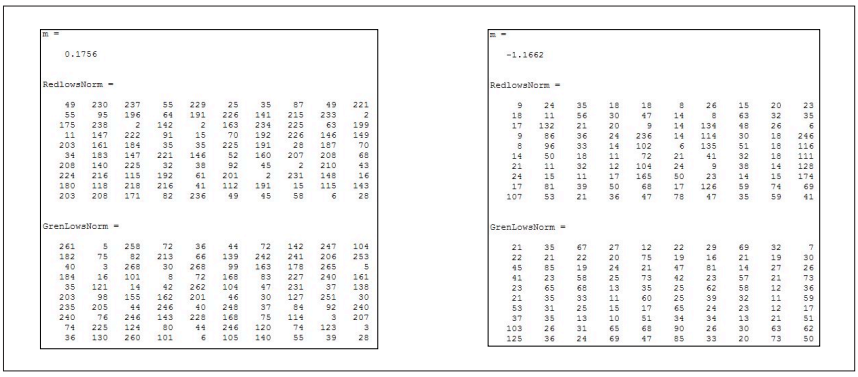
9	24	35	18	18	8	26	15	20	23
18	11	56	30	47	14	8	63	32	35
17	132	21	20	9	14	134	68	26	6
9	86	36	24	236	14	114	30	18	246
8	96	33	14	102	6	135	51	18	116
14	50	18	11	72	21	41	32	18	111
21	11	32	12	104	24	9	38	14	128
24	15	11	17	165	50	23	14	15	174
17	81	39	50	68	17	126	59	74	69
107	53	21	36	47	78	47	35	59	41

GrenLowsNorm =

21	35	67	27	12	22	29	69	32	7
22	21	22	20	75	19	16	21	19	30
45	85	19	24	21	47	81	14	27	26
41	23	58	25	73	42	23	57	21	73
23	65	68	13	35	25	62	58	12	36
21	35	33	11	60	25	39	32	11	59
53	31	25	15	17	65	24	23	12	17
37	35	13	10	51	34	34	13	21	51
103	26	31	65	68	90	26	30	63	62
125	36	24	69	47	85	33	20	73	50

Matrix 4. Red and Green Intensity for global norm of real slide image

Lowess normalisation results for ideal and real DNA microarray slide are shown in Matrix 5 and 6. First it shows (m) values, m is the mean M value for 100 spots which equal to the difference between logarithms of red and green intensities for each spot separately. (m) is equal to 0.1756 in ideal image and –1.1662 explaining why the normalisation process is important and how does the variety of c increase for the real microarray slide image. Also, it is greater than c values (for global normalisation). The difference t between the last and original results in real microarray slide is larger than the different in global normalisation.



m =

0.1756

RedlowsNorm =

49	230	237	55	229	25	35	87	49	221
55	95	196	64	191	226	141	215	233	2
175	238	2	142	2	163	234	225	63	199
11	147	222	91	15	70	192	226	146	149
203	161	184	35	35	225	191	28	187	70
34	183	147	221	146	52	160	207	208	68
208	140	225	32	38	92	45	2	210	43
224	216	115	192	61	201	2	231	148	16
180	118	218	216	41	112	191	15	115	143
203	208	171	82	236	49	45	58	6	28

GrenLowsNorm =

261	5	258	72	36	44	72	142	247	104
182	75	82	213	66	139	242	241	206	253
40	3	268	30	268	99	163	178	265	5
184	16	101	8	72	168	83	227	240	161
35	121	14	42	262	104	47	231	37	138
203	98	155	162	201	46	30	127	251	30
235	205	44	246	40	248	37	84	92	240
240	74	246	143	228	168	75	114	3	207
74	225	124	80	44	246	120	74	123	3
36	130	260	101	6	105	140	55	39	28

m =

-1.1662

RedlowsNorm =

9	24	35	18	18	8	26	15	20	23
18	11	56	30	47	14	8	63	32	35
17	132	21	20	9	14	134	48	26	6
9	86	36	24	236	14	114	30	18	246
8	96	33	14	102	6	135	51	18	116
14	50	18	11	72	21	41	32	18	111
21	11	32	12	104	24	9	38	14	128
24	15	11	17	165	50	23	14	15	174
17	81	39	50	68	17	126	59	74	69
107	53	21	36	47	78	47	35	59	41

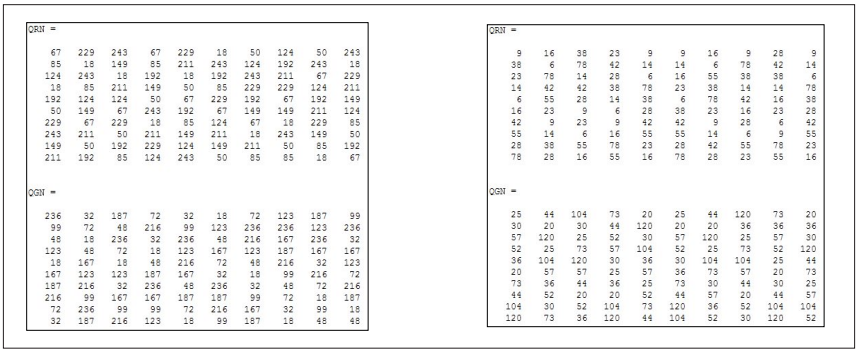
GrenLowsNorm =

21	35	67	27	12	22	29	69	32	7
22	21	22	20	75	19	16	21	19	30
45	85	19	24	21	47	81	14	27	26
41	23	58	25	73	42	23	57	21	73
23	65	68	13	35	25	62	58	12	36
21	35	33	11	60	25	39	32	11	59
53	31	25	15	17	65	24	23	12	17
37	35	13	10	51	34	34	13	21	61
103	26	31	65	68	90	26	30	63	62
125	36	24	69	47	85	33	20	73	50

Matrix 5. Red and Green Intensity for global norm of the ideal image

Matrix 6. Red and Green Intensity for global norm of real slide image

Quantile normalisation results for ideal and real DNA microarray slide are shown in Matrix 7 and 8. Quantile normalisation method differs from global and Lowess normalisations in that it does not require fixed values of (c) or (m). Rather an average of the columns after sorting the matrix in each raw as explained before in section 2. Thus, we can see in Matrix 8 that (67, 85, 124, 18 and so on) are repeated in each column of matrix QRN, and also the anther values for QGN are similar in Matrix 8. There are 10 fixed numbers repeated in each column of each matrix.



QRN =

67	229	243	67	229	18	50	124	50	243
85	18	149	85	211	243	124	192	243	18
124	243	18	192	18	192	243	211	67	229
18	85	211	149	50	85	229	229	124	211
192	124	124	50	67	229	192	67	192	149
50	149	67	243	192	67	149	149	211	124
229	67	229	18	85	124	67	18	229	85
243	211	50	211	149	211	18	243	149	50
149	50	192	229	124	149	211	50	85	192
211	192	85	124	243	50	85	85	18	67

QGN =

236	32	187	72	32	18	72	123	187	99
99	72	48	216	99	123	236	236	123	236
48	18	236	32	236	48	216	167	236	32
123	48	72	18	123	167	123	187	167	167
18	167	18	48	216	72	48	216	32	123
167	123	123	187	167	32	18	99	216	72
187	216	32	236	48	236	32	48	72	216
216	99	167	167	187	187	99	72	18	187
72	236	99	99	72	216	167	32	99	18
32	187	216	123	18	99	187	18	48	48

QRN =

9	16	38	23	9	9	16	9	28	9
38	6	78	42	14	14	6	78	42	14
23	78	14	28	6	16	55	38	38	6
14	42	42	38	78	23	38	14	14	78
6	55	28	14	38	6	78	42	16	38
16	23	9	6	28	38	23	16	23	28
42	9	23	9	42	42	9	28	6	42
55	14	6	16	55	55	14	6	9	55
28	38	55	78	23	28	42	55	78	23
78	28	16	55	16	78	28	23	55	16

QGN =

25	44	104	73	20	25	44	120	73	20
30	20	30	44	120	20	20	36	36	36
57	120	25	52	30	57	120	25	57	30
52	25	73	57	104	52	25	73	52	120
36	104	120	30	36	30	104	104	25	44
20	57	57	25	57	36	73	57	20	73
73	36	44	36	25	73	30	44	30	25
44	52	20	20	52	44	57	20	44	57
104	30	52	104	73	120	36	52	104	104
120	73	36	120	44	104	52	30	120	52

Matrix 7. R & G Intensity for Quant. Norm. of the ideal image

Matrix 8. R &G Intensity for Quant. norm of the real slide image

Finally, Print-tip normalisation gave the results for the red and green intensities for the ideal microarray image in Matrix 9 and real microarray slide in Matrix 10. M-A graphs for the results are displayed in Figure 8 and 9 respectively. PT values in Matrix 9 and 10 are represented by the normalisation values among the four intervals for each image. For example, in Matrix 9, PT equals -0.0664, 0.2457, 0.1445 and 0.2633. These values were subtracted from M (the different between logarithms of red and green intensities for each spot) according to the values of A for the same spot. These intervals are (<5, <6, <7 and else), so each interval has its own normalisation values; and that is why, at times, we can see the obvious different

between the normalised and un-normalised values in some intervals according to the values of PT. Besides that, Figure 9 represents the M-A plot for Print-tip normalization of real image slide which show more different from its original slide except by the values of PT especially in the first interval when PT= -1.5263 among the interval (A less than 5).

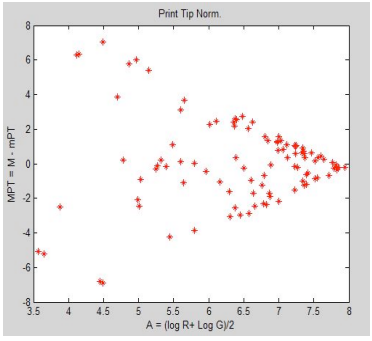


Figure 8. M-A Plot for Print tip norm of the Ideal Image

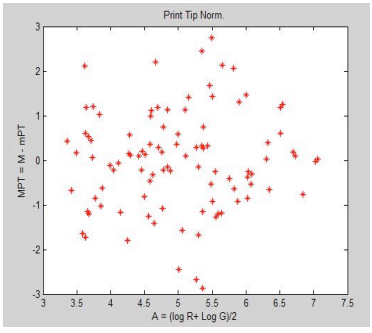


Figure 9. M-A Plot for Print tip norm of the Slide Image

PT =

-0.0664 0.2487 0.1445 0.2633

RedPTNorm =

50	250	230	54	232	24	34	88	50	214
56	96	198	65	193	220	137	209	226	3
176	258	3	143	3	165	227	219	61	216
11	144	215	99	16	71	195	220	142	145
205	157	179	34	36	219	193	28	189	71
35	178	143	214	142	51	162	200	201	67
201	136	228	32	37	89	44	3	204	43
217	218	112	187	61	195	3	224	161	15
182	115	211	210	40	108	185	14	117	155
205	202	166	83	230	50	45	56	7	30

GrenPTNorm =

258	4	266	74	35	45	74	140	244	107
180	74	81	211	66	143	249	248	212	233
39	2	247	30	247	98	168	183	273	4
189	17	105	7	66	167	83	234	247	166
34	124	15	43	259	107	47	229	36	136
201	101	159	167	208	47	30	131	259	31
243	211	44	243	41	256	38	78	95	237
247	75	254	147	225	174	69	118	2	212
73	232	128	83	45	254	123	76	121	2
35	134	268	100	6	104	138	56	36	26

Matrix 9. Red and Green Intensity for PT norm of the ideal image

PT =

-1.5263 -0.9235 -0.6295 0.5579

RedPTNorm =

11	28	32	21	21	9	29	14	23	26
21	12	51	34	43	16	9	58	36	40
19	110	24	23	11	16	111	55	29	7
11	79	34	28	130	16	105	28	21	136
9	80	31	16	94	7	112	47	21	107
16	46	21	12	60	24	38	36	21	93
20	12	36	14	96	23	11	43	16	118
28	17	12	19	137	46	26	16	17	145
16	75	36	46	56	16	116	54	61	58
89	49	24	34	43	65	43	40	49	38

GrenPTNorm =

19	31	73	24	11	19	26	75	28	6
19	19	24	18	81	17	14	22	17	26
40	103	16	21	19	41	98	12	24	23
36	25	63	22	133	37	24	61	19	133
20	78	74	11	38	22	74	63	11	39
18	38	29	9	72	22	42	28	9	70
58	27	22	13	18	71	21	20	11	18
32	31	11	9	61	37	30	12	18	61
112	28	34	71	82	98	28	32	76	74
150	39	21	75	51	103	35	18	88	54

Matrix 10. Red and Green Intensity for PT norm of real slide image

From the Matrixes and graphs discussed above, it can be observed the global and Lowes are almost similar; Print-tip, an advanced version of them, gave results that was close to Matrix 1 and 2. However, Quantile differed greatly than the correct one and its graphs fluctuate away from the goal. Furthermore, the graphs of real image Print-tip normalization shows the expected result for real slide image in Figure 9 due to the clustering around the straight line when (M = 0). These findings support the view of Smyth that the "print-tip loess normalization provides a well-tested general purpose normalization method which gives good results on a wide variety of arrays" and best combined with diagnostic plots of the data. When the diagnostic plots show that biases still remain in the data after normalization, additional steps such as quantile normalization between the arrays may be undertaken (Smyth et al., 2003).

CONCLUSION

In this paper, normalization is defined as a process to delete systematic error. Four most commonly used normalization algorithms such as Global, Lowess, Quantile and Print-tip were tested and compared to find the most suitable approach in a general normalization process. For that purpose, a Matlab code was built for each method for two slides; the ideal and real microarray slides. The results shown in the form of Matrix of red and green intensities and M-A graph show that Global, Lowess and Print-tip are more accurate in comparison with an ideal image result while Print-tip has the advantages than the other two especially in term of final graph shape.

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