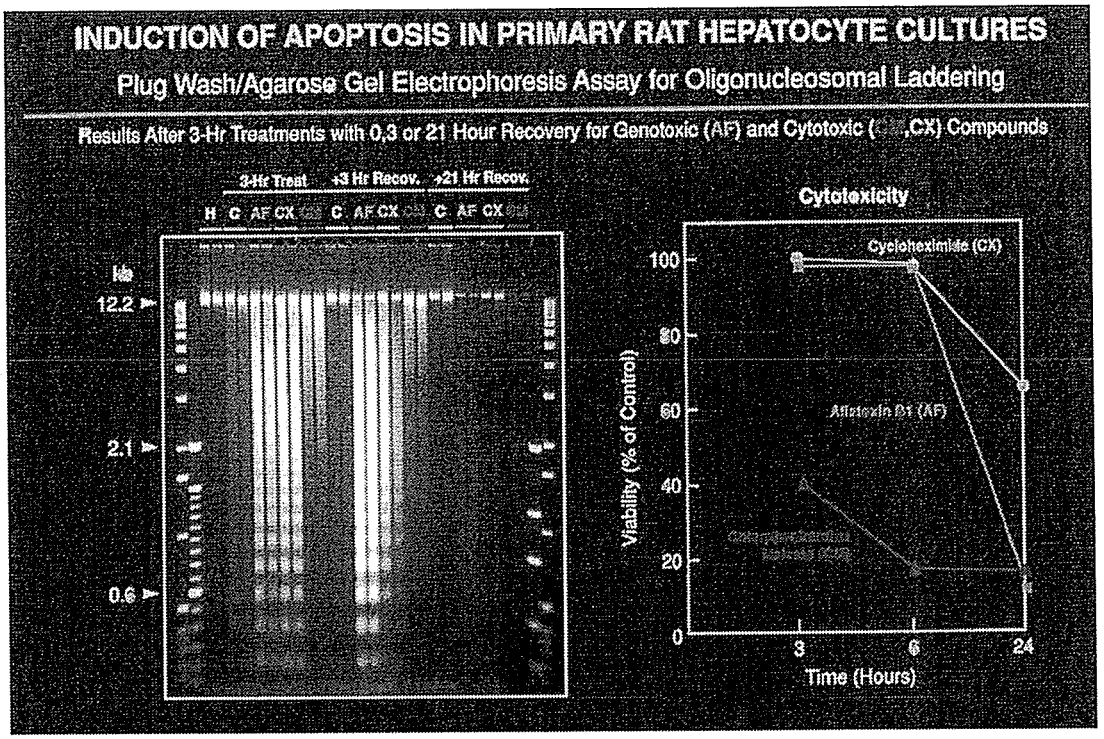
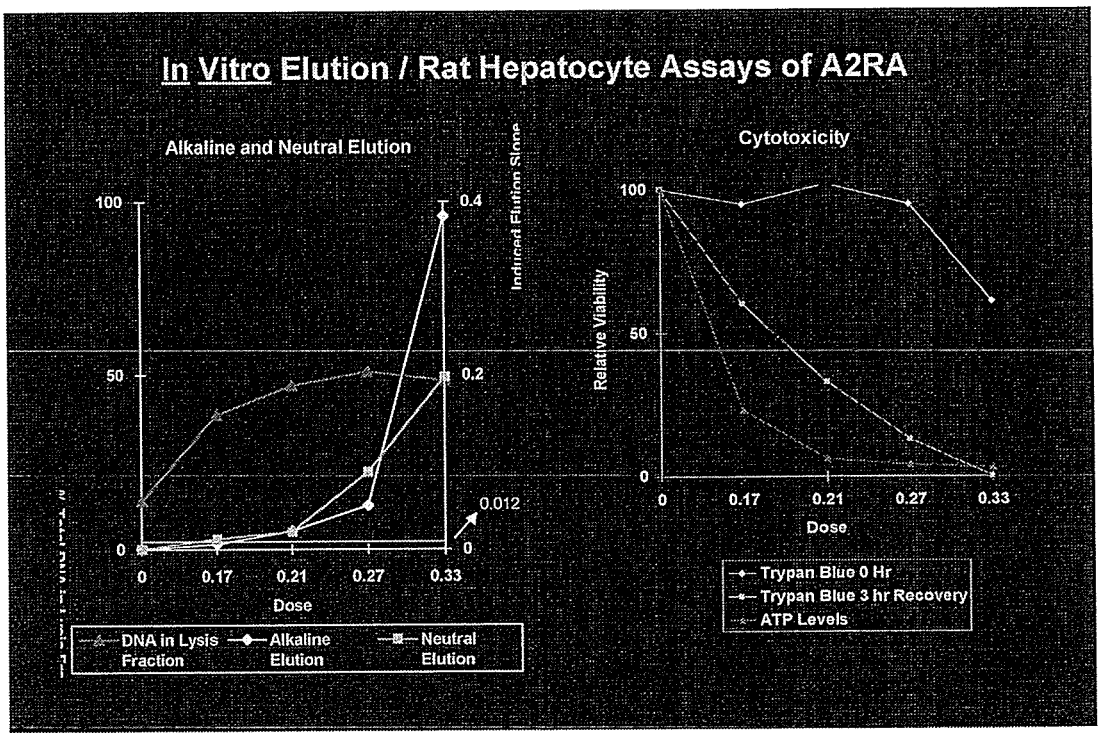
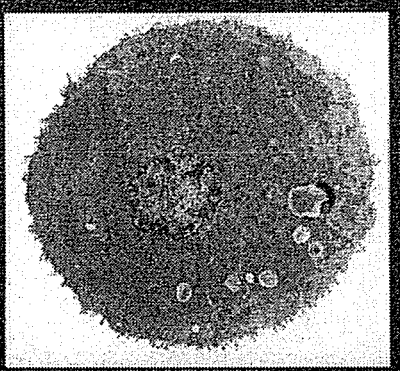
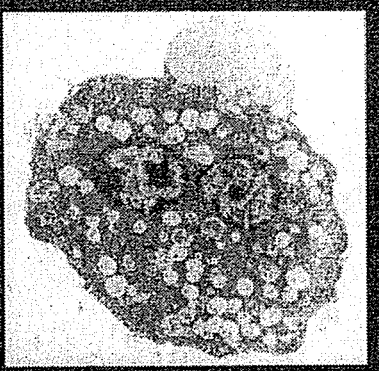

INDUCTION OF APOPTOSIS IN PRIMARY RAT HEPATOCYTE CULTURES
Plug Wash/Agarose Gel Electrophoresis Assay for Oligonucleosomal Laddering
Results After 3-Hr Treatments with 0,3 or 21 Hour Recovery for Genotoxic (AF) and Cytotoxic (CX) Compounds
3-Hr Treat
+3 Hr Recov.
+21 Hr Recov.
H C AF CX C AF CX C AF CX
kb
12.2
2.1
0.6
Cytotoxicity
Cycloheximide (CX)
Aflatoxin B1 (AF)
Viability (% of Control)
100
80
60
40
20
0
3
6
24
Time (Hours)


In Vitro Elution / Rat Hepatocyte Assays of A2RA
Alkaline and Neutral Elution
Inferred Elution Slope
100
50
0
0.4
0.2
0.012
0
0
0.17
0.21
0.27
0.33
Dose
DNA in Lysis Fraction
Alkaline Elution
Neutral Elution
Cytotoxicity
Relative Viability
100
50
0
0
0.17
0.21
0.27
0.33
Dose
Trypan Blue 0 Hr
Trypan Blue 3 hr Recovery
ATP Levels

ELECTRON MICROSCOPY OF CONTROL AND A2RA-TREATED HEPATOCYTES



Control (1% DMSO)
3 hr. Treatment



A2RA (0.29 mM)
3 hr. Treatment

Summary: Comparison of Alkaline Elution and Comet Assays

- The comet assay can provide more sensitive detection of primary DNA damage
- The assay is simpler and more widely adopted for testing and research
- Methods to recognize and quantitate DNA fragmentation in dead and dying cells (cytotoxicity associated) not fully developed.
  - overall accuracy of the assay as an indicator of genotoxic potential will benefit from development of methods to discriminate treatment-induced DNA damage in live cells from DNA fragmentation in dead and dying cells
- Alkaline elution assays have potential for high-throughput with acceptable sensitivity and minimal potential for false positives
- As with sensitive DNA adduct detection technologies, we must grapple with the question of both hazard identification and quantitative risk assessment
  - can an assay be too sensitive (eg. "good" comet vs "bad" comets, Hartman, 1999)
  - are we able to measure changes equivalent to fluctuations in background frequency of endogenous DNA damage?
  - as with DNA adduct detection methodologies, quantitative risk assessment may be problematic

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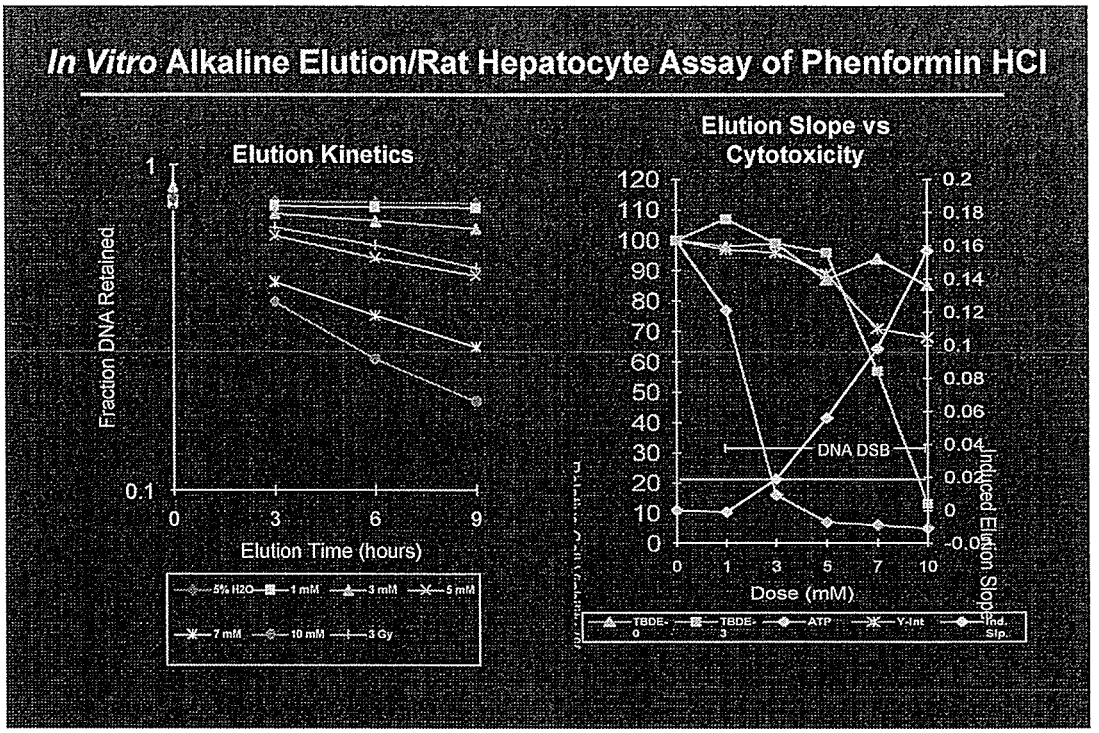
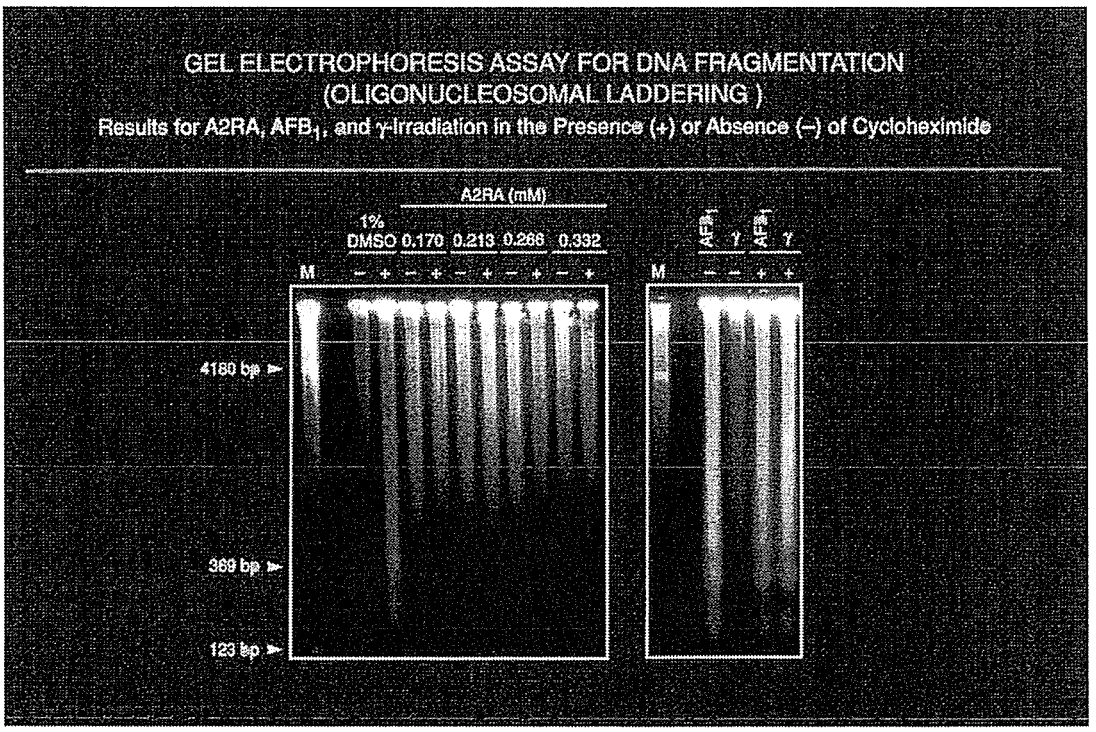
Mike Elia
Matt Bradley
Warren Nichols

John DeLuca
Sheila Galloway

Differentiating Genotoxic, Necrotic, and Apoptotic Responses in the *In Vitro* Alkaline Elution Assay in Rat Hepatocytes

- Elia et al., Mutation Research 291: 193-205, 1993
- Elia et al., Environ. Molec. Mutagen 24: 181-191, 1994
- Storer et al., Mutation Research 368: 59-101, 1996


In Vitro Alkaline Elution/Rat Hepatocyte Assay of Phenformin HCl
Elution Kinetics
Fraction DNA Retained
1
0.1
0
3
6
9
Elution Time (hours)
5% H2O
1 mM
3 mM
5 mM
7 mM
10 mM
3 Gy
Elution Slope vs Cytotoxicity
120
110
100
90
80
70
60
50
40
30
20
10
0
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
-0.02
0
1
3
5
7
10
Dose (mM)
DNA DSB
Induced Elution Slope
TBDE-0
TBDE-3
ATP
Y-Int
Ind. Slp.


GEL ELECTROPHORESIS ASSAY FOR DNA FRAGMENTATION
(OLIGONUCLEOSOMAL LADDERING)
Results for A2RA, AFB1, and γ-Irradiation in the Presence (+) or Absence (–) of Cycloheximide
A2RA (mM)
1% DMSO
0.170
0.213
0.266
0.332
M
AFB1
γ
4180 bp
369 bp
123 bp

JaCVAM/MMS seminor
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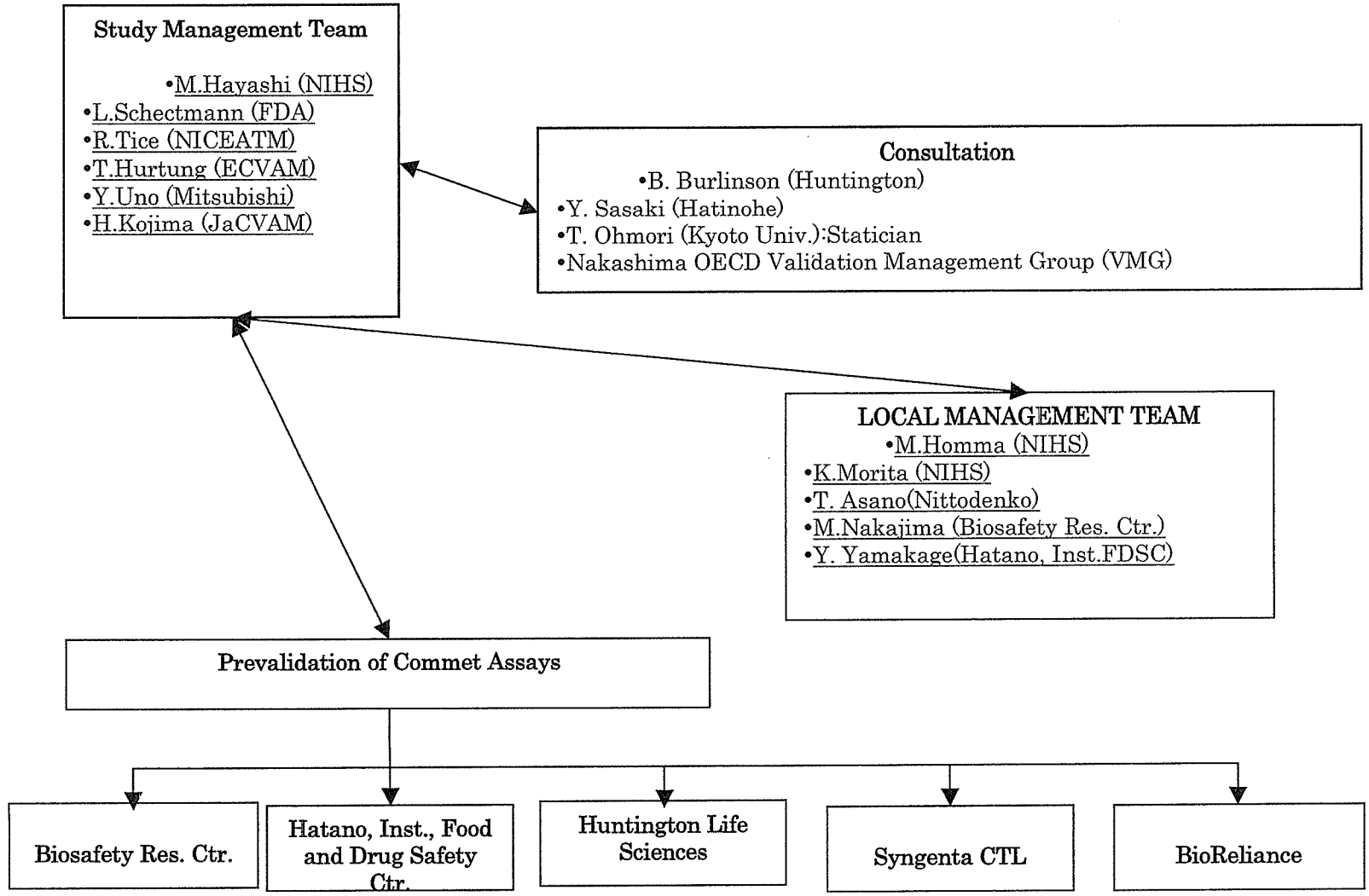
27		Leonard M. Schechtman	Food and Drug Administration
28		Raymond R. Tice	National Institute of Environmental Health Sciences
29		Brian Burlinson	Huntingdon Life Sciences
30		Patricia Escobar	BioReliance,invitrogen bioservices
31		Phil Clay	Syngenta CTL
32		Thomas Hartung	ECVAM

DRAFT INTERNATIONAL VALIDATION STUDY MANAGEMENT CHART



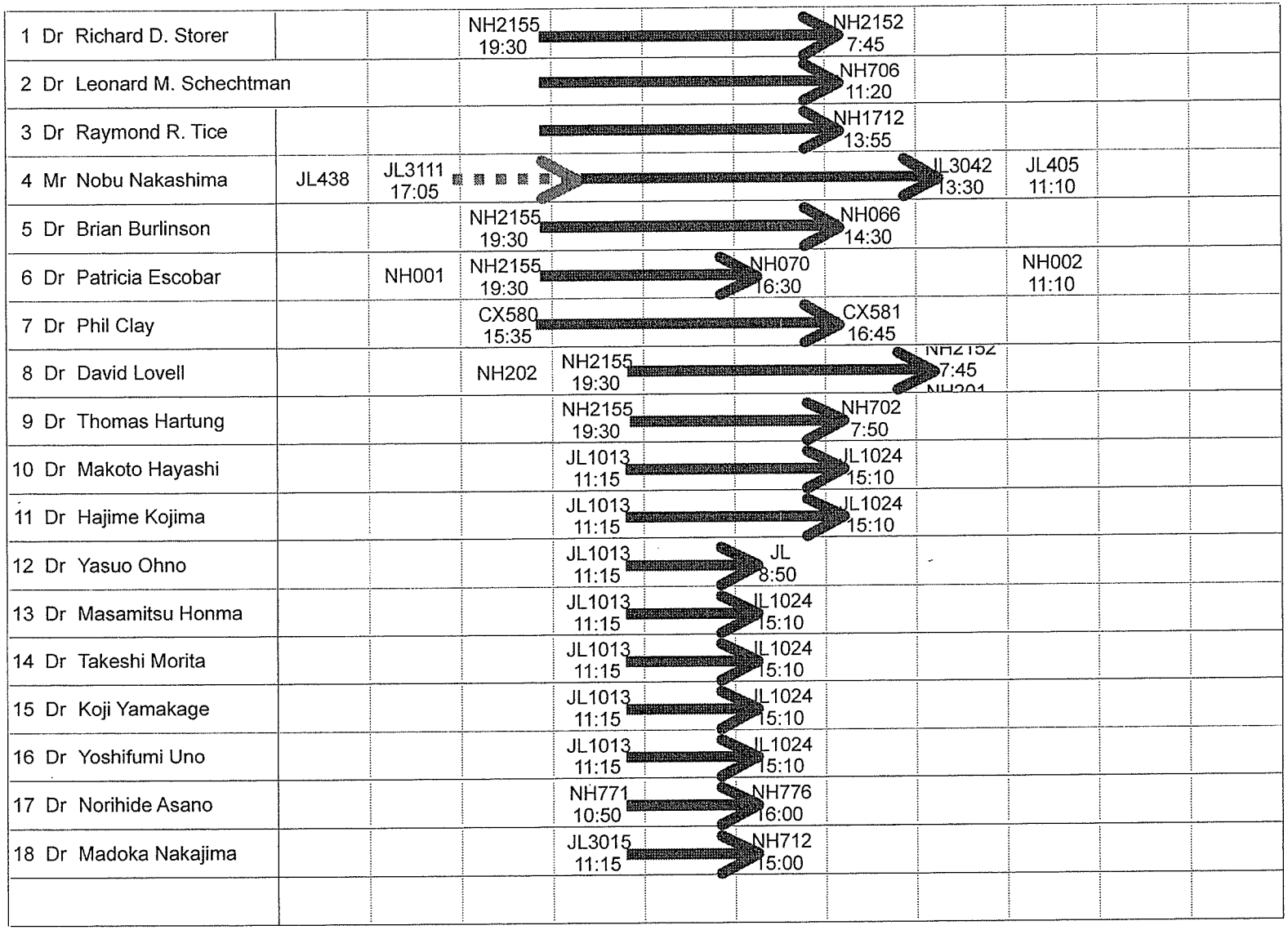
Comet Assay Meeting in August 2006

Name	Domestic Flight to Sapporo Flight Schedule						International Flight Flight Schedule						Accommodation Royton Sapporo		
	Arrival			Departure			Arrival			Departure			Check-in	Check-out	of Nights
1 Dr Richard D. Storer	NH2155	Aug-12	19:30	NH2152	Aug-16	7:45							Aug-12	Aug-16	4
2 Dr Leonard M. Schechtman		Aug-12		NH706	Aug-16	11:20							Aug-12	Aug-16	4
3 Dr Raymond R. Tice		Aug-12		NH1712	Aug-16	11:50							Aug-12	Aug-16	4
4 Mr Nobu Nakashima	JL3111	Aug-11	17:05	JL3042	Aug-17	13:30	JL438	Aug-11	13:05	JL405	Aug-18	11:10	Aug-13	Aug-17	4
5 Dr Brian Burlinson	NH2155	Aug-12	19:30	NH066	Aug-16	14:30	VS900	Aug-12	9:00	VS901	Aug-17	11:00	Aug-12	Aug-16	4
6 Dr Patricia Escobar	NH2155	Aug-12	19:30	NH070	Aug-15	16:30	NH001	Aug-12	15:20	NH002	Aug-18	11:10	Aug-12	Aug-15	3
7 Dr Phil Clay	CX580	Aug-12	15:35	CX581	Aug-16	16:45	direct flight from Hong Kong to Sapporo						Aug-12	Aug-16	4
8 Dr David Lovell	NH2155	Aug-13	19:30	NH2152	Aug-17	7:45	NH202	Aug-13	15:10	NH201	Aug-17	11:40	Aug-13	Aug-17	4
9 Dr Thomas Hartung	NH2155	Aug-13	19:30	NH702	Aug-16	7:50							Aug-13	Aug-16	3
10 Dr Makoto Hayashi	JL1013	Aug-13	11:15	JL1024	Aug-16	15:10							Aug-13	Aug-16	3
11 Dr Hajime Kojima	JL1013	Aug-13	11:15	JL1024	Aug-16	15:10							Aug-13	Aug-16	3
12 Dr Yasuo Ohno	JL1013	Aug-13	11:15	JL1024	Aug-15	15:10							Aug-13	Aug-15	2
13 Dr Masamitsu Honma	JL1013	Aug-13	11:15	JL1024	Aug-15	15:10							Aug-13	Aug-15	2
14 Dr Takeshi Morita	JL1013	Aug-13	11:15	JL1024	Aug-15	15:10							Aug-13	Aug-15	2
15 Dr Koji Yamakage	JL1013	Aug-13	11:15	JL1024	Aug-15	15:10							Aug-13	Aug-15	2
16 Dr Yoshifumi Uno	JL1013	Aug-13	11:15	JL1024	Aug-15	15:10							Aug-13	Aug-15	2
17 Dr Norihide Asano	NH771	Aug-13	10:50	NH776	Aug-15	16:00							Aug-13	Aug-15	2
18 Dr Madoka Nakajima	JL3105	Aug-13	11:15	NH712	Aug-15	15:00							Aug-13	Aug-15	2

105

Name	Aug-10 Thu	Aug-11 Fri	Aug-12 Sat	Aug-13 Sun	Aug-14 Mon	Aug-15 Tue	Aug-16 Wed	Aug-17 Thu	Aug-18 Fri	Aug-19 Sat	Aug-20 Sun

Comet Assay Meeting in August 2006



Comet Assay Meeting in December 2006

Name	Affliation	Department
1 Dr Richard D. Storer	Merck Research Laboratories	Safety Assessment, Dept. of Laboratory Sciences and Investigative Toxicology
2 Dr Leonard M. Schechtman	Food and Drug Administration	National Center for Toxicological search, Washington Operations, HFT-
3 Dr Raymond R. Tice	National Institute of Environmental Health Sc	National Toxicology Program teragency Center for the Evaluation of native Toxicological Methods
4 Mr Nobu Nakashima	Organisation for Economic Co-operation and	Environmental Health and Safety vision, Environment Directorate
5 Dr Brian Burlinson	Huntingdon Life Sciences	
6 Dr Patricia Escobar	BioReliance,invitrogen bioservices	
7 Dr Thomas Hartung	ECVAM	Institute for Health and Consumer
8 Dr Makoto Hayashi	National Institute of Health Sciences	Division of Genetics and Mutagenesis
9 Dr Hajime Kojima	National Institute of Health Sciences	Div. of Pharmacology, Biological Safety search Center,
10 Dr Yasuo Ohno	National Institute of Health Sciences	
11 Dr Masamitsu Honma	National Institute of Health Sciences	Division of Genetics and Mutagenesis, ammalian Mutagenesis Section
12 Dr Koji Yamakage	Food and Drug Safety Center	Hatano Research Institute,
13 Dr Yoshifumi Uno	Mitsubishi Pharma Corporation	Development Division
14 Dr Norihide Asano	Nitto Denko Corporation	Toxicological Research Center
15 Dr Madoka Nakajima	Biosafety Research Center, Foods, Drugs an	An-pyo Center
16 Dr Maya Ueda	Food and Drug Safety Center	Hatano Research Institute,

コメットアッセイ用画像解析ソフトによる結果のばらつきの検討

前回のリードラボ(3機関)の予備検討の際には、分析時のばらつきを抑えるために、全ての機関で作製した標本を、安評センターで観察・分析した。

今後の多機関バリデーションにおいて、画像解析ソフトウェアの差異が、どれほど影響しうるか調べる。

ソフトウェアの良し悪しを比較するものではない。

計測方法

- 陰性と陽性のコメットの画像ファイルを配布し、各機関で計測を行い、計測結果を世話人へ提出して頂く。
- 計測するコメットの数は、1個体の分析を想定して陰性・陽性それぞれ100個とする (Hartmann et al., 2003, Mutagenesis vol.18, pp45-51では、最低で100～150細胞/個体)。
- 計測指標は、テールに含まれるDNA量、Tail moment、Tail lengthとする(Hartmann et al., 2003, Mutagenesis vol.18, pp45-51)。

配布する画像について

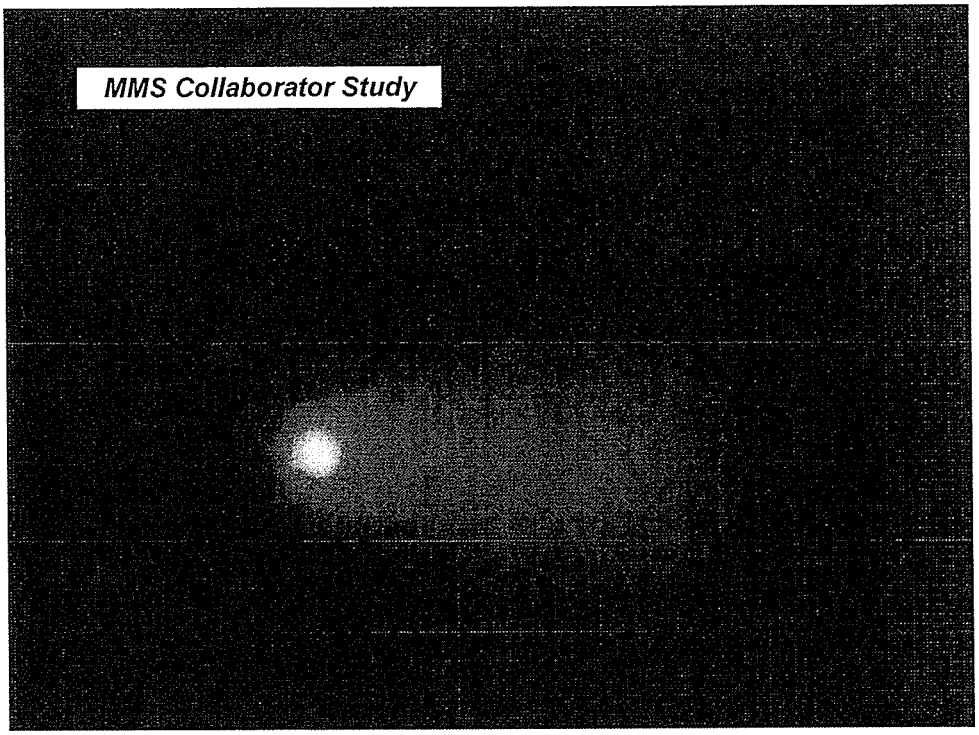
- 1枚の画像には、1細胞の画像のみが含まれるようにする。
- 画像形式の変換や、減色によって生じる像の変化をなるべく抑えるため、カラーおよびモノクロ画像(8bit～24bit)の両方を、代表的な画像形式(BMP, TIFF, JPGなど)に変換したものを配布する。それぞれの画像解析ソフトに最適な形式を一つ選び、インポートして分析する。

評価方法

1)解析に使用したソフト名およびバージョン
2)解析に使用した画像(ファイル形式)
3)各画像の%DNA in tailの測定値
4)各画像のTail momentの測定値
5)各画像のTail lengthの測定値

4)～6)の形式はエクセルやテキストファイルなど。

提出された各機関のデータについて、機関ごとに平均値を求め、上下何%ぐらいの幅に収まるか調べる。



Participants

1. Food and Drug Safety Center
2. Tanabe Seiyaku Co., Ltd.
3 The Institute of Environmental Toxicology
4. Fuji Biomedix Co., Ltd.
5.Toray Industries, Inc.
6. Canon Inc.
7. Invitrogen Co.
8.Yakult Central Institute for Microbiological Research

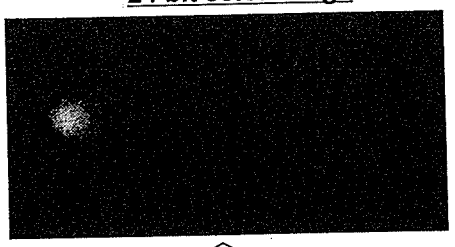
Image Analyzer used in this study

- Komet 4.0.4 & 5.5 (Kinetic imaging Ltd)
- CometAnalyzer Ver. 1.1.1 & 1.5 (Youworks)
- Cometscore Ver. 1.5 (Tri Tek)
- Comet Imager Ver. 1.20 (Carl Zeiss)
- Comet 4.1.1 (Perceptive Instruments)

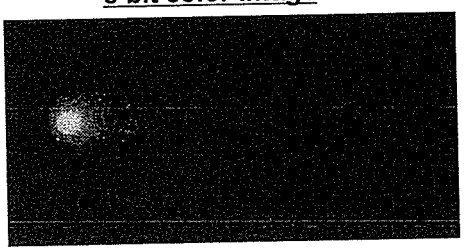
Preparation of comet images

- CHL/IU cells
  - Negative control: DMSO
  - Positive control:4-Nitroqunoline 1-oxide (4NQO)
- Stainied with ethidium bromide after electrophoresis
- Comet images were taken with a CCD camera
- Saved as 24 bit color images with BMP format
- Converted 8 bit gray and color images with 3 formats (BMP, TIF, JPG)
- Distributed 100 images
  - Negative control: 50 images
  - Positive control: 50 images
- Tail length, %DNA in tail and Morment were analysed in each image

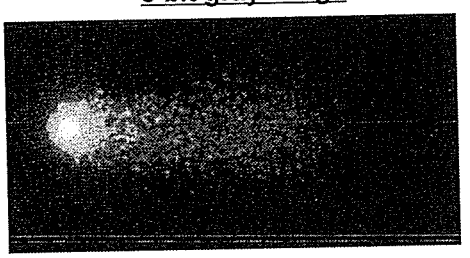
Original image (inported image in computer with a CCD camera)
24 bit color image



8 bit color image

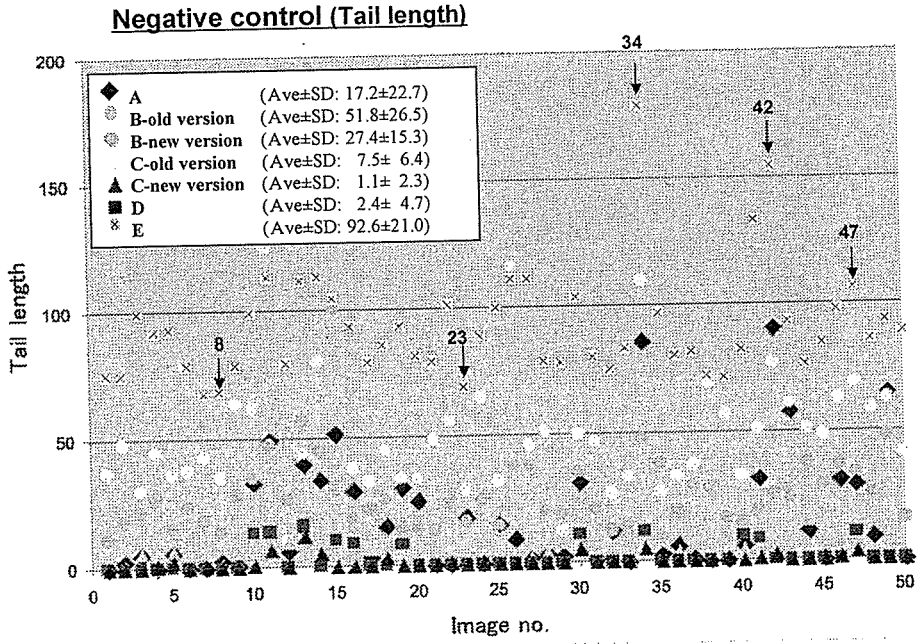


8 bit gray image



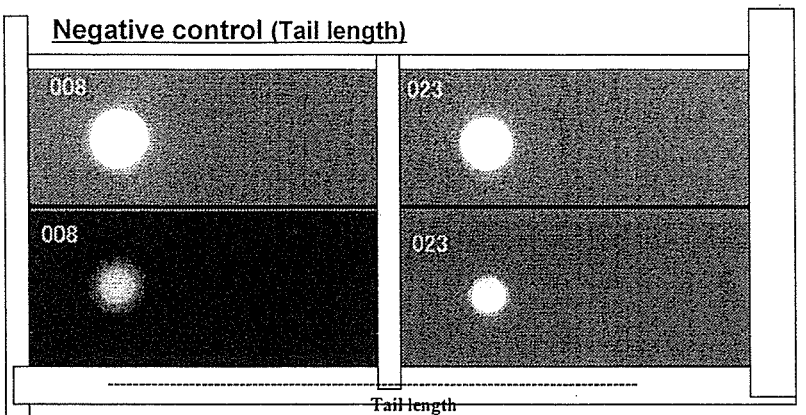
MMS/JacVAM Joint Seminar, K. Yamakage, 2006

Negative control (Tail length)



MMS/JacVAM Joint Seminar, K. Yamakage, 2006

Negative control (Tail length)

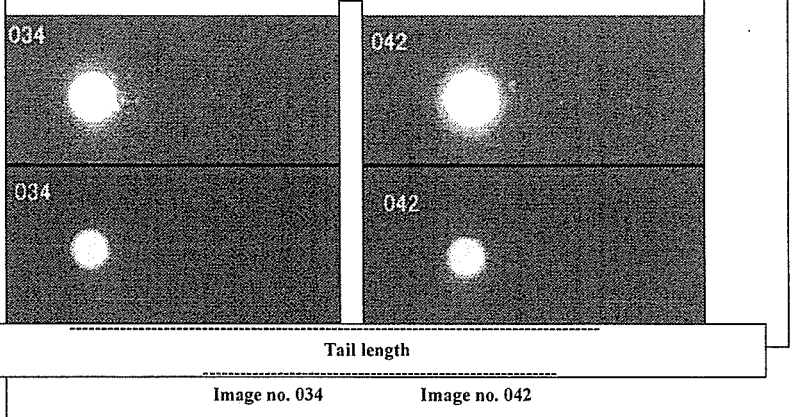


Tail length

	Image no. 008	Image no. 023
A	2.55	18.52
B-old version	35.00	29.00
B-new version	14.07	16.63
C-old version	0.64	1.91
C-new version	0.00	0.00
D	0.00	0.00
E	68.62	69.55

MMS/JacVAM Joint Seminar, K. Yamakage, 2006

Negative control (Tail length)

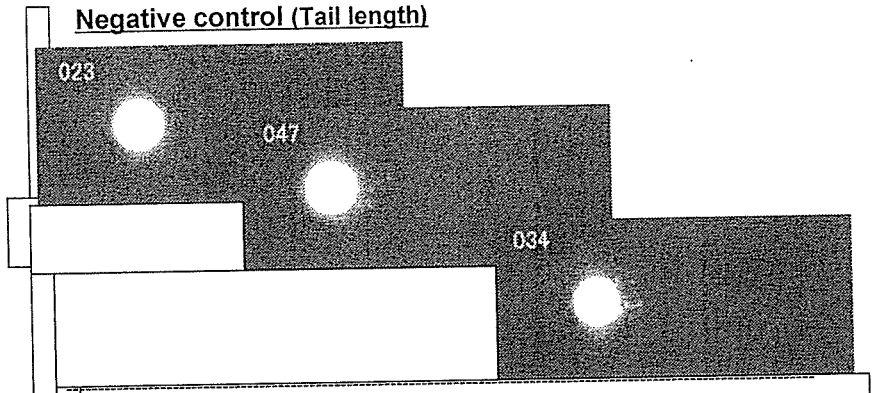


Tail length

	Image no. 034	Image no. 042
A	86.21	90.68
B-old version	110.00	77.00
B-new version	47.97	25.59
C-old version	5.11	1.91
C-new version	5.12	2.56
D	11.69	0.00
E	178.98	154.87

MMS/JacVAM Joint Seminar, K. Yamakage, 2006

Negative control (Tail length)

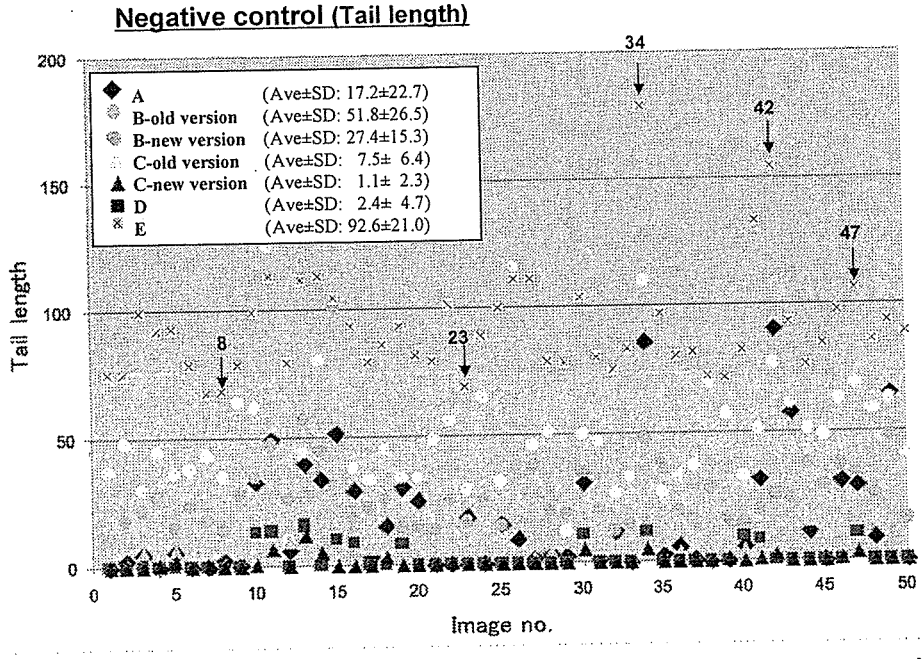


Tail length

	Image no. 023	Image no. 047	Image no. 034
A	18.52	29.37	86.21
B-old version	29.00	69.00	110.00
B-new version	16.63	33.90	47.97
C-old version	1.91	22.98	5.11
C-new version	0.00	3.20	5.12
D	0.00	10.31	11.69
E	69.55	106.64	178.98

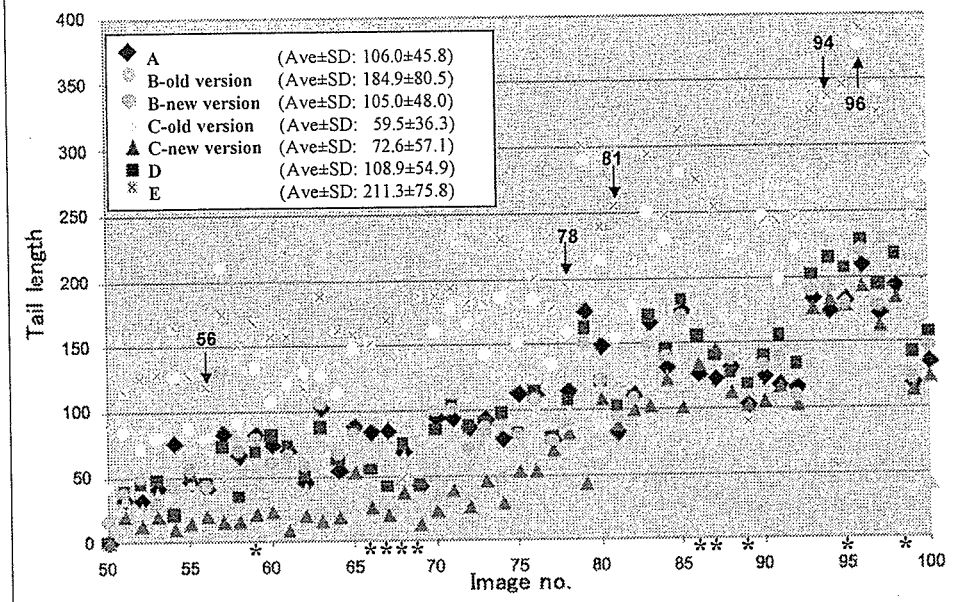
MMS/JacVAM Joint Seminar, K. Yamakage, 2006

Negative control (Tail length)



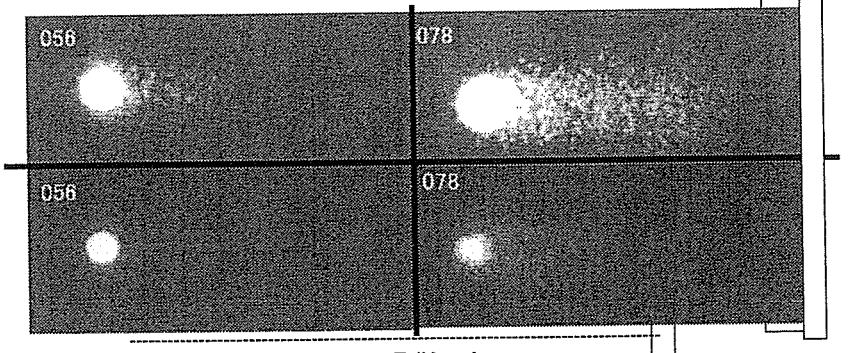
MMS/JacVAM Joint Seminar, K. Yamakage, 2006

Positive control (Tail length)



MMS/JacVAM Joint Seminar, K. Yamakage, 2006

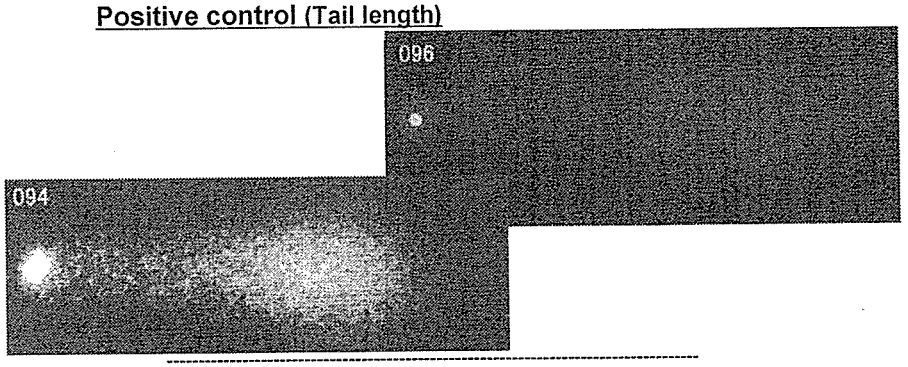
Positive control (Tail length)



	Tail length	
	Image no. 056	Image no. 078
A	42.15	114.30
B-old version	79.00	159.00
B-new version	42.86	88.27
C-old version	36.38	98.94
C-new version	21.12	81.82
D	44.69	107.00
E	118.70	119.74

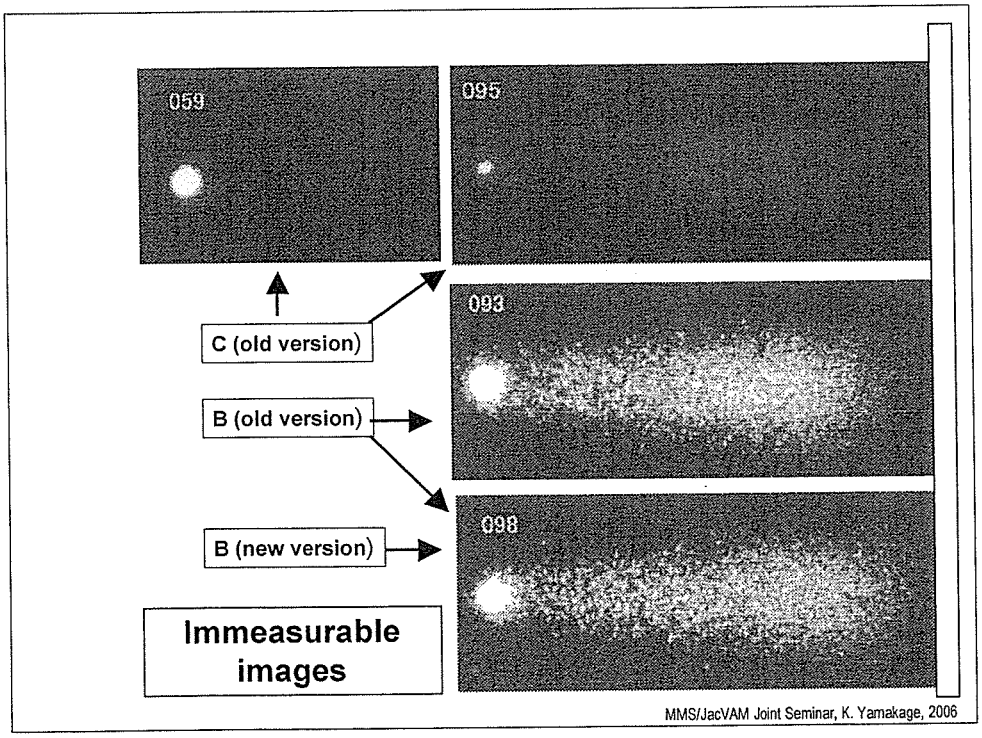
MMS/JacVAM Joint Seminar, K. Yamakage, 2006

Positive control (Tail length)

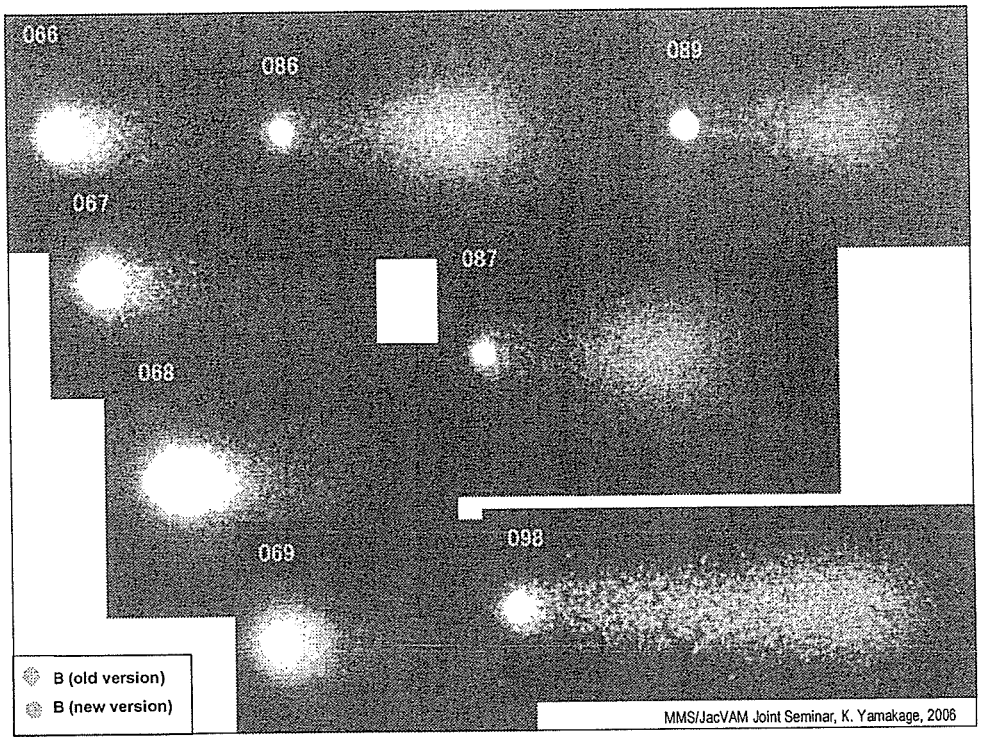


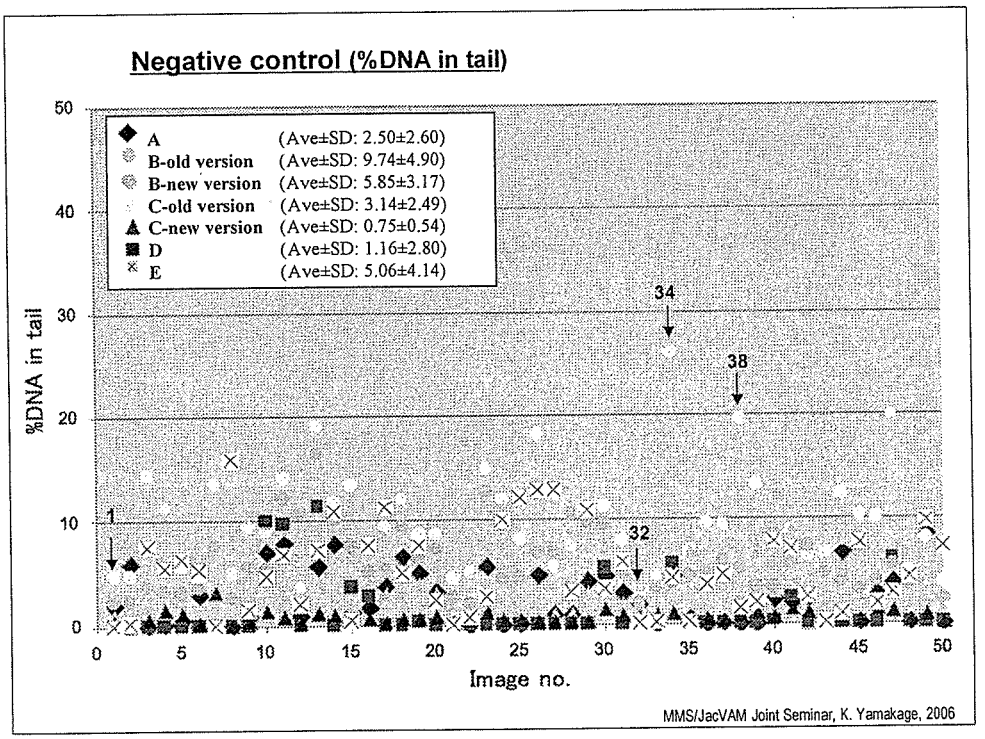
	Tail length	
	Image no. 094	Image no. 096
A	174.33	209.45
B-old version	335.00	376.00
B-new version	191.26	220.04
C-old version	38.30	44.68
C-new version	183.68	193.92
D	214.00	228.00
E	337.55	389.49

MMS/JacVAM Joint Seminar, K. Yamakage, 2006



MMS/JacVAM Joint Seminar, K. Yamakage, 2006


066
086
089
067
087
068
069
098
B (old version)
B (new version)
MMS/JacVAM Joint Seminar, K. Yamakage, 2006


Negative control (%DNA in tail)
A (Ave±SD: 2.50±2.60)
B-old version (Ave±SD: 9.74±4.90)
B-new version (Ave±SD: 5.85±3.17)
C-old version (Ave±SD: 3.14±2.49)
C-new version (Ave±SD: 0.75±0.54)
D (Ave±SD: 1.16±2.80)
E (Ave±SD: 5.06±4.14)
%DNA in tail
Image no.
34
38
1
32
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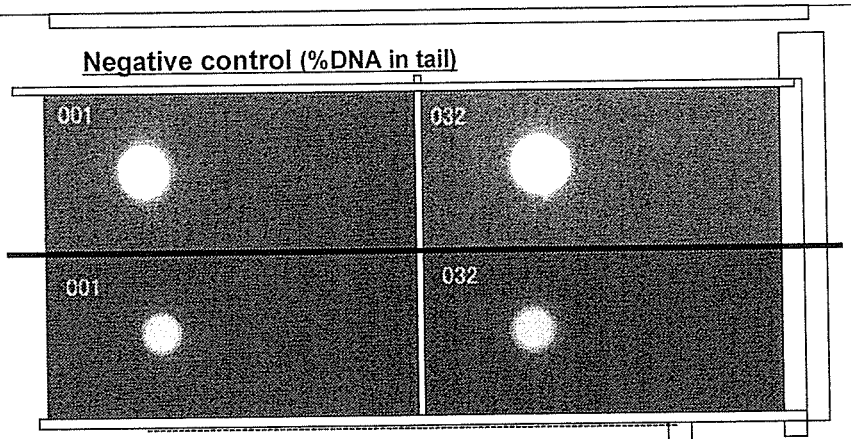
Negative control (%DNA in tail)



%DNA in tail

	Image no. 001	Image no. 032
A	2.01	1.89
B-old version	4.81	2.37
B-new version	2.26	2.05
C-old version	1.00	2.00
C-new version	0.55	0.57
D	0.00	0.00
E	0.01	0.13

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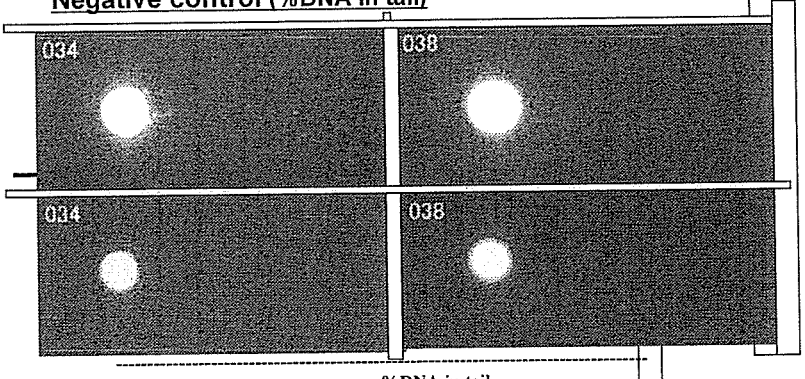
Negative control (%DNA in tail)



%DNA in tail

	Image no. 001	Image no. 032
A	4.75	0.00
B-old version	25.98	19.62
B-new version	3.88	5.31
C-old version	2.00	3.00
C-new version	0.98	0.90
D	5.75	0.00
E	4.45	1.43

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