"Early Detection is Crucial in Preventing and Treating Cancer"

American Cancer Society®

BC Cancer Agency
CARE + RESEARCH

LIFE SCANNERS

Motic
More Than Microscopy

Routine CERViscan saved my wife's life. I am thankful to our doctor and British Columbia Cancer Agency.

Ravindra S.

My Story

I had lower back ache which I thought was because of bad posture. When it didn't heal, I visited my doctor but initial treatment didn't help at all.

My husband was very worried about my health. We then consulted our gynecologist and learned that lower back pain can be one of the confusing symptoms of cervical cancer.

He then convinced me at the advice of our doctor for me to get scanned.

I was shocked when early signs of cervical cancer were detected.

My life changed that day.

With God's grace my condition was detected in pre-cancer stage and doctor was able to treat me in one day.

Had I not scanned for cervical cancer, who knows, if I would be alive to tell my story today.

"

Cervical cancer is caused by HPV which is a very common virus. Every woman should get scanned

Sarika. S.

CERViscan
Saved My Life

It's our duty to prevent our mothers, sisters and daughters from this preventable cancer which can happen to anyone.

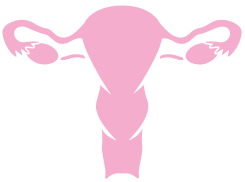
CERVICAL CANCER IS A SILENT KILLER

Usually it Has No Symptoms or They Might Be Confusing

Abnormal Bleeding

Back & Pelvic Pain

Pain During Sex



Vaginal Discharge

Belly Bloat

Constant Fatigue

Unexplained Weight Loss

The Good News

Routine screening can prevent cervical cancer

NO CUT
NO SYRINGE
NO PAIN

Soft brush sampling

Early detection can save your life

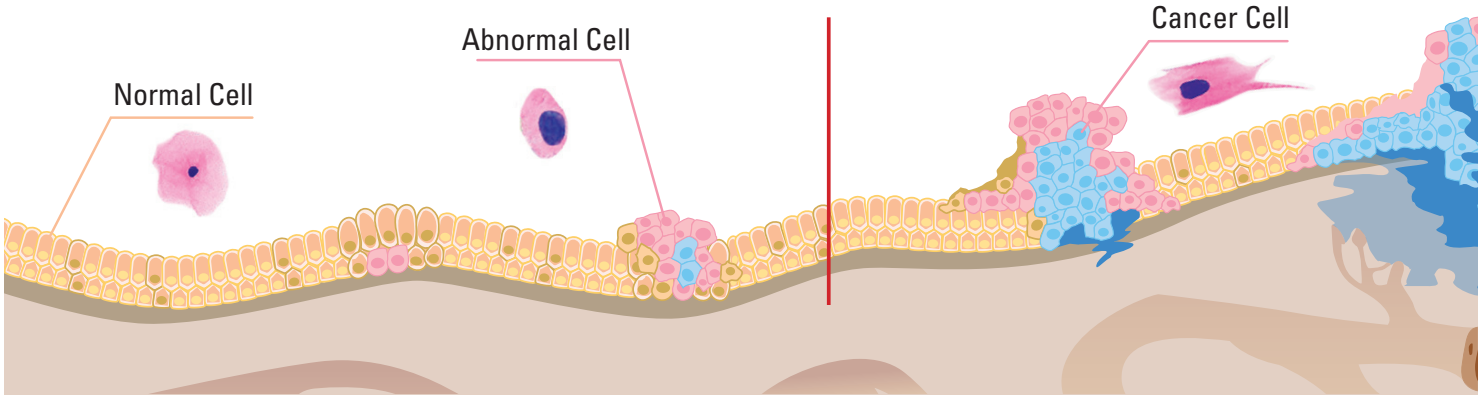
SOFT BRUSH TEST CAN SAVE YOUR LIFE

CERViscan is the world's most advanced and accurate early cancer detection test which can detect potential cancer **3-5 years before any symptoms.**

WHEN DETECTED EARLY
MORTALITY RATE

CANCER STAGE
MORTALITY RATE

0%	I	II	III	IV
	20% DIE	42% DIE	68% DIE	85% DIE



Early Cancer Detection Saves Lives

People all over the world raise awareness and celebrate benefits of CERViscan which can protect you and your loved ones from needless suffering.

BC Cancer Agency
CARE + RESEARCH

British Colombia Cancer Agency Ranked "The Most Influential Cancer Research Institution in the World."

THOMSON REUTERS

Clarivate Analytics

Motic
More Than Microscopy

- **World Leader in Advanced Microscopy & DNA Ploidy**
- Partners with Bill Gates' Global Good Project
- Red Dot Design Award Winners
- Over 2 Crore DNA Ploidy Tests Done

Early Cancer Detection
Inspire + Empower
To Save Lives

LIFE SCANNERS

CERViscan

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/DNAPloidy

DNAscan®

BEFORE SYMPTOMS

See More, Treat Early
Grow Practice

LIFE SCANNERS

Motic
More Than Microscopy

BC Cancer Agency
CARE + RESEARCH

Oral & Cervical cancer is an epidemic in India

THE TIMES OF INDIA

India's mortality to incidence ratio of 0.68 is much higher than those in rich countries, where the ratio is 0.38. The West's cancer incidence is bigger than India's.

2.5 lakh new Cancer cases in UP

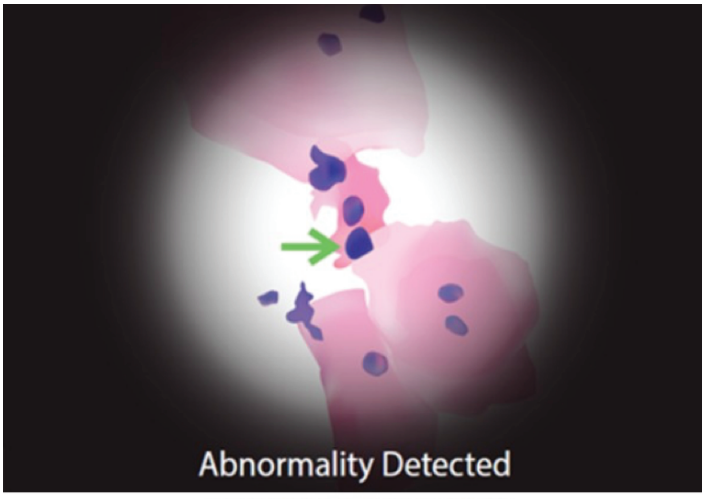
50 % of patients with the condition die within a year

50 cases of Oral Cancer in just **20 days**

According to the Deccan Herald, **the 50% patients suffering from Oral Cancer are in the primary stages that can be cured after receiving the necessary treatment.**

Detect Early & Prevent Recurrence with DNA Ploidy Testing

Regular DNA Ploidy Test for oral cancer survivors is crucial in order to prevent recurrence or detect it at very early stage.



AI Powered Quantitative DNA Analysis

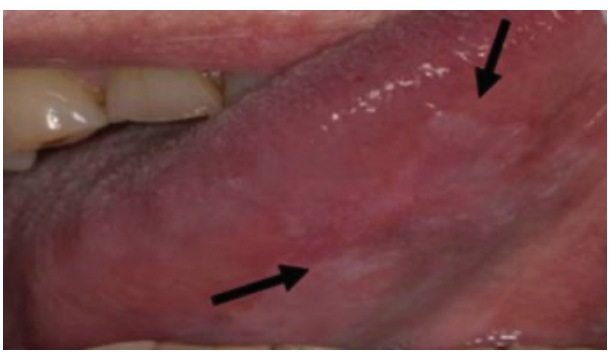
Oral cancer survivor :
My Story

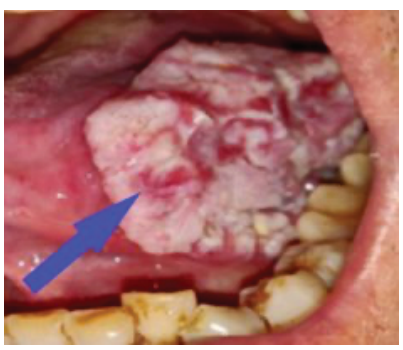
I had a mouth ulcer, it was not healing on its own and was small. My daughter in law is a nurse and was certain it will heal itself, however she bought me over the counter gels. As the gel was not helpful we visited a doctor who gave me gels, medicines & injections, but my situation was not changing.

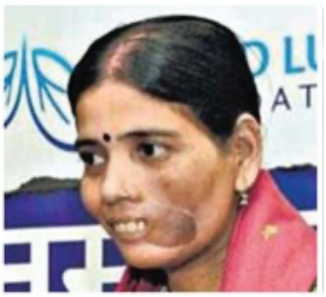
We then went to Dr Kolte, and she advised an O-DNAscan so we can take further decisions. I felt like I am not going to survive anymore, but after the scan, there were few abnormal cells and we removed the ulcer.

I am 74 year old now, confident and have atleast 10 years to live. Thank you O-DNAscan!

Lets STOP precancerous lesions into becoming T4 tumors







27 years old oral cancer victim **Sunita Tomar**

VELscan & DNA Ploidy Testing Protocol

Pre-Surgery

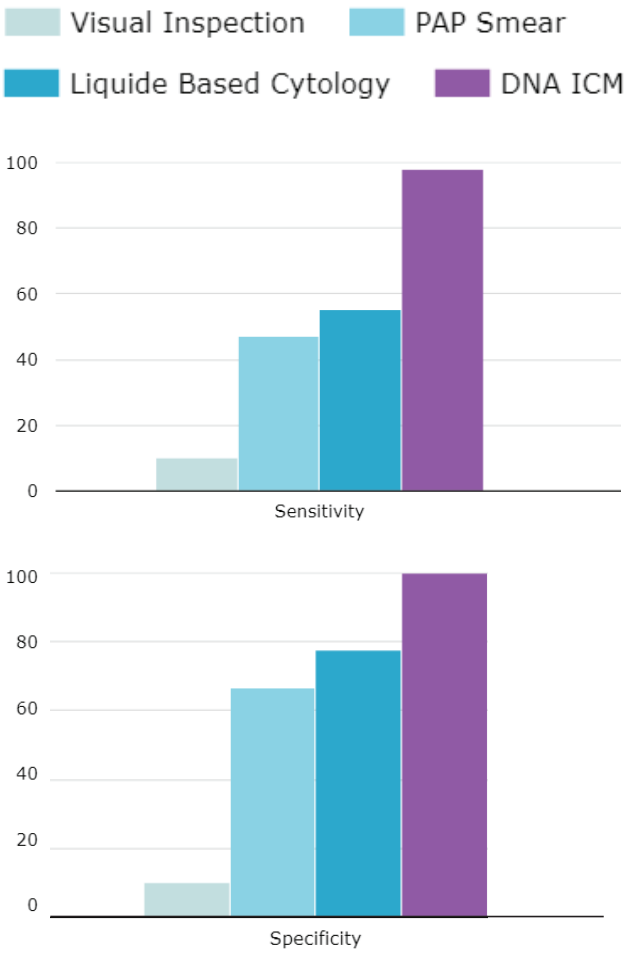
DNA Ploidy test is done to identify if aneuploidy, which is proven biomarker for cancer, is present in suspicious oral tissue.

Post-Surgery

DNA Ploidy test is done to confirm there is no abnormal cells left in a surgical site, once surgical site is healed.

Routine Screening

Routine annual screening conducted to monitor biological cell behavior in order to prevent loco-regional recurrence or detect it very early.

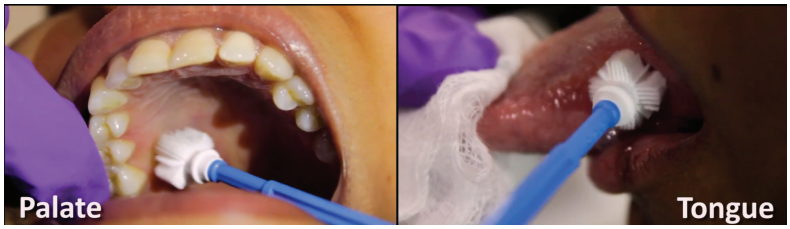


AI-Powered DNA Ploidy Test has the highest accuracy for a non-invasive diagnostics:

Sensitivity 98%
Specificity 100%

How to collect sample?

- Oral sample: 10 rotations and scraping motion using brush for adequate sample collection.
- Cervical Sample: Same as PAP sample collection.



Why current practices struggle?

Late Detection

70%

Reported in Stage 3 & 4

Inaccurate Diagnosis

60%

Discordance

Lack of Post Treatment follow up

40%

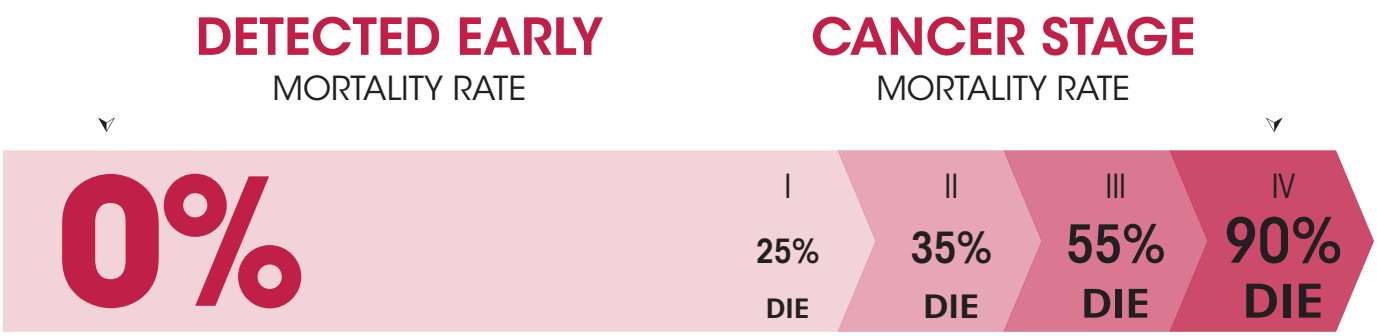
Recurrence rate

Why do DNA Ploidy test FIRST ?

- **98% Sensitivity and 100% Specificity**
- **PAP and LBC PAP test accuracy is low**
- **HPV DNA test does not measure abnormal or cancer cells**
- **Merely prescense of HPV doesn't mean anything, since body's own immune system will clear it in 90%+ of cases**

"Early Detection is Crucial in Preventing and Treating Cancer."

American Cancer Society®



DNA PLOIDY TEST DETECTS ABNORMAL CELLS BEFORE THEY TURN CANCEROUS

Proven Benefits

Aneuploidy is a proven biomarker for cancer for more than 100 years.

Quantified DNA Ploidy Painless & Accurate Diagnosis on a Molecular Level

AI-Powered DNA Ploidy test can detect aneuploidy in suspicious oral cells, **up to 2 years earlier than cytology or histology alone.**

Affordable and Accessible Pathology Imaging and Workflow Solutions from

Motic®

DIGITAL PATHOLOGY

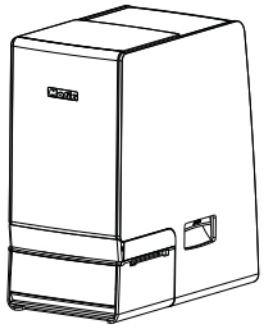
Whole Slide Image Scanning to...

Motic whole slide scanners and image software allow users to:

- Access diagnostic expertise more efficiently through sending scanned slide images
- Obtain second opinion consultations without shipping slides
- Output slide images in multiple open formats for easy access and sharing
- Bundle confidential patient data securely with our proprietary .ds format
- Toggle side-by-side slide display for IHC or reference image use

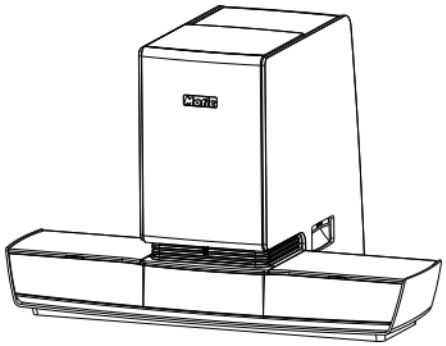
CONTENTS

MOTIC'S LINE OF WHOLE SLIDE IMAGING SCANNERS PROVIDES SOLUTIONS TAILORED FOR A VARIETY OF LAB ENVIRONMENTS:



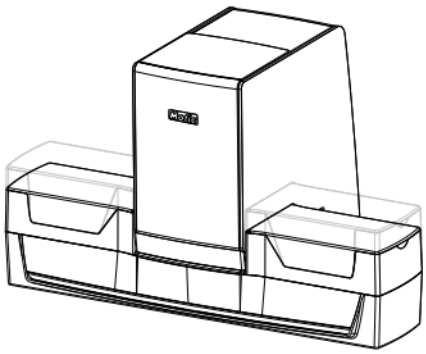
The MoticEasyScan One

Our slimmest and most affordable model, offering 1-slide capacity and a compact footprint that sits discreetly at any workbench. Optional live view mode coupled with efficient scanning makes the One a perfect choice for small, low-throughput labs.



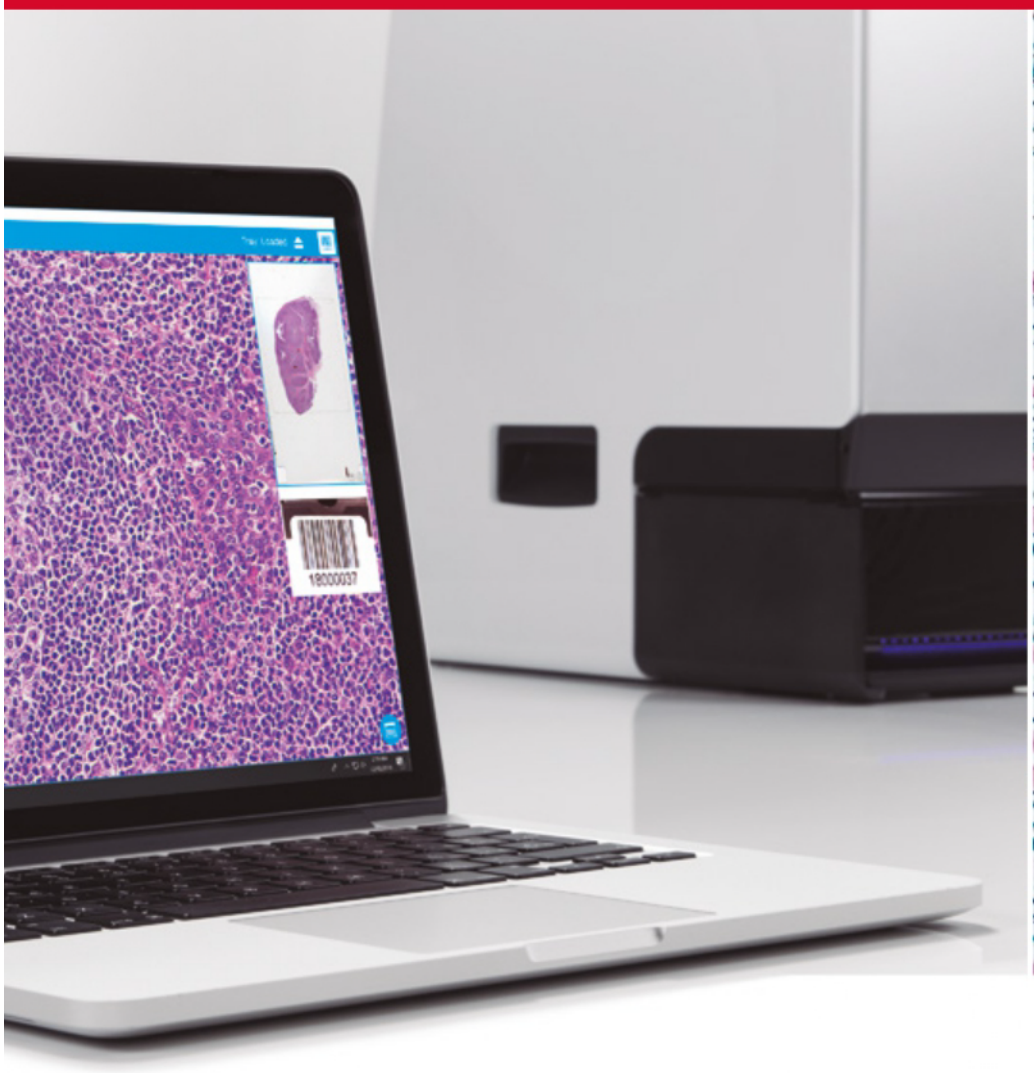
The MoticEasyScan Pro

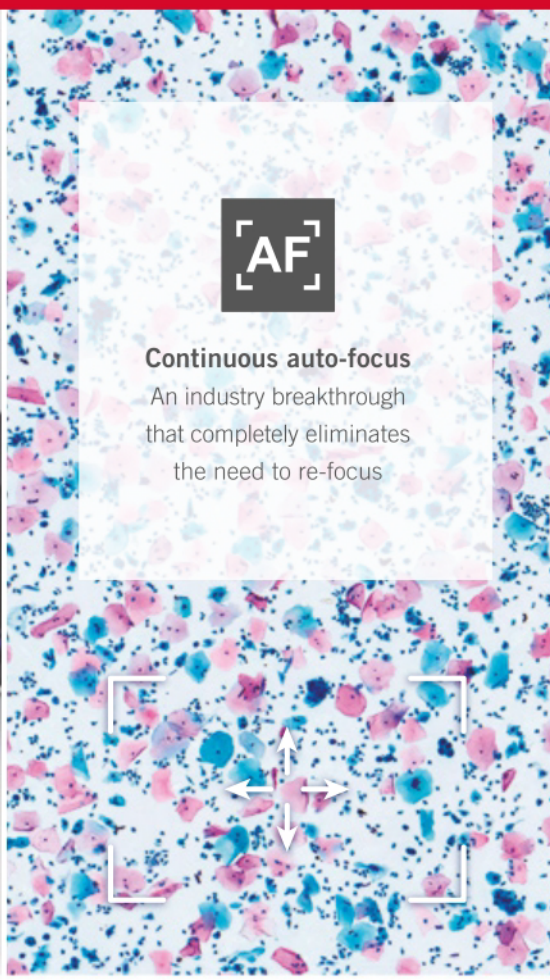
The ideal scanner for midsized clinics and networked labs. The MoticEasyScan Pro features a 6-slide capacity tray and optional live view mode for a robust, all-in-one scanner optimized for teleconsultations and remote frozen sections.



The MoticEasyScan Infinity

Our most powerful scanner, offering uninterrupted, continuous scanning for 60- or 100-slide high-throughput needs. Add slides at any time in the scanning process without disruption. Perfect for research, data acquisition, education, and archival uses.

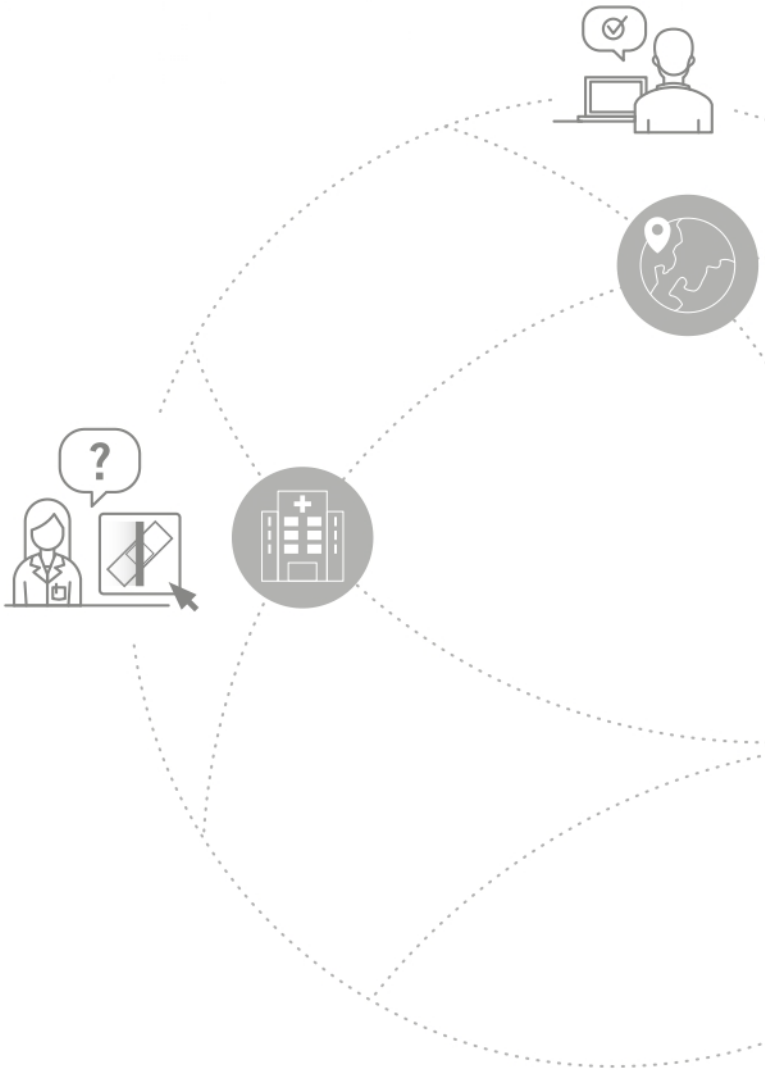




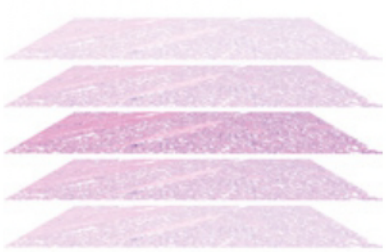
FS-Live

Diagnose from Anywhere

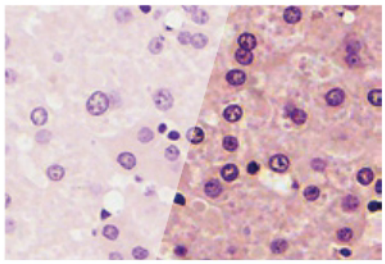
The FS-Live Telepathology System transforms the MoticEasyScan One and Pro scanners into live microscopes that can be piloted remotely offsite. Eliminate travel time, support multiple labs from one location, and keep control entirely in the pathologist's hands.



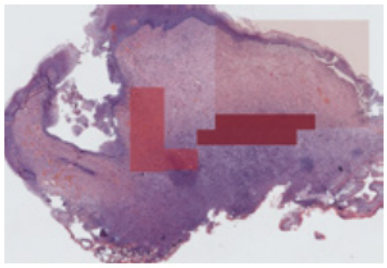
Z-stack snapshot
Capture and compile multiple depths of focus from a single slide into a composite image



Gamma adjustment
Digitally lighten over-stained nuclei to reveal structural details



Macro heatmap
Tracks which areas on the slide have been viewed



2 view modes
Default for pixel-to-pixel resolution, or full view mode for faster scanning

On-screen tools
Place markers, take measurements, and communicate with collaborators, all in the same window

Hotkeys
For an ergonomic, distraction-free workflow

FS-LIVE IS BUILT FOR

- FROZEN SECTIONS
- FNA
- CYTOLOGY
- ROSE
- TUMOR BOARDS

KEY FEATURES:

- Continuous autofocus completely eliminates the need to refocus, no matter the size or thickness of the sample
- Onscreen measurement, add-marker, heatmap, and notepad features put all the necessary tools at the pathologist's fingertips
- 8 different magnification levels
- No need to restain, as contrast adjustment digitally corrects over- or under-staining
- One-click snapshot and Z-stacking to capture and share images without interrupting workflow
- Manually pan through cell layers for cytology applications
- Generates a macro-preview within 2 seconds of tray loading
- Pilotable live view of the slide populates within 5 seconds of tray loading

DSServer

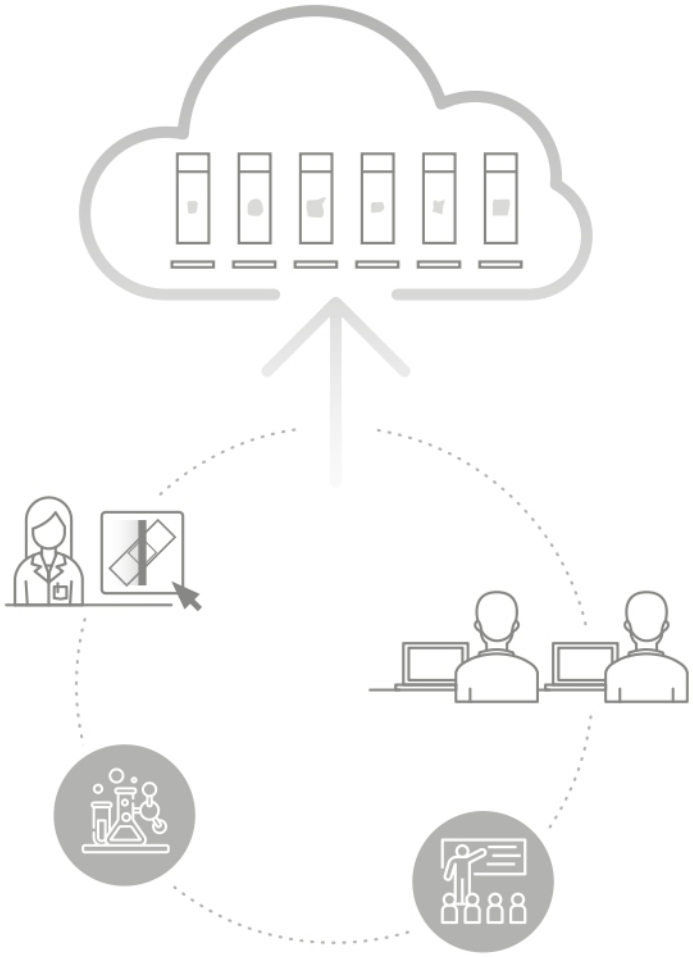
Support Pathology Research

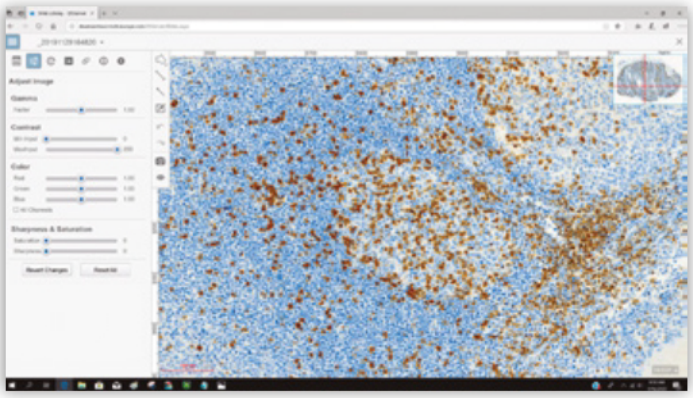
High-throughput scanning coupled with Motic's digital slide management software allow academic and industry researchers to:

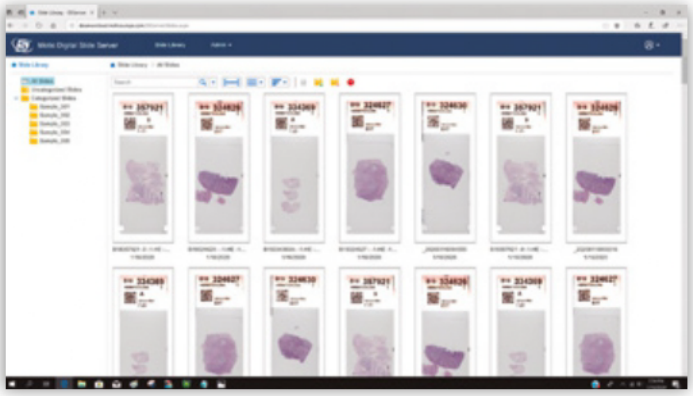
- Create and manage large data sets for analysis and machine learning
- Streamline workflow with automated barcode metadata capture
- Annotate slides with built-in markup features
- Easily search their entire slide database for library management
- Quantify results with measurement marking, size estimates, and counting
- Compare side by side displays (for IHC or reference image use) or view multiple images
- Encrypt slides for data security

WITH DSSERVER:

- Flexible cloud- or local network-deployable slide library management software
- Conference tools for group discussion and education (including logins and access management)
- Tools for analysis, including measurement, screenshot, annotation, and more
- One time fee for a perpetual license, because you shouldn't have to pay extra to see your own slides



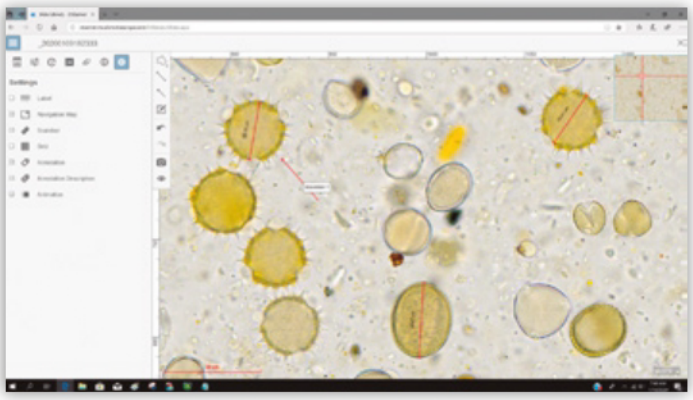




Bring your archive or classroom into the future

Slide management and storage for archiving and education allow users to:

- Digitize rare, delicate, or historic slides
- Eliminate the risk of broken slides
- Offer students identical slide information, accessible 24/7
- Reduce the physical footprint required for storing glass slides



Scanning modes

- Automatic tissue area detection (with manual override) saves time by removing blank glass from the scan area.
- Intuitive and easy to use. Once the tray is loaded, initiate scanning with a single click.

Four scanning modes offer incredible versatility to suit every purpose and sample thickness:

STANDARD AUTOFOCUS MODE

Scans in a single, high-definition plane.

HIGH PRECISION MODE

Pans through wavy specimens to locate the ideal focus plane, then scans to output a single layer.

Used for wavy slides or when high precision images are required.

Z-STACK MODE

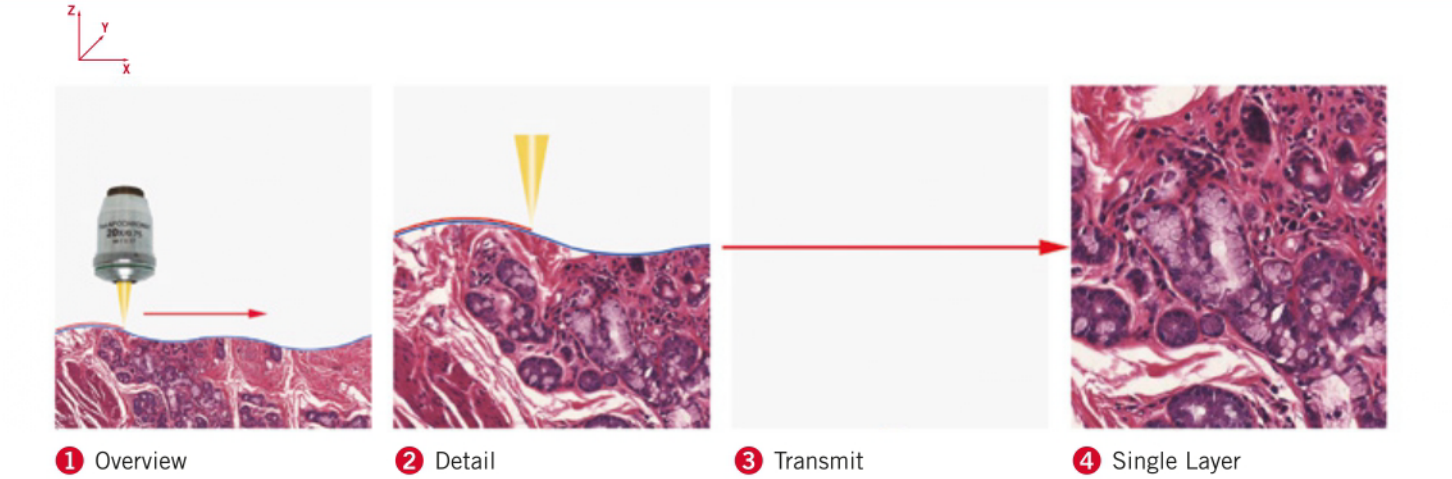
Scans multiple layers of a thick specimen and then collates into a 3D reconstruction.

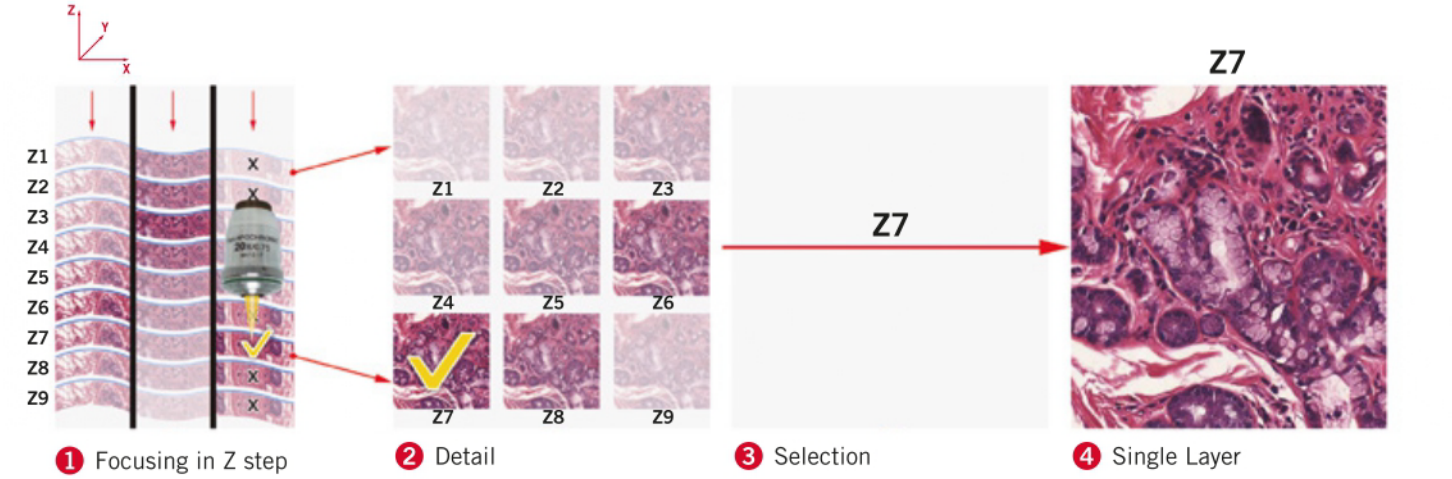
Used to create 3D reconstruction of slide layers.

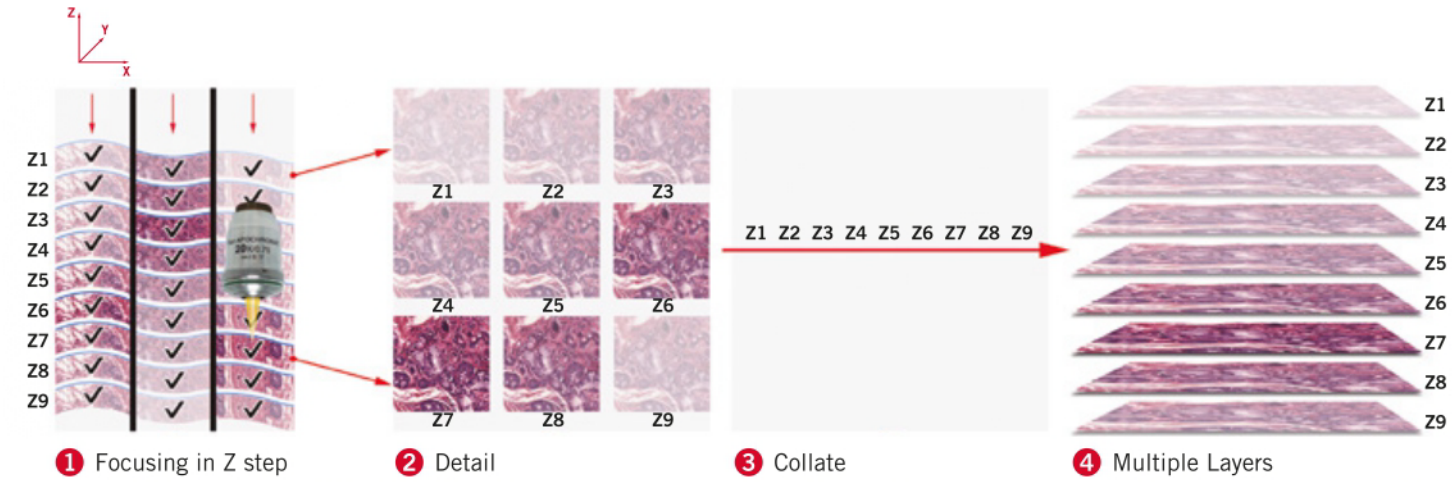
EXTENDED DEPTH OF FIELD MODE

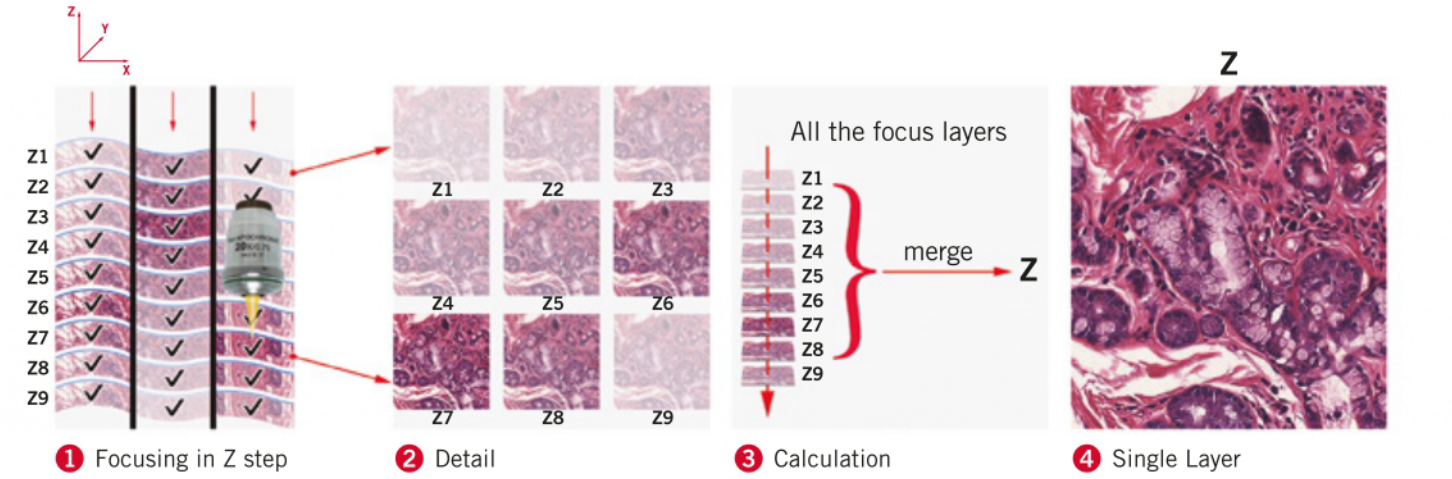
Scans multiple layers of a thick specimen and then merges all those layers into one final, fully in-focus image.

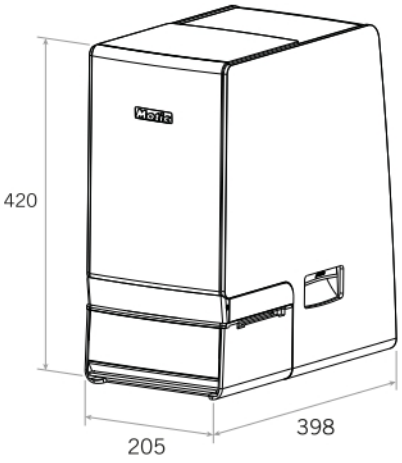
Used for thick slides with low light transmission.

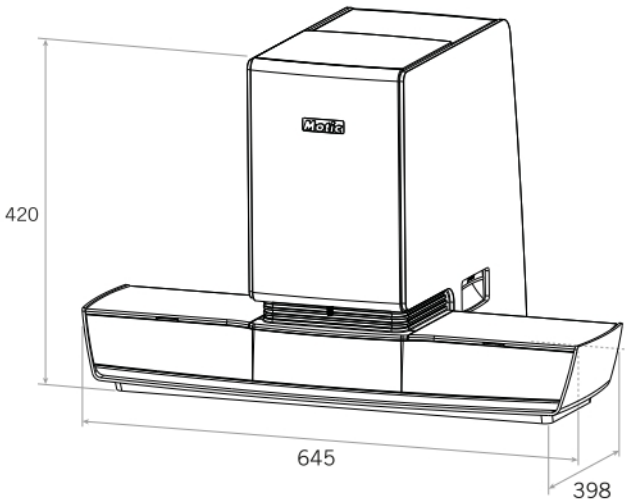

1 Overview
2 Detail
3 Transmit
4 Single Layer


Z1
Z2
Z3
Z4
Z5
Z6
Z7
Z8
Z9
Z7
Z7
1 Focusing in Z step
2 Detail
3 Selection
4 Single Layer

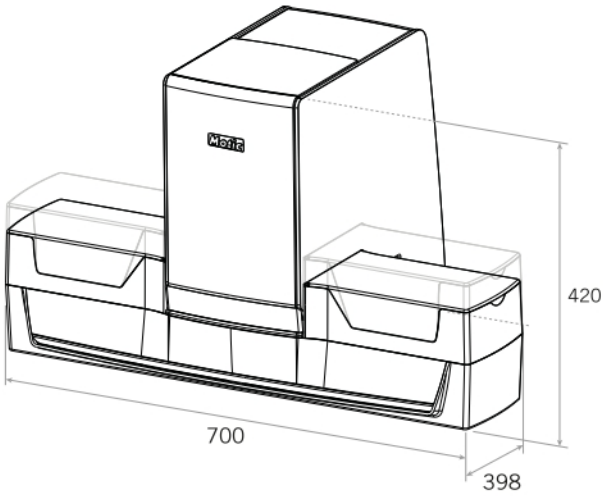

Z1 Z2 Z3 Z4 Z5 Z6 Z7 Z8 Z9
1 Focusing in Z step
2 Detail
3 Collate
4 Multiple Layers


All the focus layers
merge
Z
Z
1 Focusing in Z step
2 Detail
3 Calculation
4 Single Layer



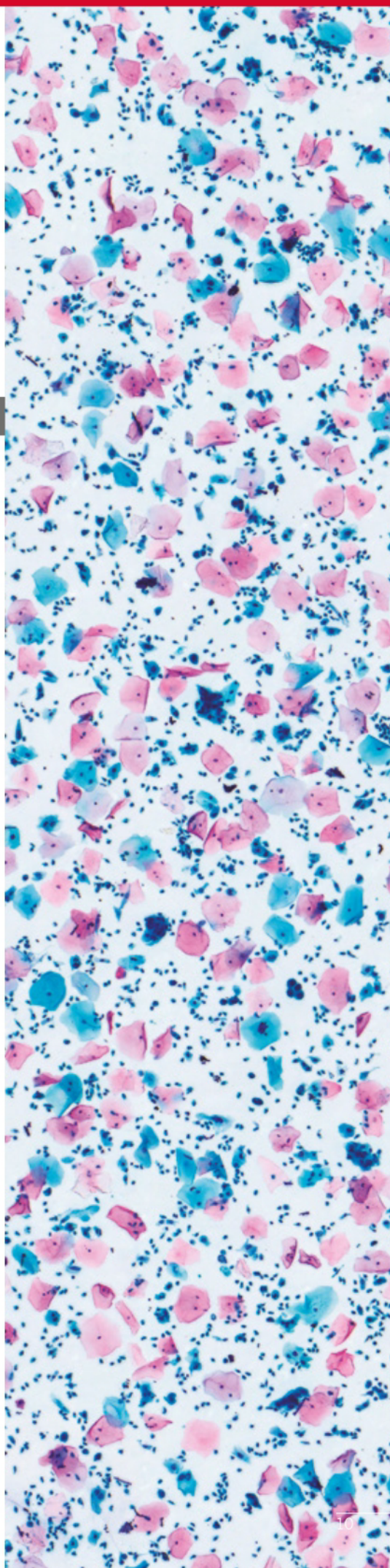


Product Series	MoticEasyScan One	MoticEasyScan Pro
Model	**MoticEasyScan One**	**MoticEasyScan Pro 6**
Objectives CCIS®	S Apo Objective 10X/0.3	S Apo Objective 10X/0.3
	Plan APOCHROMAT 20X/0.75	Plan APOCHROMAT 20X/0.75
	S Apo Objective 40X/0.75	S Apo Objective 40X/0.75
Scanning time	Standard mode: 60s (20X - with 10X objective)	Standard mode: 60s (20X - with 10X objective)
(15x15mm - full tissue)	Standard mode: 160s (40X - with 20X objective)	Standard mode: 160s (40X - with 20X objective)
	Standard mode: 640s (80X - with 40X objective)	Standard mode: 640s (80X - with 40X objective)
Resolution	20X: 0.52µm/pixel	20X: 0.52µm/pixel
	40X: 0.26µm/pixel	40X: 0.26µm/pixel
	80X: 0.13µm/pixel	80X: 0.13µm/pixel
Focusing Technique	Real-time autofocus	Real-time autofocus
Scanning camera	5.0 MP (2/3" high speed Sensor)	5.0 MP (2/3" high speed Sensor)
Nosepiece	3 hole	3 hole
Light source	10W LED (Lifetime: 25,000 Hours)	10W LED (Lifetime: 25,000 Hours)
Slide capacity	1 Slide	6 Slides
Slide Tray	1 Slide Capacity	6 Slide Capacity
Slide dimensions	76 x 26mm	76 x 26mm
Slide tolerances (mm)	Length: +0/-1, Width: +0/-1	Length: +0/-1, Width: +0/-1
Scanning mode	Normal (Real-time autofocus)	Normal (Real-time autofocus)
	High precision (High precision autofocus)	High precision (High precision autofocus)
	EDF (Extended depth of field)	EDF (Extended depth of field)
	Z-Stack (Three Dimensional stacking)	Z-Stack (Three Dimensional stacking)
Barcode Support	1D: Interleaved 2 of 5, Code 39, Code 128	1D: Interleaved 2 of 5, Code 39, Code 128
	2D: Data Matrix, QR Code	2D: Data Matrix, QR Code
Computer*	Not included / Optional	Included
	Minimum Specifications:	All-in-one business PC with 4K monitor and Windows OS
	Intel Core i7-7700	Intel Core i7-7700 / 16GB Memory
	16GB Memory	128GB SSD & 1TB SATA Disk
	128GB SSD & 1TB SATA Disk	Wireless Keyboard and Mouse
	Windows 10 Professional 64-bit	Windows 10 Professional 64-bit
Monitor	Not included / Optional	Included: All-in-One 23.8'' LED 4K resolution
Interface	USB 3.0	USB 3.0
Included software	DSAssistant	DSAssistant
	EasyScanner software (for MoticEasyScan One)	EasyScanner software (for MoticEasyScan Pro)
Optional software	DSAConference, DSServer, FS-Live Telepathology System	DSAConference, DSServer, FS-Live Telepathology System
Optional Modules	No	Large Mode (76x50mm slide)
Dimensions	205 x 398 x 420mm	645 x 398 x 420mm
Net weight	12.6 kg	16 Kg



MoticEasyScan Infinity		Product Series
MoticEasyScan Infinity 60	**MoticEasyScan Infinity 100**	**Model**
S Apo Objective 10X/0.3		**Objectives CCIS®**
Plan APOCHROMAT 20X/0.75		
S Apo Objective 40X/0.75		
Standard mode: 60s (20X - with 10X objective)		**Scanning time**
Standard mode: 160s (40X - with 20X objective)		(15x15mm - full tissue)
Standard mode: 640s (80X - with 40X objective)		
20X: 0.52µm/pixel		**Resolution**
40X: 0.26µm/pixel		
80X: 0.13µm/pixel		
Real-time autofocus		**Focusing Technique**
5.0 MP (2/3" high speed Sensor)		**Scanning camera**
3 hole		**Nosepiece**
10W LED (Lifetime: 25,000 Hours)		**Light source**
60 Slides	102 Slides	**Slide capacity**
6 Slide Capacity (10 trays)	6 Slide Capacity (17 trays)	**Slide Tray**
76 x 26mm		**Slide dimensions**
Length: +0/-1, Width: +0/-1		**Slide tolerances (mm)**
Normal (Real-time autofocus)		**Scanning mode**
High precision (High precision autofocus)		
EDF (Extended depth of field)		
Z-Stack (Three Dimensional stacking)		
1D: Interleaved 2 of 5, Code 39, Code 128		**Barcode Support**
2D: Data Matrix, QR Code		
Included		**Computer***
All-in-one business PC with 4K monitor and Windows OS		
Intel Core i7-7700 / 16GB Memory		
128GB SSD & 1TB SATA Disk		
Wireless Keyboard and Mouse		
Windows 10 Professional 64-bit		
Included: All-in-One 23.8'' LED 4K resolution		**Monitor**
USB 3.0		**Interface**
DSAssistant, DSServer		**Included software**
EasyScanner software (for MoticEasyScan Infinity)		
DSAConference		**Optional software**
Large Mode (76x50mm slide)		**Optional Modules**
700 x 398 x 420mm		**Dimensions**
33 Kg	33.4 Kg	**Net weight**

**The computer configuration may change in accordance with the technical progress, without notice and without obligation*



Motic®

Canada | China | Germany | Spain | USA

Motic Digital Pathology addresses the growing global pathology care gap by making digital medicine approachable for hospitals, labs, and doctors everywhere. We promote adoption of telepathology through our innovative, cost-effective solutions developed directly in conjunction with partner pathologists. As a business unit of Motic, a leader in the field of optics since 1988, we are part of a global company innovating for a better tomorrow.

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Motic Instruments (Canada)
130 - 4611 Viking Way. Richmond, BC V6V 2K9 Canada
Tel: 1-877-977 4717 | Fax: 1-604-303 9043

Motic Deutschland (Germany)
Christian-Kremp-Strasse 11, D-35578 Wetzlar, Germany
Tel: 49-6441-210 010 Fax: 49-6441-210 0122

Motic Hong Kong (Hong Kong)
Unit 2002, L20, Tower Two, Enterprise Square Five
38, Wang Chiu Road, Kowloon Bay, Kowloon, Hong Kong
Tel: 852-2837 0888 | Fax: 852-2882 2792

Motic Europe (Spain)
C. Les Corts 12, Pol. Ind. Les Corts. 08349 Cabrera de Mar, Barcelona, Spain
Tel: 34 93 756 62 86 | Fax: 34 93 756 62 87

Designed in Barcelona (Spain)
April 2020

Official Distributor:

Certificate CN07/31104

The management system of

Motic China Group Co., Ltd.

Motic Building, Torch Hi-Tech Industrial Development Zone, Xiamen City, Fujian Province, 361006, P.R. China

has been assessed and certified as meeting the requirements of

ISO 13485:2016
EN ISO 13485:2016

For the following activities
Design and Manufacture of optical & digital microscope and Digital Microscopy Image Scanning System and Digital Microscopy Image Scanning Analysis System for in vitro diagnostic purpose

This certificate is valid from 19 July 2022 until 18 July 2025 and remains valid subject to satisfactory surveillance audits.
Issue 10. Certified since 18 July 2007.

Authorised by

SGS United Kingdom Ltd
Rossmore Business Park, Ellesmere Port, Cheshire, CH65 3EN, UK
t +44 (0)151 350-6666 - www.sgs.com

UKAS MANAGEMENT SYSTEMS 0005

PHOENIX TESTLAB GmbH

CONFIRMATION OF COMPLIANCE

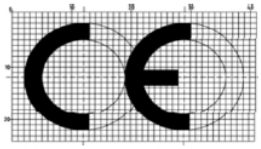
Number: 15-211034

Applicant:	Motic China Group Co., Ltd
Address:	MOTIC BLDG, TORCH HI-TECH INDUSTRIAL DEV ZONE XIAMEN FUJIAN, 361006, CN
Product Description:	Digital Slide Scanning System
Product Designation	Model Name: EasyScan Brand Name: Motic
Manufacturer:	Motic China Group Co., Ltd
Address:	MOTIC BLDG, TORCH HI-TECH INDUSTRIAL DEV ZONE XIAMEN FUJIAN, 361006, CN

Applied Specifications / Standards	Documentary Evidence	Result Of Test
EN 61326-1:2013	T1851293 01	Conform
EN 61326-2-2:2013	T1851293 01	Conform
EN 61000-3-2:2014	T1851293 01	Conform
EN 61000-3-3:2013	T1851293 01	Conform
EN 61010-1:2010	T1851293 03	Conform
EN 61010-2-032:2012	T1851293 03	Conform

The test report(s) has demonstrated that the tested sample(s) complies with the requirements of the above mentioned standards.

On the premise that the manufacturer has been carried out the conformity assessment and the relevant documents demonstrate the conformity to the applicable European Directives, e.g. the Quality Management System of the company, Technical Documentation, the applied harmonized standards and the Declaration of Conformity (which clearly states that the basic protection requirements were met), the company is obligated to affix the above described equipment with the 'CE' Marking that shall consist in the initial 'CE' taking the following form:



The 'CE' marking must have a height of a least 5 mm. If the 'CE' marking is reduced or enlarged the proportions given in the above graduated drawing must be respected.

This confirmation of compliance does not constitute a declaration of conformity. The manufacturer has sole and ultimate responsibility for the conformity of the product to the directives 2006/95/EC, 2004/108/EC and further applicable Directives.

2015-10-01

Signed by Alan Lane
Product Certification Department

Phone +49(0)5235-9500-42
Fax +49(0)5235-9500-28

PHOENIX TESTLAB GmbH
Königswinkel 10
D-32825 Blomberg, Germany
www.phoenix-testlab.de

Certificate CN05/00178

The management system of

Motic China Group Co., Ltd.

Unified Social Credit Code: 91350200612018359W
Business Registration Address: Motic Building, Torch park, Torch Hi-Teach Industrial Development Zone, Xiamen
Business Operation Address: Motic Building, Torch park, Torch Hi-Teach Industrial Development Zone, Xiamen City, Fujian Province, P.R. China

has been assessed and certified as meeting the requirements of

ISO 9001:2015

For the following activities

Design and manufacture of optical microscopes and digital microscopes

This certificate is valid from 07 April 2023 until 06 April 2026 and remains valid subject to satisfactory surveillance audits.
Issue 8. Certified since 07 April 2005

Authorised by
Jonathan Hall
Global Head - Certification Services

SGS United Kingdom Ltd
Rossmore Business Park, Ellesmere Port, Cheshire, CH65 3EN, UK
t +44 (0)151 350-6666 - www.sgs.com

The certification information can be verified on the web site of Certification and Accreditation Administration of the People's Republic of China
www.cnca.gov.cn

Motic® MICROSCOPES
Motic Hong Kong Limited

Date: 31-OCT-2023

PROPRIETARY ARTICLE CERTIFICATE

To whom so ever it may concern

We hereby certify that Motic EasyScan Pro-6 (Digital Slide Scanner) is a proprietary item of Motic. Motic is the sole manufacturer of this type of item. To best of our knowledge and understanding, there is no other manufacturer in the world who manufactures same or similar product with same performance and specification as listed in our product brochure (as attached below).

It also includes an artificial intelligence-based DNA scanner system that empowers pathologists and doctors to use proven DNA ICM technology to deliver accurate results (for early detection of oral and cervical cancers. It is based on the principle of tumor aneuploidy). Sample collection method is same as the routine standard protocol of liquid brush-based cytology.

We are using our inhouse developed patented technology for manufacturing Motic EasyScanPro-6.

Name: Patrick Pek

Title: General Manager

Seal:

Date: 31-OCT-2023

Unit 1712, Exchange Tower, 33 Wang Chiu Road, Kowloon Bay, Kowloon
Tel: (852) 2837 0888 Fax: (852) 2882 2792 Email: info@motic.com.hk

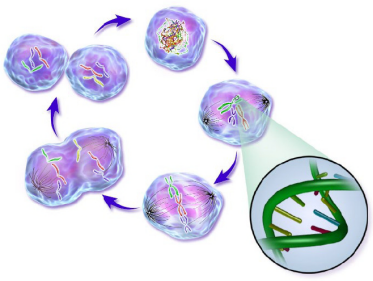
DNA Ploidy Test

Technology developed by British Columbia Cancer Research, Canada.

BC Cancer Agency
CARE + RESEARCH

DNA Ploidy Test is a Diagnostic Quantitative test based on DNA Aneuploidy.

What is DNA Aneuploidy?



Aneuploidy is the presence of an abnormal number of chromosomes in a cell. It means that when cells were dividing some error happened and number of chromosomes was lost or gained, it affected the DNA quantity within a cell.

It can happen due to many factors such as genetics, pollution, bad habits, virus and others.

If these damaged cells keep dividing, it can develop into cancer.

DNA Ploidy Test

DNA Ploidy Test can detect aneuploidy in squamous epithelium cells of oral mucosa **up to 2 years earlier than cytology or histology alone.**

AI Powered Quantitative DNA Analysis

How?

It is so because cytology and histology is based on morphological analysis of sample, so very early changes that happen within cell nucleus are not yet visible through microscope even for the most experience pathologist.

DNA Ploidy test enables pathologist to provide the most accurate diagnosis based on quantitative and objective results.

DNA Ploidy Test has very high accuracy:

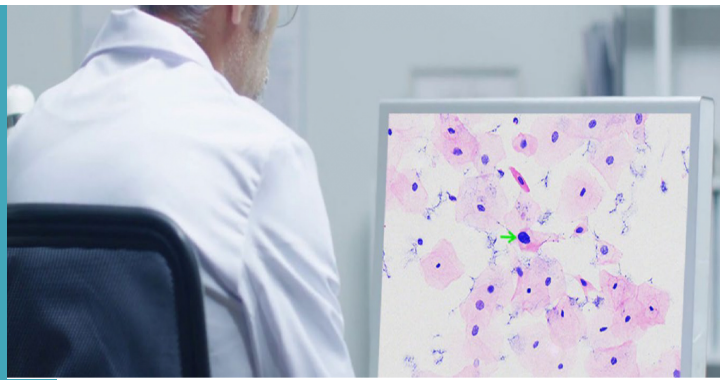
Sensitivity 98%

Specificity 100%

Technology

Developed by
British Columbia Cancer Research, Canada

Peer reviewed by MD Anderson Cancer Center, US



Internationally standardized by 4th Consensus Reports of the European Society for Analytical Cellular Pathology (ESACP).

Certified by:

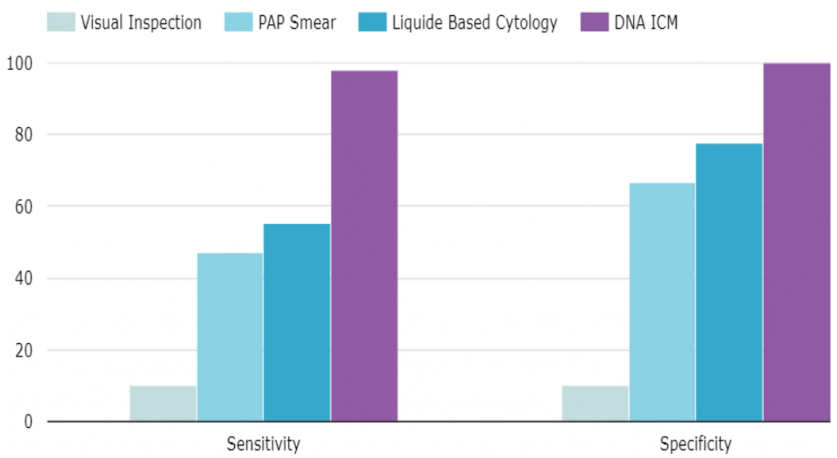
Health Canada (Health Regulator)

Gemeinsamer Bundesausschuss (Health Regulator)

Conformité Européenne (Health & Safety Regulator EEA)

China Food and Drug Administration (Health Regulator)

Central Drugs Standard Control Organization (Indian Regulator for Diagnostic Devices)

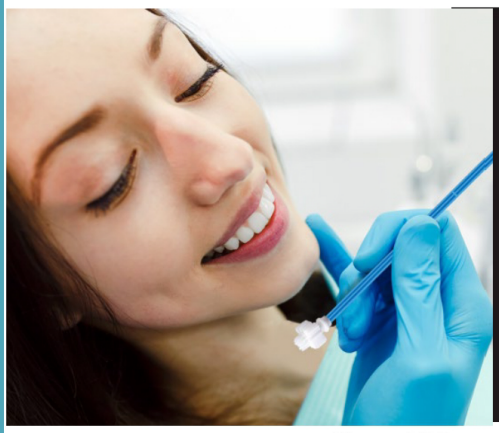


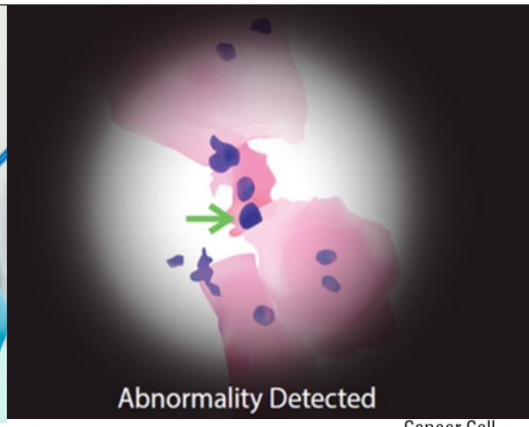
Aneuploidy is a proven biomarker for cancer for more than 100 years.

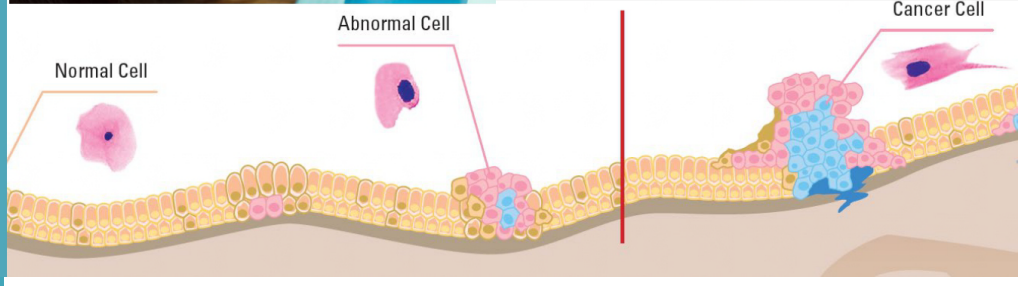
Now the power to access this is with you.

- Highest Accuracy
- Molecular Diagnostics
- Quantitative Results
- Non-Invasive
- Cost-Effective
- Early Detection of Cancer
- Assesses biological behavior of cells
- Reduce needless biopsies
- Saves Lives

DNA Ploidy Test is a quantitative diagnostic test which measures cell aneuploidy helping detect abnormalities up **to 2 years** before cytology or histology alone. So cancer development can be stopped.







DNA Ploidy Collection Kit

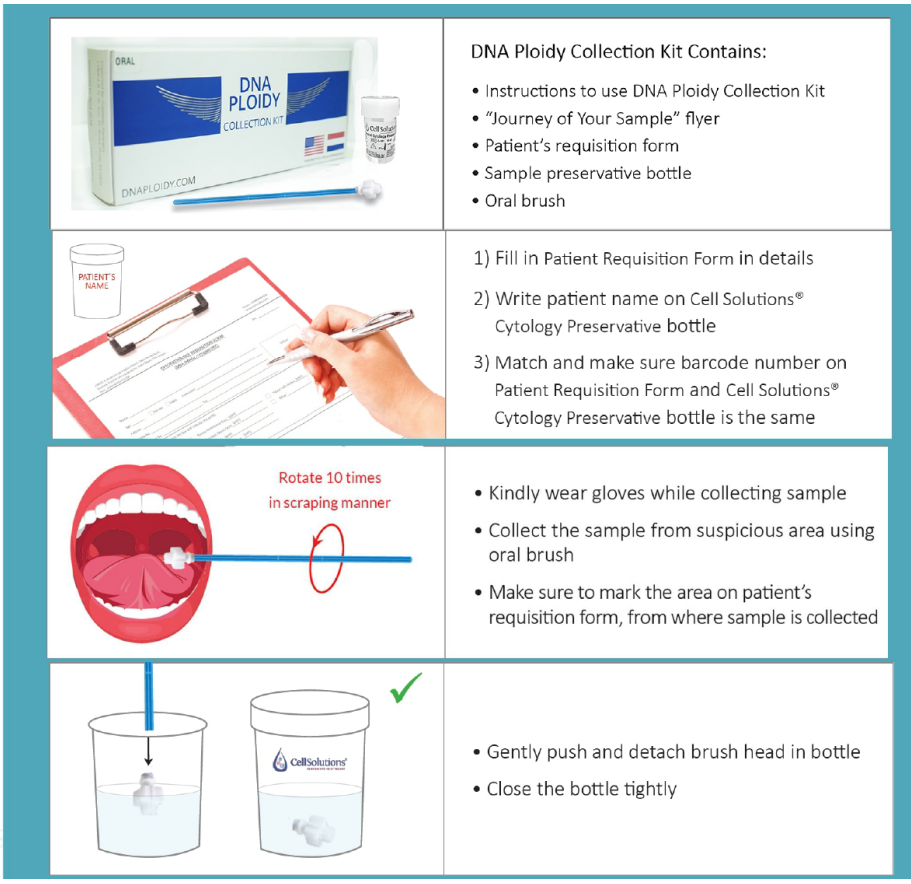
Sample collection is exactly same as LBC PAP sample collection process.

DNA Ploidy Collection Kit includes the highest quality sample collection tools. It is one of the most important elements that determines success of DNA Ploidy test.

DNA Ploidy Collection Kit:

World's most advanced cytology preservative technology from **CellSolutions®** (US) securely holds cells from specimen drawn by high cellularity **Rovers® Orcellex® Brush** (The Netherlands).

- New, patented design to enable optimal collection, storage and release of cell material
- Significantly reduces the number of inadequate smears.
- Avoids bleeding and pain.



DNA Ploidy Test Applications

1. **Screening**
2. **Diagnostics**
3. **Treatment**

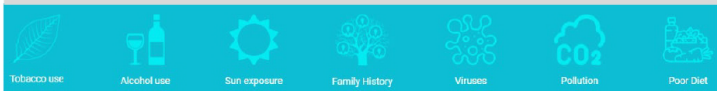
We recommend to use **VELscope Vx** for advanced visualization, so oral abnormalities that are not visible in a white light can be detected and sample collection site may be assessed more accurately. **However, using VELscope Vx is NOT a prerequisite for DNA ploidy testing.**

With DNA Ploidy Testing:

- **Cancer can be stopped from developing in pre-cancerous stage.**
- **Early detection leads to successful treatment.**
- **Cancer recurrence can be prevented.**

Risk factors increase oral cancer risk, though **25% of patients with oral cancer had NONE** of these risk factors. Most of these patients are younger than 40 years of age. Current air and food pollution levels in India puts everybody at high risk to develop oral cancer.

Thus everybody should be screened for oral cancer regularly despite of habits or no showing symptoms.



Sample Collection - Site

1. Routine screening for asymptomatic patients and no abnormalities found with VELscan:

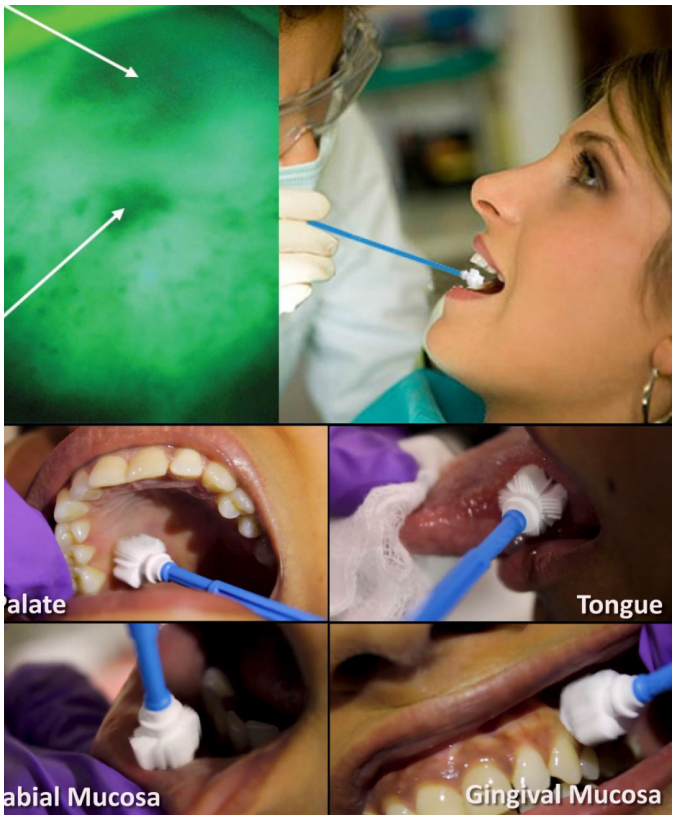
Brush over the most common sites for oral cancer to occur: Tongue, Floor of the mouth, gums, etc.

2. With deleterious habits and no visible lesion – ask patient about the placement of tobbaco / guthkha quid. Collect sample from that area.

3. OPMD - Clinically apparent lesions

4. Abnormalities detected using VELscope

Oral potentially malignant disorders (OPMD) may progress to oral cancer. As per WHO guidelines, OPMD (e.g., leukoplakia, erythroplakia, oral lichen planus, oral submucous fibrosis) must be investigated further.



World Health Organization

1. Screening

Application of DNA Ploidy Test	Indication Of Use	Benefits
Routine screening for asymptomatic people	Routine screening with DNA Ploidy test is done routinely for healthy asymptomatic people to check for abnormalities that may be indicative of development of oral cancer. Prefer with VELscan, but not prerequisite for DNA Ploidy Test. VELscan every 6-12 months. DNA Ploidy test every 1-3 years	Oral cancer can happen to everyone. Risk factors increase the risk, **however 25% of oral cancer cases report none of these risk factors.**
Regular screening for high risk patients	- Tobacco use/ Betel nut use - Alcohol use - Family history of cancer - Immunosuppression - Upper aerodigestive tract problems - HPV infection - Age older than 40 years	DNA Ploidy test can detect abnormal changes in oral mucosa that can lead to development of **oral cancer up to 2 years earlier** than cytology or histology alone. When abnormal cells are detected early, in pre-cancerous stage lifestyle change, or simple treatment can help to stop abnormal changes and prevent cancer from developing.
Cancer Patients Family Screening	Relatives of oral cancer patients should be advised to undergo oral screening regularly.	Oral cancer can be happening due to genetic or environmental reasons, such as habits, HPV, pollution, so relatives of oral cancer patients are at the greater risk to develop this disease themselves.

2. Diagnostics

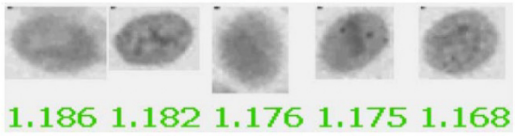
Application of DNA Ploidy Test	Indication Of Use	Benefits
Primary Diagnosis **Oral Potentially Malignant Disorders (OPMD)** (e.g., leukoplakia, erythroplakia, oral lichen planus, oral submucous fibrosis)	Irrespective of the clinical appearance, suspicious findings should be primarily investigated with a non-invasive DNA Ploidy test to assess the presence of early abnormal molecular changes and to rule out underlying malignancy.	In early stages pathologist can be unable to see underlying malignancy through microscope, but it can be detected with quantitative DNA Ploidy test up to 2 years earlier than cytology and histology alone.
Second Opinion to primary diagnosis	DNA Ploidy test is AI-powered test which overcome limitations and bias of clinical and morphological sample analysis and provide the highest quality second opinion to the patient.	DNA Ploidy test provides the most accurate, objective and non-biased second opinion to a patient and doctor.
Supplement Biopsy diagnosis	To maximize diagnostic accuracy DNA Ploidy is done to supplement biopsy report with quantitative and objective information about biological cell behavior in molecular level and provide doctor with valuable information necessary for successful treatment plan.	DNA Ploidy test increases diagnostic accuracy overcoming such limitations as: - Human Error - Subjectivity - Wrong sampling area - Low accuracy - Technical limitations

3. Treatment: OPMD, Oral Pre-Cancers, Oral Cancers

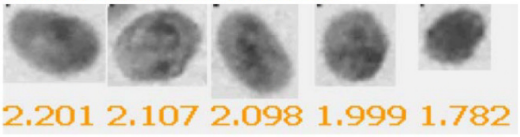
Application of DNA Ploidy Test	Indication Of Use	Benefits
Before Treatment (Surgery, Radiation, other)	DNA Ploidy test is done before treatment to identify aneuploidy and measure biological cell behavior in identified potentially malignant, pre-cancerous or cancerous area. DNA Ploidy report before treatment sets the base line of aneuploidy level in before treatment which is crucial to plan treatment and follow-up progress of the treatment.	DNA Ploidy test report supplements histopathological, MRI/CECT and other required reports with quantitative and objective information about biological cell behavior in molecular level.
After Treatment	Once treatment site is healed, DNA Ploidy test is done to confirm there is no abnormal cells left in a treatment site that could develop into recurrent disease. Non-invasive DNA Ploidy test is recommended every 3-6 months for 3 years after surgery. Later, life-long annual testing.	Non-invasive testing will provide accurate results with no painful experience to a patient and healed treatment site will not be disturbed.
Lifelong Follow-up	Non-invasive DNA Ploidy test should be done annually to monitor biological cell behavior and prevent loco-regional recurrence or detect abnormal changes happening in molecular level very early.	Oral cancer patients are in a lifelong risk to develop recurrent disease; thus regular monitoring is crucial. DNA Ploidy test is the most accurate, non-invasive test available today to provide the valuable report to a patient.

DNA Ploidy Test Report Interpretation

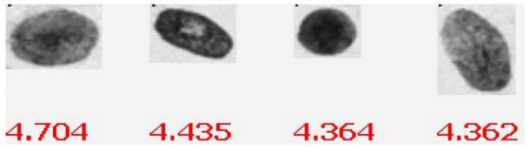
- Normal cells ID (DNA Index): 0 - 1.2 (Green colour)



- Cycling or proliferating cells: ID 1.2 – 2.5 (Yellow colour)



- Abnormal cells: ID ≥ 2.5 (Red colour)



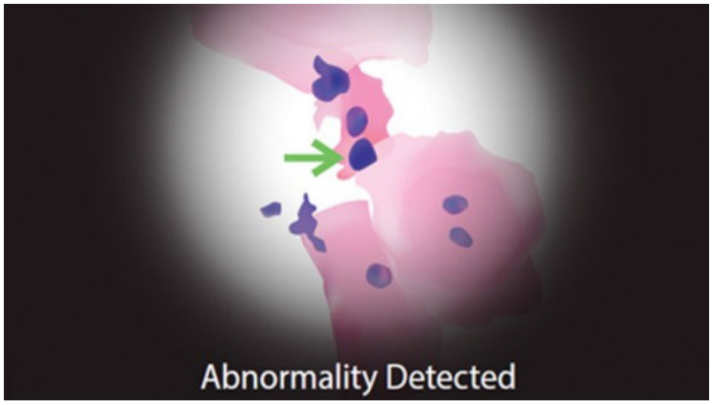
Medium Alert - cell proliferation: 5-10 %

High Alert - cell proliferation: >10%

DNA DIAGNOSIS	RECOMMENDATION
No cells were detected with abnormal DNA amount	Routine screening is recommended.
1-2 cells were detected with abnormal DNA amount (DI>2.5)	Repeat DNA Ploidy test in 3 to 6 months.
5%-10% of the cells are proliferating (going through the cell cycle, DI = 1.252.5)	Repeat DNA Ploidy test in 3 to 6 months.
3 or more cells were detected with abnormal DNA amount (DI>2.5)	Biopsy is recommended to confirm malignancy and determine the grade and stage of tumor.
Over 10% of the cells are proliferating (going through the cell cycle, DI = 1.252.5)	Biopsy is recommended to confirm malignancy and determine the grade and stage of tumor.
The sample is unsatisfactory	Repeat DNA Ploidy test as soon as possible.

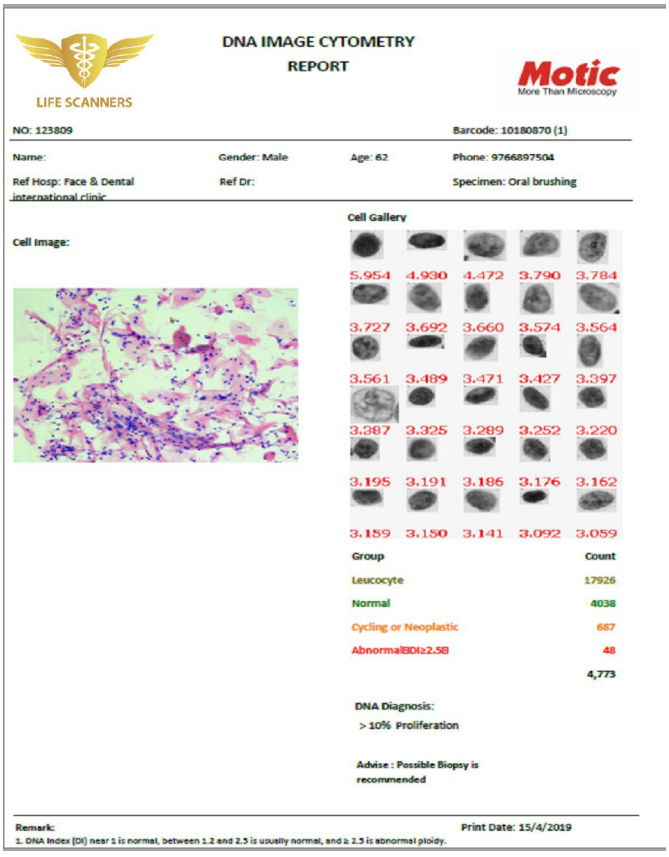
DNA Ploidy Test Report

1. **Cell Gallery**
2. **DNA Index**
3. **Biological Cell Behaviour - Proliferating**



DNA Ploidy Test is a quantitative diagnostic test which measures cell aneuploidy helping detect abnormalities up **to 2 years** before cytology or histology alone.

This deep learning and AI-Powered system has been studied globally with hundreds or research papers published over 40 years.



DNA IMAGE CYTOMETRY REPORT

LIFE SCANNERS

Motic
More Than Microscopy

NO: 123809 Barcode: 10180870 (1)

Name: Gender: Male Age: 62 Phone: 9766897504

Ref Hosp: Face & Dental international clinic Ref Dr: Specimen: Oral brushing

Cell Image:

Cell Gallery

5.954	4.930	4.472	3.790	3.784
3.727	3.692	3.660	3.574	3.564
3.561	3.489	3.471	3.427	3.397
3.387	3.325	3.289	3.252	3.220
3.195	3.191	3.186	3.176	3.162
3.159	3.150	3.141	3.092	3.059

Group	Count
Leucocyte	17926
Normal	4038
Cycling or Neoplastic	687
AbnormalBDI≥2.5B	48
	4,773

DNA Diagnosis:
>10% Proliferation

Advise : Possible Biopsy is recommended

Remark: Print Date: 15/4/2019

1. DNA Index (DI) near 1 is normal, between 1.2 and 2.5 is usually normal, and ≥ 2.5 is abnormal ploidy.

CANCER
CYTOPATHOLOGY

Diagnostic and Prognostic Use of DNA Image Cytometry in Cervical Squamous Intraepithelial Lesions and Invasive Carcinoma

Alfred Böcking, M.D., Ph.D.[1]
Vu Quoc Huy Nguyen, M.D., Ph.D.[2]

[1] Institute of Cytopathology, Heinrich-Heine University Düsseldorf, Düsseldorf, Germany.

[2] Department of Obstetrics and Gynecology, Hue University Medical School and Central Hospital, Hue, Vietnam.

In the fight against cervical malignancy and its precursors, several adjuvant diagnostic methods have been proposed to increase the accuracy of cytologic and histologic diagnoses. Because chromosomal aneuploidy has been accepted as an early key event in tumorigenesis caused by genetic instability, the cytometric equivalent of chromosomal aneuploidy detected by DNA image cytometry (DNA-ICM) may serve as a marker of neoplasia. During the last decade, the appearance of a new generation of hardware with high processing and storage capacities, together with the development of appropriate software, has facilitated the development of high-performance DNA-ICM systems. International consensus on the clinical application of DNA-ICM has been reached. According to the statements of Task Force 8 of the International Consensus Conference on the Fight Against Cervical Cancer, indications for DNA-ICM include the identification of prospectively malignant cells in squamous intraepithelial lesions (SILs) and atypical squamous cells of undetermined significance (ASCUS). The European Society of Analytical Cellular Pathology consensus reports on DNA-ICM have provided standardized technical details on performance, terms, and algorithms for diagnostic data interpretation and quality-assurance procedures. Increasing biologic evidence and clinical data have confirmed the utility of DNA-ICM as an adjuvant method suitable for determining the diagnosis and prognosis of cervical intraepithelial lesions and invasive carcinoma. Patients with ASCUS and low-grade SIL diagnoses that reveal DNA euploidy may return for normal screening intervals, whereas the detection of DNA aneuploidy indicates that these lesions should be removed. Formerly a research tool, today, standardized DNA-ICM has become a useful and low-cost laboratory method to establish objectively and reproducibly an early diagnosis of prospectively progressive cervical intraepithelial lesions at a high-quality level. DNA-ICM may further contribute to the monitoring of treatment in patients with invasive cervical malignancies. ***Cancer (Cancer Cytopathol)* 2004;102:41–54.** *© 2003 American Cancer Society.*

KEYWORDS: DNA image cytometry, cervical cytopathology, squamous intraepithelial lesion, cervical invasive carcinoma.

In patients with borderline lesions (mild and moderate dysplasias) of the uterine cervix, cytomorphology alone often is not sufficient for the early and definite cytologic detection of malignancy. Cervical dysplasia describes squamous cells or tissues that potentially may lead to malignancy but do not exhibit sufficient evidence for a definite assumption. Resulting from weakness of morphologic criteria to identify malignant transformation in epithelial cells early and unequivocally, dysplasias are not a disease entity. The widely accepted assumption is that the higher the grade of dysplasia is, the higher the

Dr. Nguyen was supported by a grant from the Düsseldorf Entrepreneurs Foundation (Düsseldorf, Germany).

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Received April 16, 2003; revision received September 23, 2003; accepted September 23, 2003.

DOI 10.1002/cncr.11889

probability of progression to carcinoma is.[1] However, as a result of insufficient morphologic criteria, neither histologic nor cytologic evaluation can predict whether a lesion will progress to cancer in an individual patient.[2] Rates of regression and progression of cervical dysplasia (positive and negative predictive values) are quite different from one study to another.[1] Insufficient interobserver reproducibility in diagnostic cytology and histology represents another dilemma in the microscopic diagnosis of precancerous cervical intraepithelial lesions.

Because the diagnosis of cervical squamous intraepithelial lesions (SILs) is not only poorly reproducible but also of limited biologic meaning for the individual patient, the number of resulting control procedures usually is high. These range from repeated cytologic smears and biopsies to unnecessary operations (conizations). Missed early diagnoses of cancers may result from cytomorphologic uncertainties. This also results in unnecessary costs and avoidable anxiety for the patients.

In the late 1970s, zur Hausen suggested that there may be an association between human papillomavirus (HPV) and cervical carcinoma.[3] Large numbers of subsequent epidemiologic, clinicopathologic, and molecular studies have linked the presence of specific types of HPV to the development of anogenital carcinoma and its precursors. A recent study estimated the worldwide HPV prevalence in cervical carcinomas at 99.7%.[4] Today, it is widely accepted that HPVs play a critical role in the pathogenesis of most cervical carcinomas and their precursor lesions. The infection of cervical epithelial cells with HPV itself is necessary but still insufficient for neoplastic progression. Other factors, especially genetic alterations, are needed to enter into the process of neoplastic transformation.[5]

Several adjuvant diagnostic methods currently are proposed to increase the diagnostic accuracy and reproducibility of cytology and histology. These range from clinical procedures, like colposcopy, to laboratory methods, like assays for the detection of HPV DNA and HPV typing[6] or DNA image cytometry (DNA-ICM).[1,7,8] In the current review, we focus on the technical performance and application of DNA-ICM in diagnosis and prognosis of cervical SILs and invasive cervical carcinoma.

Biologic Background

Chromosomal aneuploidy is defined as numeric and/or structural aberrations. It is an early key event in tumorigenesis caused by genetic instability.[7,9] The cytometric equivalent of chromosomal aneuploidy, DNA aneuploidy, serves as a marker of neoplasia by assessing large-scale genomic alterations resulting from genetic instability.[9] DNA-ICM is capable of monitoring the effect of cytogenetic tumor progression on nuclear DNA content. Quantitation of DNA aneuploidy, therefore, may serve as a prognostic marker.[9,10]

Chromosomal aneuploidy has been found in most cervical squamous carcinomas and recently even in high-grade SILs.[11–13] Aberrations of chromosome 1 have been found only in SILs that progressed to invasive carcinoma.[11] The finding of aneuploidy qualifies SIL as high grade, which requires further clinical management.[9,14,15]

The hypothesis that chromosomal aneuploidy itself may be a cause of cancer was proposed first by Boveri at the beginning of the 20th century.[16] During recent decades, this hypothesis has been ignored, because most efforts have been centered on the hypothesis of somatic gene mutations.

Increasing numbers of recently published scientific articles on the relation between aneuploidy and cancer pathogenesis have added to the current understanding about this issue. Chromosomal aneuploidy seems to play a crucial role in cancer development. A recent study using in situ hybridization to analyze cervical intraepithelial neoplasias (CINs) has provided sufficient evidence that chromosome 1, 7, and X aneusomies in SILs are associated with progression toward cervical carcinoma.[17] Using a molecular cytogenetic approach termed comparative genomic hybridization (CGH), Heselmeyer et al. found that the gain of chromosome 3q that occurs in HPV type 16 (HPV-16)-infected aneuploid cells was a pivotal genetic aberration at the transition from severe dysplasia/carcinoma in situ to invasive cervical carcinoma.[18] Another study that was published by this working group in 1997 demonstrated that, in patients with advanced-stage (Stage IIb–IV) invasive cervical squamous carcinoma, a recurrent pattern of chromosomal aberration was observed.[19] The most commonly seen aberration, again, was the gain of chromosome arm 3q (in 23 of 30 tumors). During progression to advanced-stage disease, additional chromosomal aberrations are acquired, namely, the gain of chromosome arms 1q and 5p and the loss of chromosomal band 2q36–37. Alterations of many other regions also have been detected in various proportions of cervical malignancies, as shown in Table 1.

From a cytogenetic viewpoint, several categories of chromosomal aberration may occur at different grades of tumor progression[21] that may be divided into primary, secondary, and tertiary aberrations. Primary chromosomal aberrations are the first and are detectable by light microscopy after neoplastic transformation. Usually, one or a few chromosomes are concerned. They mostly result in a peridiploid stem-

TABLE 1
Genetic Changes in Invasive Cervical Carcinomas[a]

Gain of genetic material		Loss of genetic material	
Region	Frequency (%)	Region	Frequency (%)
1q	25–45	1q	20–30
1p	22–33	2q	20–33
3p	50	3p13–25.3	39–52
3q	35–77	3q	22
5p	26–34	4p16	20–44
6p	27	4q21–35	20–53
8q	41	5q	20–40
11q	20	6q	22–38
15q	34–41	8p	23–30
17p	30–35	11p15	28
17q	12–45	11q22–24	7–51
20p	23	13q	27–45
20q	16	18p	20–30
Xq	34	18q12.2–21.2	24–37
		19p	20–30
		Xq	44

[a] Adapted from Zhang, 2002.[20]

line. Secondary chromosomal aberrations follow the primary aberrations during tumor progression. Rather regularly, tumor type-specific chromosomes are affected, and additional stemlines result. Their net effects on nuclear DNA content mostly are detectable by DNA-ICM. Tertiary chromosomal aberrations occur during further tumor progression; these also are caused by genetic instability. Due to the final loss of stemlines and a large variation in DNA values, their effects on DNA content usually are detectable by DNA cytometry.

A codiscoverer of viral oncogenes in the 1970s, Peter Duesberg, recently observed that the correlation between aneuploidy and malignancy is strong and is evident in almost all solid malignancies. He also observed that the aneuploidy-malignancy correlation explains the both growing list of nonmutagenic carcinogens and why human oncogenes cannot turn human cells into cancer cells.[22] Duensing and coworkers, in a series of experiments on genomic instability, found that high-risk HPV may cause aneusomy through two mechanisms.[23,24] One mechanism involves the ability of viral protein E7 to uncouple the duplication of the centrosome from the cell division cycle, probably by targeting the pRb pathway. The second mechanism is the disturbing effect of E6 on the checkpoint function of the cell cycle by degrading p53. Another study supported this centrosome aberration hypothesis and demonstrated a connection between the severity of the lesions, centrosome aberration, and aneuploidy.[25] The progression from Grade 1 CIN to CIN 2, CIN 3, and invasive carcinoma seems to be related to increasing numbers of cells with abnormal centrosome replication and increasing deviation of histograms from the normal DNA-diploid pattern. Most recently, Bollmann and coworkers found that atypical squamous cells of undetermined significance (ASCUS) with DNA rare events > 9c DNA content unit were found exclusively in combination with high-risk HPV infection.[26] These findings support the concept that DNA aneuploidy represents an objective, very early, and highly specific marker of (prospective) neoplasia and that its detection in epithelial dysplasias identifies those lesions that most likely will progress to histologically manifest malignant disease.

Principle of Method

The terms *DNA-ICM* and *DNA flow cytometry* (*DNA-FCM*) should be used as descriptors to designate the type of nuclear DNA measurement performed. The amount of DNA in the nucleus of a cell (called the '2 c' or 'diploid amount' of DNA) is specific to the type of organism in question. However, within the animal kingdom, with 3 major exceptions, all healthy cells in a given organism contain the same amount of DNA. These include 1) cells that have undergone meiosis in preparation for sexual reproduction and thus contain only 1 c, a haploid amount of DNA, typical of a gamete; 2) cells that are carrying out DNA synthesis in preparation for cell division (mitosis) and thus, for a short period, contain between 2 c and 4 c of DNA; and 3) cells that are undergoing apoptosis and have begun to loose pieces of fragmented DNA. The cell cycle has been divided into different phases: cells in G0 phase are not cycling at all; cells in G1 phase are either recovering from recent division or preparing for the initiation of another cycle; cells are said to be in S phase when they actually are in the process of synthesizing new DNA; cells in G2-phase are those that have completed DNA synthesis and thus possess double the normal amount of DNA; and cells in M phase are in mitosis, undergoing chromosomal condensation and organization that occurs immediately before cytokinesis (resulting in the production of 2 daughter cells, each with 2 c of DNA).[27] In DNA histograms, the following cell cycle phases can be distinguished from each other[9]: 1) G0/G1 phase fraction, all nuclei that belong to a peak that does not represent a duplication of a lower peak (Fig. 4); 2) G2/M phase fraction, all nuclei that belong to a peak in the duplication region of a G0/G1-phase fraction; and 3) S phase fraction, all nuclei with integrated optical density (IOD) values between those of the corresponding G0/G1 phase fraction and its G2/M phase fraction counterpart that do not belong to other stemlines.

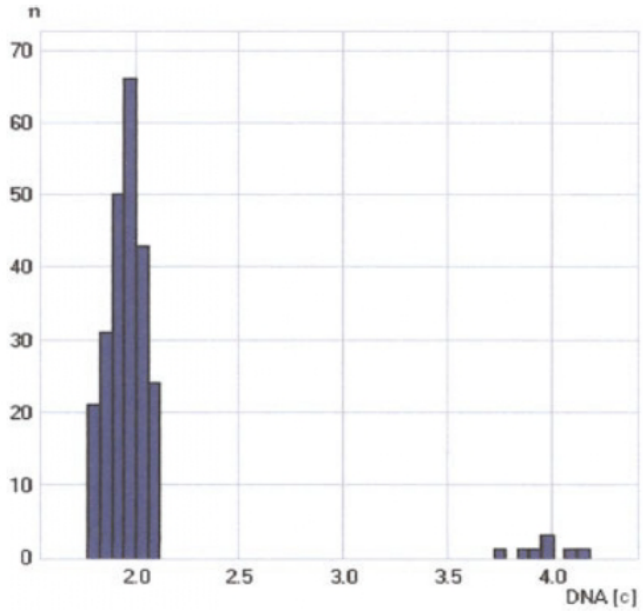

FIGURE 1. DNA histogram showing a euploid pattern (stemline at 2.0 DNA content units [c]; 9 c–exceeding events = 0).

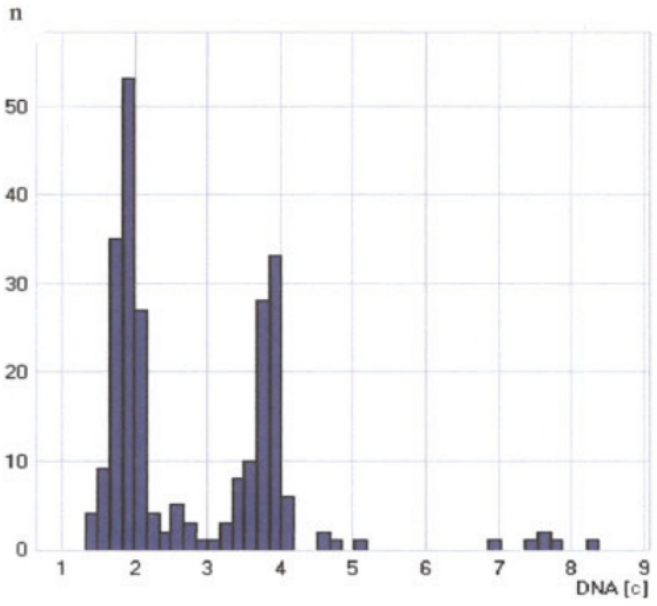

FIGURE 2. DNA histogram indicating a polyploid pattern (stemlines at 2.0 DNA content units [c]) and 4.0 c; 9 c–exceeding events = 0).

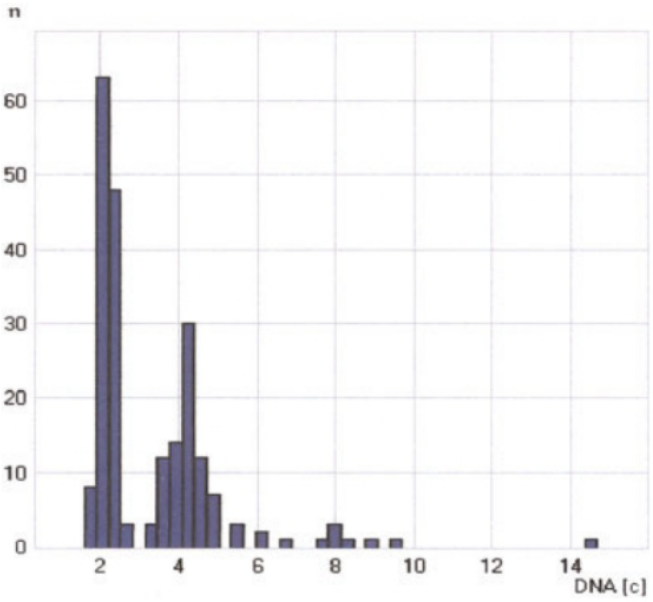

FIGURE 3. DNA histogram showing single-cell aneuploidy (9 DNA content unit [c]–exceeding events = 2).

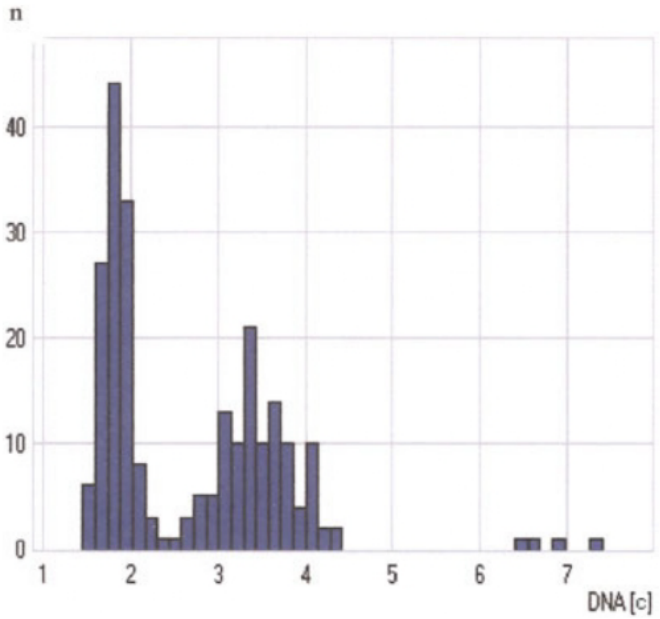

FIGURE 4. DNA histogram indicating stemline aneuploidy (stemlines at 1.7 DNA content units [c] and 3.4 c; 9 c–exceeding events = 0).

The nuclear DNA content cannot be measured directly by cytometry. After quantitative DNA staining, the nuclear IOD is the cytometric equivalent of its DNA content in DNA-ICM. Therefore, the DNA content is expressed in a *c scale*, in which 1 c is half of the mean nuclear content of cells from a normal (non-pathologic), diploid population in G0/G1 cell cycle phase (Figs. 1–5).[9] Although 'chromosomal ploidy' theoretically is detectable by cytogenetic methods in each single cell, its DNA content cannot be equated with a certain chromosomal outfit; therefore, the term *DNA ploidy* is the expression of the typical, large-scale genomic status of a cell population. The quantity of nuclear DNA may be influenced by the following mechanisms: replication, polyploidization (Fig. 2), gain, or deletion. Each mechanism affects the size or the number of chromatids. Viral infections may change the nuclear DNA content that is detectable by

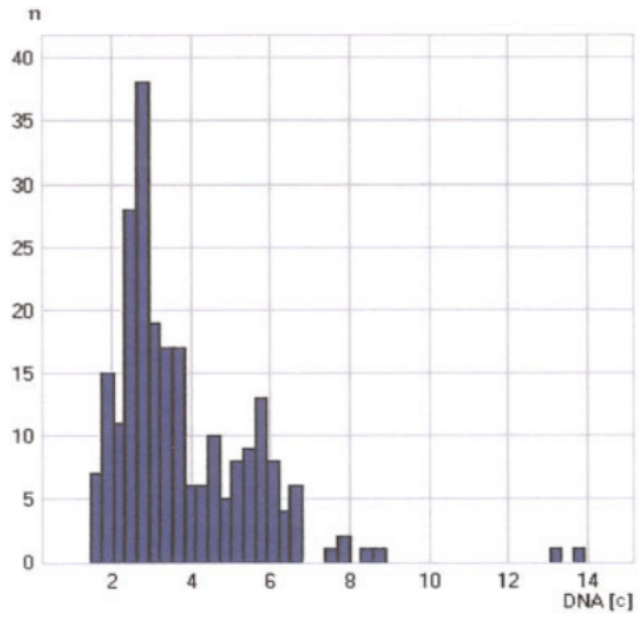

FIGURE 5. DNA histogram showing stemlines and single-cell aneuploidy (stemlines at 3.0 DNA content units [c] and 6.0 c; 9 c–exceeding events = 2).

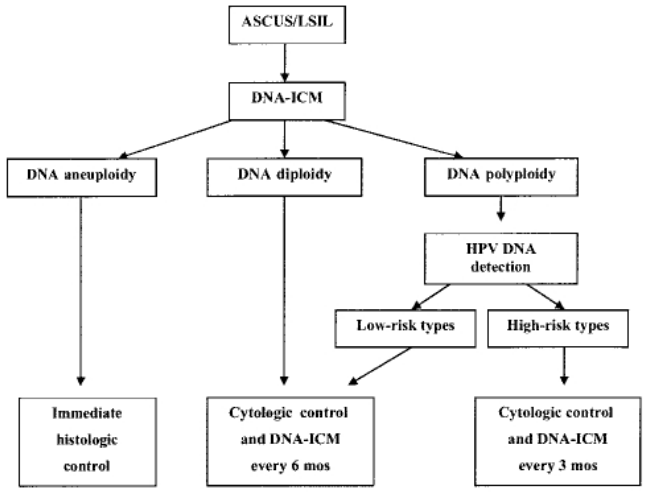

FIGURE 6. Quality-control flow chart for Papanicolaou smears of atypical squamous cells of undetermined significance/low-grade squamous intraepithelial lesions (ASCUS/LSIL).[8] DNA-ICM: DNA image cytometry; HPV: human papillomavirus.

DNA-FCM and DNA-ICM. Among others, the unspecific effects of cytostatic or radiation therapy, vitamin B12 deficiency, apoptosis, autolysis, and necrosis on nuclear DNA content also play a role. Furthermore, the DNA content of a cell is changed regularly throughout the cell cycle.[9] All of these effects have to be taken into consideration when a diagnostic interpretation of DNA histograms is performed. The basic objective of DNA-ICM is to identify DNA stemlines outside the euploid (diploid, tetraploid, or octaploid) regions as abnormal (or aneuploid) at a defined statistical level of significance (Figs. 4,5). Furthermore, DNA-ICM should provide information about the number of abnormal (aneuploid) DNA stemlines (Fig. 5), the polyploidization of euploid or aneuploid DNA stemlines (Fig. 2), the occurrence of rare cells with an abnormal high DNA content (most likely resulting from genomic alterations) (Figs. 3,5), and cell cycle fractions.

DNA-ICM results in nuclear IOD values in arbitrary units (AU), equivalent but not identical to nuclear DNA content, and the quantitation of nuclear DNA requires a rescaling of IOD values compared with the IOD values from cells with known DNA content, so-called reference cells. By means of reference cells, the AU scale is transformed into a reference unit scale (e.g., 2 c, 4 c, 8 c). Internal reference cells should be used because they have the advantage of sharing all preparatory steps with the analysis cells in the clinical specimens.

Because most interpretations of DNA measurements are population based, the results usually are displayed as DNA histograms (Figs. 1–5). The bin size of such histograms should be adapted to the precision of the actual measurements, i.e., the lower the variability in the reference cells peak, the smaller should be the bin size of histogram classes.

The resolution of DNA-ICM is defined by its precision, which allows the differentiation of 2 separate peaks, and its accuracy in recognizing a peak in an abnormal position using the European Society of Analytical Cellular Pathology (ESACP) consensus thresholds for the minimum precision of measurements, which, given the coefficient of variation (CV) of reference cells, should be $< 5\%$, enabling the recognition of an abnormal DNA stemline that deviates $> 10\%$ from the normal diploid position.[9] That means that at least 10% of the genomic DNA has to be lost or gained before this deviation can be detected reliably by means of DNA-ICM. However, in DNA-ICM, a single cell with an abnormally high DNA content ($> 10\%$ above the genomic DNA in G2/M-phase) also can be detected reliably.

Indications for DNA-ICM

In cervical pathology, the application of DNA-ICM is mostly in the differential diagnosis of cytologically doubtful cases, namely, ASCUS or low-grade SIL (LSIL) (the Bethesda system) or Papanicolaou III (Pap III) or Pap IIID (Munich II nomenclature) in German-speaking countries. The effectiveness of diagnostic DNA-ICM for identification of neoplasia/malignancy in cervical preneoplastic diseases has been established sufficiently.[7] For grading the malignancy of invasive

cervical carcinoma, the number of scientific articles still is limited, and their conclusions are not unequivocal.[28–32]

During the last 5 years, international consensus on standardized application of diagnostic DNA-ICM has been reached. Four consecutive ESACP consensus reports provided the scientific methodology for DNA-ICM.[9,33–35] According to the statements of the International Consensus Conference on the Fight Against Cervical Cancer Task Force 18, the indication for DNA-ICM is the identification of potentially malignant cells in SILs and ASCUS.[15]

Instrumentation and Software

DNA quantitations of Feulgen-stained samples first were performed with cytophotometry using ultraviolet light. During the 1960s and 1970s, FCM and scanning microscope photometers were developed, but these instruments still were expensive, required special operational skills, and were available only in special research laboratories. On the other hand, cytometric measurements performed with these systems were too time consuming. Many efforts have been undertaken since the 1970s to develop image-analysis systems for DNA cytometry using the scanning capabilities of video cameras. DNA-ICM is based on the physical principle of light absorption measurement of Feulgen-stained nuclei using a video camera. The camera is provided with a charge-coupled device (CCD) sensor. The CCD is subdivided into thousands of tiny square or rectangular potential wells (6–15 μm per side), each behaving as an independent, true photometric unit that transforms single photons in photoelectrons. Thus, with appropriate enlargement, each nucleus is divided into hundreds of independent, single photometric measurements, which are summarized to give the final integrated optical density.[36] Video-image cytometers are highly efficient, very easy to use, and cost less than one-third of the cost of the previously used instruments. This lead to a wide expansion of instrument development for research and clinical applications. The first generations of DNA-ICM systems used for diagnostic purposes were SAMBA (Unilog, France),[37] LEYTAS (Leyden, Netherlands),[38] CAS (Becton Dickinson, San Jose, CA),[39] TAS-plus (Leitz, Wetzlar, Germany),[40] Roche Pathology Workstation (Roche Image Analysis),[7] AccuMed Cyto-Savant (AccuMed, Chicago, IL),[41] CYTOMETER CM-1 (Hund, Wetzlar, Germany),[42] and MIAMED-DNA (Leitz).[43]

The appearance of a new generation of high-resolution video cameras and personal computers with high processing and storage capacities facilitated the development of high-performance DNA-ICM systems. Several systems currently are in use, including CYDOK (Hilgers; Koönigswinter, Germany),[44] ACAS (Ahrens, Bargteheide, Germany),[45] and AUTOCYTE QUIC DNA (TriPath, Burlington, NC).[46]

The most important optical problems that have great influence on densitometric measurements are glare and shading errors. In most instruments from the first generation, these problems were not solved. During the last decade, glare and shading corrections in most of the commercially available devices are provided by software procedures.[44,47] Because there is a wide variety of DNA-ICM instruments today, we propose using only instruments that meet ESACP performance standards[9,33–35] in clinical routine work to reach a quality-assured and standardized DNA cytometric diagnosis. Appropriate tests and performance standards have been published to assure sufficient densitometric linearity.[36]

Laser scanning cytometry is a new method of image analysis that was developed during the late 1980s and became available commercially in the early 1990s.[48] The method applied to the analysis of populations of more than 100,000 cells per smear is capable of detecting rare DNA events.[26] Its advantage is fast performance and, thus, excellent sample throughput, comparable to that of FCM. Based on the automated scanning principle, all normal cells also are measured; thus, the representative nature of comparably small numbers of dysplastic cells may not be preserved. The visual verification of relevant nuclei or the exclusion of artifacts may be determined only after both the measurement and a restaining step.

Sample Preparation and Staining

Cytologic smears from the ectocervix or endocervix and from the endometrium or imprints (touch preparations) may be investigated. Additional core biopsy samples or paraffin-embedded tissue samples may be used for DNA cytometric investigation after enzymatic cell preparation.

It has been found repeatedly that for diagnostic and prognostic purposes, DNA-ICM is unsuitable when performed directly on tissue sections.[49,50] The measurement of sectioned, individual nuclei of unknown size and shape (such as dysplastic and neoplastic nuclei) does not indicate the amount of DNA (the so-called tomato-salad phenomenon). The proposed procedure for these samples, therefore, is the preparation of monolayer smears by enzymatic cell separation.[7]

Feulgen staining is recommended strictly for absorption cytometry. Other protocols, including Pap staining, are unsuited for DNA-ICM. Usually, prestained Pap smears or monolayer preparations from paraffin blocks undergo rehydration in decreas-

ing ethanol concentrations and refixation in buffered 10% formalin. Hydrolysis in 5 N HCl then is allowed to take place at 27 °C for 60 minutes, followed by staining in Schiff reagent for another 60 minutes ay room temperature, rinsing in SO_2-water, and dehydration at increasing ethanol concentrations. The slides then are covered and kept away from light until measurement.[7]

Sampling, Measurement, and Scaling

Basic performance standards

The precision of DNA-ICM measurements must at least allow the identification of DNA stemline(s) as aneuploid if they deviate $> 10\%$ from the diploid domain (2 c) or the tetraploid domain (4 c), i.e., if they are outside 2.0 c $\pm$ 0.2 c or 4.0 c $\pm$ 0.4 c.[9] To achieve this goal with an error probability (P) < 0.05, the test statistics require a measurement performance described by the following: 1) a CV of the ratios between modal IOD values of reference cells and nonpathologic G0/G1 cells in a series of measurements of $< 5\%$; 2) a relative standard error of the mean (rSEM; rSEM = CV/ $\sqrt{n}$) of reference cells in each sample of $< 1.5\%$; 3) linear regression of the mean IOD versus transmission with $< 1\%$ deviation from (slope = 1); and 4) linear regression of IOD ratios versus theoretical ratio with $<$ 1% deviation from (slope = 1). Furthermore, a DNA stemline should be identified as polyploid within the duplication position of a G0/G1 phase fraction $\pm$ 0.2 c (at 4.0 c) and $\pm$ 0.4 c (at 8.0 c), respectively, with an error probability $P < 0.05$ if the CV of the ratios between modal IOD values of nonpathologic G0/G1 phase and G2/M phase fractions in a series of measurements is $< 2.5\%$ (Fig. 2).

The different aspects of the measuring process and of diagnostic interpretation should regularly be subjected to quality-control measures to assure a consistently high level of quality in the diagnostic procedure. Appropriate protocols for such quality assurance have been developed and described elsewhere.[35]

Densitometric measurement

One important step in setting up the system is to check for glare phenomena. Glare errors should be corrected by software procedures. Measurements of reference cells are needed for rescaling densitometric measurements. Internal reference cells should be used, as discussed above. Lymphocytes, granulocytes, and normal epithelial or stroma cells usually are analyzed as internal standards. At least 30 reference cells should be measured. The CV of the reference cell population should not exceed 5% (CV = standard deviation of the mean $\times$ 100).[7] Nuclei to be measured are sampled in a systematic, random manner. Only diagnostically relevant cells should be measured, i.e., cells of a certain cytologic entity (e.g., all tumor cells or all dysplastic cells) that can be identified by their morphology. A selective sampling for rare nuclei characterized by a high DNA content is allowed only if the occurrence, per se, is of diagnostic relevance.

Terms and Diagnostic Algorithms

According to ESACP consensus reports, simple and complex algorithms and diagnostic or prognostic classification strategies for DNA histogram interpretation may be used for the following purposes, depending on the material under investigation and the diagnostic or clinical questions: 1) diagnosis of neoplasia, 2) prognostication of neoplasia, and 3) monitoring of therapy.[9,33] Histogram classifications should not be based on subjective interpretations but should be defined by algorithms.

Diagnostic purposes

The reports of ESACP on standardization of diagnostic DNA-ICM proposed the definitions and algorithms for diagnostic interpretation of DNA histograms that are described in Tables 2,3.[9,33,34]

Prognostic purposes

According to the ESACP consensus reports,[9,34] classification of the entire DNA histogram based on the position of DNA stemlines may be of prognostic value. The terminology for grading is applicable only to neoplasias, either proven by morphologic investigations or in cases of DNA aneuploidy. The terms defined in Table 4 may be used to classify the histogram for prognostic purposes.

Clinical Application in Cervical Premalignant Lesions and Invasive Cervical Carcinoma

Diagnosis of malignancy

DNA aneuploidy is the cytometric equivalent of chromosomal aneuploidy and may serve as a marker of neoplasia by assessing large-scale genomic alterations resulting from genomic instability.[9] Using video image analysis principles on Feulgen-stained smears, DNA-ICM is capable of detecting DNA aneuploidy in the form of aneuploid stemline(s) (Fig. 4,5) and/or rare DNA events (Fig. 5; Table 3)

In a series of 276 Pap smears, 73% of DNA aneuploid, mild or moderate cervical dysplasias developed into carcinoma in situ or higher lesions, and the histologic diagnosis remained dysplastic in 17%. For the detection of invasive lesions, DNA aneuploidy had a sensitivity of 99% and a positive predictive value of 86%.[14]

DNA-ICM has been proposed repeatedly as an adjunctive diagnostic and prognostic method for eval-

TABLE 2
Terms and Algorithms in Diagnostic Interpretation[a]

DNA histogram
Frequency distribution of IOD values obtained by quantitative DNA stains and rescaled by IOD values from reference cells in 'c' units; the class width should be twice the standard deviation of the IOD of the G0/G1 phase fraction of reference cells
DNA histogram peak
A statistically significant local maximum in a DNA histogram
Modal value of a histogram peak
The most frequent value in the peak, i.e., the mean value of the histogram class containing the highest number of nuclei; this is close or equal to the mean value of a fitted Gaussian curve according to the principle described above
DNA stemline
The G0/G1 cell phase fraction of a proliferating cell population (with a first peak and a second doubling peak or with nuclei in the doubling region)
DNA stemline ploidy
The modal value of a DNA stemline in the unit c
DNA euploidy
the type of DNA distribution that cannot be differentiated from the distribution of normal cell populations (resting, proliferating, or with polyploidization)
Diploid euploidy
DNA stemlines with a modal value between 1.8 and 2.2 c possess diploid euploidy
Tetraploid euploidy
DNA stemlines with a modal value between 3.6 and 4.4 c possess tetraploid euploidy
DNA aneuploidy
Types of DNA distributions that are different at a statistically significant level from the distributions of normal (resting, proliferating, or with polyploidization) cell populations; DNA aneuploidy either can be seen as DNA stemline aneuploidy or can be indicated by 'rare events'
DNA stemline aneuploidy
Stemline(s) with modal values < 1.80 c or > 2.20 c and < 3.60 c or > 4.40 c possess stemline aneuploidy
'Rare events' in DNA histograms
These are abnormal cells and often are called 5 c– or 9 c–exceeding events (5c-EE or 9c-EE), with nuclear DNA contents higher than the duplicate or quadruplicate of a normal G0/G1 phase population (i.e., not belonging to G2/M phase); they likely represent nonproliferating abnormal cells with different chromosomal aneuploidies and abnormally high number of chromosomes
DNA single-cell aneuploidy
Because most preneoplastic or invasive lesions of uterine cervix are caused by human papillomavirus, which is known for its effects on nuclei causing polyploidization, the 5c-EE cutoff level may not be appropriate for a diagnosis of DNA aneuploidy; thus, DNA single-cell aneuploidy in cervical pathology is defined by the presence of at least 1 cell with a DNA content > 9 c (9c-EE ≥ 1) per slide (Chatelain et al.[52])
Polyploidization
The (repeated) doubling of a chromosomal set
Euploid DNA polyploidization
The occurrence of peaks in the duplication ($\times 2$, $\times 4$, $\times 8$) regions of euploid stemlines; in human tissues, the highest peak usually is at 2 c
Aneuploid polyploidization
The occurrence of peaks in the duplication regions of aneuploid stemlines

IOD: integrated optical density; c: DNA content units.
[a] See Böcking and Haroske et al.[9]

TABLE 3
Algorithms for the Differentiation of Euploidy from Aneuploidy in DNA-Histograms from the Uterine Cervix

DNA-euploid	diploid: 1.8c <= STL <= 2.2c, 5cEE = 0 polyploid: 1.8c <= STL <= 2.2c or 3.6c <= STL <= 4.4c, 9cEE = 0
DNA-aneuploid	stemline - aneuploidy: STLs neither diploid nor polyploid single cell - aneuploidy: 9cEE > 0

STL: DNA-stemline; 5cEE: 5c Exceeding-Events; 9cEE: 9c Exceeding-Events.

TABLE 4
DNA Histogram Classification for Prognostic Purposes[a]

Classification	Definition
Peridiploid	A single DNA stemline with a modal DNA value between 1.8 and 2.2 c
Peritetraploid	A single DNA stemline or a stemline additional to a peridiploid stemline with a modal DNA value between 3.6 and 4.4 c
X-ploid	A single DNA stemline or a DNA stemline additional to a peridiploid or peritetraploid stemline with a modal DNA value outside the thresholds mentioned above ('X' will be substituted by the DNA ploidy value of this stemline, e.g., peritriploid, hyperdiploid, etc.)
Multiploid	Occurrence of more than one abnormal DNA stemline (often called 'Manhattan skyline')

c: DNA content units.
[a] See Böcking[7] and Haroske et al.[9]

uating patients with cervical intraepithelial lesions and invasive cervical carcinoma.[10,14,31,51–55] Many authors have reported high positive predictive values for the development of in situ or invasive carcinoma out of mild-to-moderate cervical dysplasias with proven DNA aneuploidy, varying from 84% to 100%.[14,52,53,56] Intervals between the detection of DNA aneuploidy and histologic follow-up in these studies were up to 3 years.

The frequency of aneuploid DNA patterns in different types of invasive carcinoma of the uterine cervix was investigated by Kashyap and Bhambhani. An increasing trend toward DNA aneuploidy was observed from well differentiated squamous cell carcinoma (SCC) (64%), to moderately differentiated SCC (71%), to poorly differentiated SCC (83%). Eighty-five percent of all endocervical adenocarcinomas in that study also showed aneuploid DNA patterns.[57]

It also has been found that DNA-ICM is useful as an adjuvant diagnostic procedure in patients with endocervical adenocarcinoma. The presence of nuclei with DNA contents > 9 c was observed exclusively in adenocarcinomas with a sensitivity of 96% and a specificity of 100%, indicating that this parameter is suited best for differentiating between malignant and nonmalignant endocervical epithelia.[58]

TABLE 5
Predictive Values of Diagnostic DNA Image Cytometry in Mild or Moderate Cervical Dysplasia for Grade ≥ 3 Cervical Intraepithelial Neoplastic Lesions

Author	No. of patients	Follow-up (mos)	Predictive value (%)	
			Positive	Negative
Böcking et al., 1986[14]	37	21.3	84	86
Chatelain et al., 1989[52]	18	≤ 42.0	100	83
Kashyap et al., 1990[53]	42	30.8	93	67
Bollman and Böcking, 1996	497	—	95	98
Hering et al., 2000[54]	193	≤ 36.0	85	77

From the data presented in Table 5, proposals for clinical consequences of DNA-ICM results may be formulated as follows: 1) a high negative predictive value of 95% in cervical smears with ASCUS and LSIL diagnoses revealing DNA euploidy allows patients to return to normal screening intervals; and 2) positive predictive values of 46% for patients who have CIN 3 or higher-grade lesions after 2 months and up to 100% after 3 years for patients who have ASCUS and LSIL with DNA aneuploidy allow the removal of lesions by conization or loop electrical excison procedure (LEEP).

Prognosis of malignancy

In addition to its diagnostic application, DNA-ICM may be used as a prognostic tool for patients with cervical carcinoma. Although controversy over the prognostic value of DNA-related parameters exists,[28–30] several recent studies have provided more promising data.

In a study of postirradiation cytologic smears from 46 patients with cervical carcinoma, Davey and coworkers found that DNA histograms were correlated significantly with patient outcome.[50] A peridiploid histogram generally denoted a healthy outcome, whereas multiploidy was correlated most often with postirradiation dysplasia. The highest positive predictive value was for DNA aneuploidy; 92% of patients developed recurrences or postirradiation dysplasia. According to the authors, once an abnormal histogram pattern occurs, the patient should be evaluated more aggressively for early diagnosis and management of postirradiation dysplasia and/or local recurrence.

Results from a recent study by Grote et al. in patients with Stage IB/II cervical carcinoma confirmed the high prognostic value of standardized DNA-ICM.[31] A DNA stemline ploidy > 2.2 c was correlated with an unfavorable prognosis. In a Cox proportional hazards regression model, it was found that DNA stemline ploidy and the 5 c–exceeding rate (5c-ER) had prognostic value in both presurgical analysis and postsurgical analysis. In another study of 163 pathologic Stage T1b1–T2b invasive cervical squamous carcinomas, tumors with a DNA index > 1.70 and a 5c-ER $> 11\%$ represented the poor prognostic group.[32] Thus, in addition to clinical and histopathologic parameters, DNA cytometric indices may contribute to the prognostic evaluation of an individual patient.

It also has been reported that DNA ploidy is a valid indicator of response to radiotherapy in women with cervical malignancies. In 1991, Yu et al. found that tumors with DNA aneuploidy were more radiosensitive than diploid tumors.[59] They concluded that there is a direct correlation between DNA content and radiosensitivity in invasive cervical carcinoma.

Because changes in DNA histograms may indicate therapeutic effects,[60,61] DNA-ICM may have a role in monitoring the response to chemotherapy of patients with invasive cervical carcinoma. To achieve a final conclusion, further studies on this topic will be needed.

The follow-up course and corresponding management strategy for patients with cervical lesions that occur during pregnancy have a great impact not only on the women themselves but also on the outcome of pregnancy. To date, there has been no valid consensus on the follow-up interval and management strategy for such lesions in pregnant women. Except for invasive lesions, which require immediate, appropriate management, DNA-ICM performed in women with ASCUS and SILs may provide relevant information on the prospective biologic behavior of these lesions and may help to establish an appropriate follow-up course for each individual pregnant woman. Women who have cytologic high-grade lesions with a high DNA grade (multiploid aneuploidy) of malignancy in early pregnancy probably should undergo surgical interventions, whereas for women who have cytologic low-grade lesions with a low DNA grade (single cell or single stemline aneuploidy) of malignancy in late pregnancy, colposcopic/cytologic follow-up may be the appropriate management.

Quality assurance of cytologic and histologic diagnoses

In cases of equivocal cytologic diagnoses, the cytometric detection of DNA aneuploidy speaks in favor of the presence of (prospectively) malignant cells, especially when there are discrepancies between cytologic and histologic diagnoses. In a series of 170 seemingly false-positive routine cervical smears, DNA aneuploidy was found in 47 smears without histologic explanation.[56] Using DNA aneuploidy as a solid marker of malignancy in uterine epithelia, the authors classified those

cases as histologically false-negative, and not false-positive, cytologic diagnoses. In four cytologically suspicious oral lesions, Remmerbach and coworkers demonstrated that the histologic diagnosis had to be revised after the detection of DNA aneuploidy.[62]

DNA-ICM also may be used as a method for quality control in histologic diagnosis. In patients with positive cytologic tumor cell diagnoses and negative histologic follow-up, the detection of DNA aneuploidy should motivate the pathologist to work-up more thoroughly a given conization or biopsy specimen. If no malignancy is found, then the patient should undergo surgery again to remove more cervical tissue and to find the cancer that was missed previously in histologic follow-up.

Combination with other diagnostic methods

Adjuvant diagnostic methods currently proposed to increase the diagnostic accuracy of cervical cytology and histology are assays for the detection of HPV and for HPV typing[6,63] and DNA-ICM.[1,7,8] In general, proposed approaches, such as administering HPV tests to women with mild dysplasia to determine whether treatment is necessary, have shown varying levels of effectiveness and are relatively costly.[64–67] Testing for high-risk HPV types actually yielded a wide variation in positive predictive values for the detection of high-grade SIL from 13% to 33.5%.[68–72] Using the Hybrid Capture II test (Digene, Gaithersburg, MD), several studies reported that the specificity of high-risk HPV detection of histologic high-grade SIL ranged from 58% to 85%.[68,69,73] Transient incident infection is common, especially in young women.[70] Thus, specificity remains a concern with HPV testing for primary screening, and more research is needed to determine optimal approaches.[66,74] A recent study in South Africa suggested that specificity may be improved by adjusting the level of HPV DNA used to define a positive result.[75]

Because, to date, DNA-ICM has provided more encouraging data on its diagnostic validity, combining DNA-ICM with HPV testing in patients with high-risk HPV types may improve the specificity of the HPV test and provide more information on the prospective behavior of an individual HPV-positive lesion. In a series of 50 patients with of mild, moderate, and severe cervical dysplasia, Kashyap et al. found that 44.0% of patients showed an aneuploid DNA pattern, whereas positivity for high-risk HPV-16 DNA was found in 46.0% of patients. Follow-up data revealed that 72.7% of patients who had an aneuploid DNA pattern and were positive for HPV-16 experienced progression to carcinoma in situ, compared with only 4.4% of patients who were positive for HPV-16 and had a euploid DNA pattern.[76]

For an individual patient with a diagnosis of ASCUS or LSIL, the detection of high-risk HPV may lead to uncertainty in management strategy for the health care provider and anxiety for the patient. In such cases, DNA-ICM can subsequently be performed to provide more information on the prospective biologic behavior of the lesion and, thus, an assured management strategy that is reasonable for both the health care provider and the patient.

Latency period

Because it may require an interval period ranging from several months up to 10 years for a mild or moderate squamous dysplasia, as diagnosed cytologically on a Pap smear, to develop into histologically proven carcinoma, simultaneously obtained histologic diagnoses may not be adequate to decide correctly on the positive predictive value of cytologic diagnoses or adjuvant methods. In 2001, Sudbø et al. showed that the progression rate from oral dysplasia to invasive oral carcinoma based on the detection of DNA aneuploidy was only 10% after 1 year but increased significantly, to 90%, after 5 years.[77] Thus, this interval should be taken into account in the evaluation of the diagnostic and prognostic value of DNA-ICM.

Reproducibility of DNA-ICM

The reproducibility of a method includes two aspects: intraobserver agreement and interobserver agreement. Features that have an impact on reproducibility are the objectivity of diagnostic criteria, the number of diagnostic categories, the study population, and the experience of the individuals who perform the test. Interobserver variability has important implications for diagnostic error and, thus, patient care and medical litigation issues. In cancer screening and diagnosis, cytologic and histologic investigations have contributed greatly to the fight against malignant disease worldwide. Despite its success in screening and early detection of cervical premalignant lesions, the diagnostic accuracy of histology and cytology in pathology of the uterine cervix still has limits. Histomorphologic and cytomorphologic criteria for the diagnosis of different grades of precancerous SILs (dysplasias), even carcinoma in situ of the uterine cervix, are neither defined objectively nor agreed upon internationally. These facts may explain the insufficient intraobserver and interobserver reproducibility of histologic and cytologic diagnoses in pathology of the uterine cervix as reported by many authors.[78–80]

Because the correct performance of DNA-ICM requires cytomorphologic knowledge, it is possible that

both intraobserver reproducibility and (especially) interobserver reproducibility of DNA measurements are not negligible. To date, only a limited number of studies have dealt with the reproducibility of DNA grading. In 1989, Böcking et al. reported intraobserver and interobserver reproducibility rates of 83.9% and 82.2–86.7%, respectively, for DNA grading of malignancy in breast and other cancers.[81] Recently, Nguyen and coworkers reported an interobserver correlation of 94.1% ($\kappa = 0.84$) in DNA measurements performed on 202 routine ASCUS-positive smears (unpublished own data). The comparably high value of interobserver agreement achieved in that study was at least 20% higher than the rates reported in the literature for morphologically based histologic or cytologic diagnoses of cervical dysplasias. Explanations for this high value may include both the high standardization of DNA measurements and diagnostic data interpretation and the objectivity of the method. Compared with the subjective assessment of conventional morphology-based diagnostic methods, DNA histogram interpretation is based on well defined algorithms with proven diagnostic validity in cervical pathology (Figs. 1–5). Therefore, it may be assumed that when it is performed according to standardized procedures, DNA-ICM represents a reliable method with high interobserver reproducibility.

Economic Aspects

DNA-ICM may contribute to the avoidance of costs that result from repeated and unnecessary cytologic, histologic, and clinical controls, as well as surgical procedures in patients with DNA-euploid ASCUS/LSIL. If DNA aneuploidy is detected, then the indication for a surgical intervention (conization, LEEP-conization, etc.) should be assumed, also to avoid the costs that occur when advanced carcinomas require treatment. DNA-ICM is paid for by German health insurance companies in cases of cytologically doubtful diagnoses (approximately 50 Euros per measurement). It should be pointed out that DNA-ICM is a rather time-consuming method (30–40 minutes per measurement) that requires performance skills and, thus, appropriately trained personnel.

Quality Control of Diagnostic DNA-ICM

Because a wide variety of hardware and software for diagnostic DNA-ICM exists, and because different algorithms may be used for diagnostic data interpretation, there is a need for 1) standardization of measurement performance, terminology, and diagnostic interpretation of data and 2) quality-control procedures. To obtain results with high diagnostic accuracy and prognostic validity, all steps involved in DNA-ICM should be performed following a standardized procedure. The 1997 ESACP consensus reports on diagnostic DNA-ICM reserved Part II for dealing with the quality-control issue.[35] The respective steps are specimen preparation and staining, instrumentation, sampling and densitometric measurement, scaling process, and measurement interpretation.

Additional important steps in quality-control procedures in diagnostic ICM are accreditation of measurement devices and the set-up of a remote-control server.[35,82] In the framework of the Prototype Reference Standard Seiden (PRESS) project,[83] several tests have been developed and designed to control the basic technical characteristics of ICM instrumentation; these tests involve inexpensive measures using defined density filters. These different tests have to be performed upon delivery of the machinery by the industrial provider using the appropriate software and at yearly intervals by users. The first system that was officially accredited by the ESACP was the ACAS system (Ahrens). Accreditation of other systems that are in use is encouraged.

The development of the quantitation server EUROQUANT[84] is an important step toward quality control and standardization in diagnostic DNA-ICM.[82] This system is a remote server that was designed to act both as a teleconsultation system and as a cytometric workstation that can be used through Internet technology. Its main functional components are 1) measurement of the performance of DNA ploidy analysis obtained by every end user in an individual case through all steps, leading to methodological recommendations; 2) confirmation or revision of the diagnostic interpretation of the data obtained by the user in individual cases; and 3) rescaling of DNA data for exchange with multicenter data bases.

Concerning quality control, this tool may be used at different levels of the measurement and diagnostic process. All appropriate protocols for quality assurance recommended by ESACP have been implemented into this server. It works completely independently of any commercially available cytometric device. Its functionality is based on the latest scientific knowledge and consensus agreement in the field. Thus, the server is an objective tool for an international methodologic standard.

Conclusions

Increasing amounts of biologic evidence and clinical data confirm the utility of DNA-ICM as an adjuvant method suitable for the diagnosis and prognosis of cervical intraepithelial lesions and invasive cervical carcinoma. Formerly a research tool, standardized DNA-ICM has become a useful and low-

cost laboratory method for objectively and reproducibly establishing an early diagnosis of prospectively progressive cervical intraepithelial lesions at a high-quality level. It may further contribute to the assessment of relevant prognostic parameters and to the monitoring of treatment for patients with invasive cervical carcinoma.

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Identification of Progressive Cervical Epithelial Cell Abnormalities Using DNA Image Cytometry

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Received February 23, 2004; revision received July 20, 2004; accepted August 9, 2004.

BACKGROUND. The objectives of the current study were to compare the capabilities of conventional cervical cytology and of DNA image cytometry (DNA-ICM) in the prediction of progressive or regressive behavior in atypical squamous cells (ASC), low-grade squamous intraepithelial lesions (LSIL), and atypical glandular cells (AGC).

METHODS. One hundred ninety-six women with Papanicolaou (Pap) smears that yielded diagnoses of ASC, LSIL, or AGC were included in a prospective cohort study. Slides were classified according to the Bethesda system. DNA-ICM was performed according to the consensus reports of the European Society of Analytical Cellular Pathology.

RESULTS. Reference standard verification was available in 108 patients. The rate of DNA aneuploidy in Pap smears increased significantly from cervical intraepithelial neoplasia 1 (CIN1) (54%) and CIN2 (64.3%) to CIN3 or greater (CIN3+) (83.3%) in subsequent biopsies ($P < 0.05$). Using ASC, LSIL, and AGC as input cytologic diagnoses and $\geq$ CIN2 as the output histologic diagnosis, the positive predictive values (PPVs) for conventional cytology and DNA-ICM were 35.2% and 65.9%, respectively ($P < 0.001$). The negative predictive value (NPV) of DNA-ICM was 85.0%. When $\geq$ CIN3 was used as the output histologic diagnosis, conventional cytology had a PPV of 22.2%. The PPV and NPV of DNA-ICM were 43.9% and 93.3%, respectively.

CONCLUSIONS. The results of the current study confirmed the prognostic validity of DNA image cytometry for differentiation between progressive and regressive lesions in patients with ASC, LSIL, and AGC diagnoses. ***Cancer (Cancer Cytopathol)* 2004;102:373–9.** *© 2004 American Cancer Society.*

KEYWORDS: DNA image cytometry, atypical squamous cells, squamous intraepithelial lesion, atypical glandular cells, cervical cytology.

Cervical dysplasias are heterogeneous lesions, particularly with respect to their clinical behavior. Neither histologic nor cytologic evaluation can predict whether dysplastic cells will progress to carcinoma in an individual patient. Approximately 15–30% of all women who have low-grade squamous intraepithelial lesions (LSILs) on cervical cytology will have moderate or severe cervical intraepithelial neoplasia (CIN) identified on a subsequent cervical biopsy.[1,2] Consequently, in cervical cancer screening, large numbers of control procedures are performed, including conizations without evidence of CIN2, CIN3, or invasive carcinoma.

DNA aneuploidy, as measured by DNA image cytometry (DNA-ICM), represents the quantitative cytometric equivalent of chromosomal aneuploidy and has been accepted internationally as a well standardized marker of neoplastic cell transformation.[3–6] Various studies have demonstrated that it indicates either invasive carcinoma

DOI 10.1002/cncr.20644
Published online 3 November 2004 in Wiley InterScience (www.interscience.wiley.com).

or prospectively neoplastic development in cervical dysplasia.[7–11] In addition, there is growing molecular biologic evidence that aneuploidy may play a causal role in carcinogenesis.[12] The International Consensus Conference on the Fight Against Cervical Cancer International Academy of Cytology (IAC) Task Force 8 recommended DNA-ICM as a useful adjunctive method for identifying cervical intraepithelial lesions, which require further clinical management.[13]

To the best of our knowledge, all previous studies on DNA-ICM of cervical dysplasias have been retrospective.[7–11] The objective of the current study was to investigate whether DNA-ICM significantly improved the diagnostic accuracy of Papanicolaou (Pap) testing in a prospective cohort of women with atypical squamous cells (ASC), LSIL, or atypical glandular cells (AGC).

MATERIALS AND METHODS

Patients

Between June 1996 and November 2003, Pap smears from 274 women yielded diagnoses of ASC, LSIL, or AGC at the Institute for Cytopathology, University of Düsseldorf (Düsseldorf, Germany). Cytologic samples were obtained consecutively from routine input at this institution and represent $< 3\%$ of the total workload, which is in line with diagnostic practice in Germany.[14] The diagnostic category ASC was used restrictively. In 78 patients, DNA-ICM was not performed because this procedure was not requested by the patients' gynecologists. The remaining 196 patients, who were referred from a total of 28 different institutions, were included in the current study (ASC, $n = 35$; LSIL, $n = 130$; AGC, $n = 31$). More than 1 diagnostic cytology/DNA-ICM study was performed in 24 of 196 patients. For these 24 patients, only the first DNA histogram was considered for the current study to avoid biases resulting from multiple testing of the same patient. The median patient age was 39 years (range, 16–78 years).

Sample Processing and Assessment

Samples from the uterine cervix were obtained using an Ayre spatula or a Cervex Brush (CooperSurgical, Trumbull, CT). Colposcopy generally was not performed. The specimens were fixed in alcohol, subjected to Pap staining, and screened by two of the authors (H.J.G. and H.V.Q.N.) or by medical technical assistants. Each abnormal smear also was examined by an experienced cytopathologist (A.B.) before a final diagnosis was rendered. The 1991 and 2001 Bethesda systems were used for cytologic classification.[15,16] The diagnoses of specimens that had been evaluated before publication of the 2001 Bethesda system were updated according to the revised nomenclature in the final compilation of the data.

Directly after morphologic investigation, the smears underwent destaining and restaining according to the method described by Feulgen.[17] Measurements of nuclear DNA content were performed as described previously using a computer-based image analysis system consisting of a Zeiss Axioplan 2 microscope (Zeiss, Jena, Germany) with a 40× objective (numeric aperture, 0.75; Köhler illumination) and a charge-coupled device black-and-white video camera with 572 lines of resolution (VariCam CCIR; PCO Computer Optics, Kehlheim, Germany).[18]

The software package used in the current study was the AutoCyte QUIC-DNA-Workstation (AutoCyte Inc., Burlington, NC), which provides shading and glare correction. The latter was performed at a rate of 2.2%. In each case, at least 30 intermediate squamous cells with normal appearance were measured as internal reference cells. Using squamous cells as an internal reference, latent human papillomavirus (HPV) infection must be considered as a potential cause of a slightly changed peridiploid DNA content.[6,19] Because a clonal change would be unlikely in cells with normal appearance, latent viral infection should increase the coefficient of variation of reference cells rather than shifting the respective DNA histogram peak. The former potential confounder was limited in the current study, because the coefficient of variation for reference cells was always $\leq 5\%$. At least 200 epithelial cells with abnormal (i.e., hyperchromatic), enlarged, or polymorphic nuclei were measured, starting with encircled areas. To increase the detection rate of 9*c*–exceeding events (9cEEs), all Feulgen-stained smears were checked during measurement. All technical instruments and all software used in the study met the standard requirements of the consensus reports of the European Society for Analytical Cellular Pathology (ESACP).[3–6]

A number of parameters were assessed for diagnostic interpretation.[3–6] *DNA stemline* is the G0/G1 cell phase fraction for a proliferating cell population (with a first peak and a second doubling peak or with nuclei in the doubling region). *DNA stemline ploidy* was defined as the modal value of a DNA stemline in *c* units (*c* = DNA content). DNA stemline aneuploidy was assumed if the modal value of a stemline was $< 1.80c$ or $> 2.20c$ and $< 3.60c$ or $> 4.40c$. *Rare DNA events* included the 9cEEs, which were defined as the number of cells with a DNA content $> 9c$. *Single-cell aneuploidy* was diagnosed when at least 1 cell per slide had DNA content $> 9c$ (9cEE > 1).[8] Single-cell aneuploidy was not applicable in patients who had a his-

tory of radiotherapy, because radiotherapy can induce polyploidization in excess of 9*c*.[20]

Follow-Up

Follow-up information was retrieved from a questionnaire, which was sent to patients' gynecologists. The reference standard was histologic examination. Histologic diagnoses were classified according to the CIN system.[21] In accordance with American Cancer Society guidelines, comparison with cytologic results was acceptable as a reference standard under defined circumstances.[22] We considered cytologic follow-up of at least 6 months to be equivalent to the reference standard if at least 2 consecutive Pap smears agreed with respect to the presence or absence of progressive disease. Final data retrieval of follow-up histology and cytology was carried out in December 2003.

Statistical Analysis

The diagnostic validity of cytologic or DNA-ICM categories within the study sample was evaluated by calculating prevalence rates and predictive values. Differences in proportions were evaluated using the chi-square test and the McNemar paired chi-square test. The level of significance was set at $P < 0.05$.

RESULTS

Descriptive Statistics

Among the 196 patients examined, 89 had a history of abnormal pathologic findings with respect to the genital tract preceding the Pap smear investigated in the current study. These findings included uterine malignancy (cervical carcinoma, $n = 8$; endometrioid carcinoma, $n = 1$; leiomyosarcoma of the uterus, $n = 1$; non-Hodgkin lymphoma, $n = 1$), hysterectomy for other reasons ($n = 3$), conization ($n = 8$), and abnormal Pap smear findings made elsewhere ($n = 67$). Altogether, hysterectomy and/or irradiation had previously been performed in 14 patients (hysterectomy, $n = 10$; irradiation, $n = 7$). Reference standards were available for 108 patients (biopsy, $n = 2$; conization, $n = 49$; curettage, $n = 2$; hysterectomy, $n = 6$; cytologic follow-up of at least 6 months with at least 2 concurring Pap smears, $n = 50$). With two exceptions, all patients who were without progressive disease on cytologic follow-up presented with two normal Pap smears as controls; the two exceptions were patients who had irradiation-induced dysplasia, which remained stable. In 34 patients, cytologic follow-up did not meet the criteria for equivalence to the reference standard (i.e., duration > 6 months, with 2 concurring Pap smears). Fifty-four patients were lost to follow-up because they did not revisit their gynecologist for further diagnostic procedures.

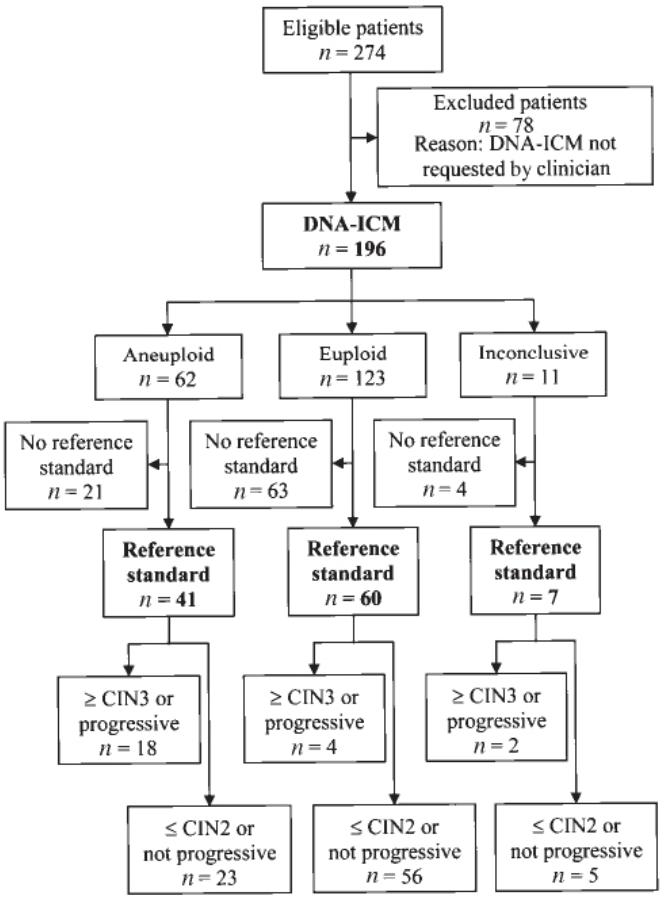

FIGURE 1. Flow chart illustrating the flow of participants within the study. ICM: image cytometry; CIN: cervical intraepithelial neoplasia; *c*: DNA content.

The median interval between the initial cytologic/DNA-ICM diagnosis and its histologic verification was 3 months (range, 1–30 months). In patients who had cytologic findings that were equivalent to the reference standard, the median follow-up was 25 months (range, 6–66 months). Figure 1 depicts the design of the study and the flow of participants using the standardized flowchart suggested by the Standards for Reporting of Diagnostic Accuracy initiative.[23]

Prevalence of DNA Aneuploidy

The overall prevalence of DNA aneuploidy in the current prospective cohort of 196 patients with ASC, LSIL, or AGC was 31.6% (Table 1). The prevalence of DNA aneuploidy differed with respect to cytologic subgroup and was correlated with the grade of histologically proven CIN, although this trend failed to reach statistical significance (chi-square ≤ 1.55; $P \geq 0.11$). The highest rate of stemline aneuploidy was observed in patients with AGC (chi-square ≥ 3.73; $P < 0.05$). Altogether, single-cell aneuploidy was the most frequent type of aneuploidy detected in 85.5% of all patients who had abnormal DNA distributions (Table 2).

TABLE 1
DNA Aneuploidy in the Various Cytologic Subgroups

		DNA-ICM (%)				Outcome (PPV [%])		
Cytology	No. of cases	Single-cell aneuploidy	STL aneuploidy	Single-cell and STL aneuploidy	Total	N_{RS}	≥ CIN2	≥ CIN3
ASC	35	7 (20.0)	1 (2.9)	1 (2.9)	7 (20.0)	7	1 (14.3)	1 (14.3)
LSIL	130	39 (30.0)	11 (8.5)	7 (5.4)	43 (33.1)	84	29 (34.5)	17 (20.2)
AGC	31	7 (22.6)	7 (22.6)	2 (6.5)	12 (38.7)	17	8 (47.1)	6 (35.3)
Total	196	53 (27.0)	19 (9.7)	10 (5.1)	62 (31.6)	108	38 (35.2)	24 (22.2)

DNA-ICM: DNA image cytometry; PPV: positive predictive value; STL: stemline; N_{RS}: number of samples with reference standard available; CIN: cervical intraepithelial neoplasia; ASC: atypical squamous cells; LSIL: low-grade squamous intraepithelial lesion; AGC: atypical glandular cells.

TABLE 2
Positive and Negative Predictive Values for DNA Image Cytometry

				Outcome (%)	
DNA-ICM finding	No. of cases	No. of aneuploid samples (%)	N_{RS}	≥ CIN2	≥ CIN3
Aneuploid	62	62/62 (100.0)	41	65.9[a]	43.9[a]
Single-cell aneuploidy	53	53/62 (85.5)	34	61.8[a]	41.2[a]
STL aneuploidy	19	19/62 (30.7)	13	92.3[a]	76.3[a]
Single-cell and STL aneuploidy	10	10/62 (16.1)	6	100.0[a]	100.0[a]
Euploid	123	—	60	85.0[b]	93.3[b]

DNA-ICM: DNA image cytometry; N_{RS}: number of samples with reference standard available; CIN: cervical intraepithelial neoplasia; STL: stemline.
[a] Positive predictive value.
[b] Negative predictive value.

TABLE 3
Prevalence of DNA Aneuploidy in Association with Histologic Follow-Up

Histology	No. of cases	Single-cell aneuploidy (%)	STL aneuploidy (%)	Single-cell and STL aneuploidy (%)	All aneuploidy (%)
WNL	8	2 (25.0)	—	—	2 (25.0)
CIN1	13	6 (46.2)	1 (7.7)	—	7 (53.9)
CIN2	14	7 (50.0)	2 (14.3)	—	9 (64.3)
CIN3	23	13 (56.5)	10 (54.2)	6 (26.9)	19 (82.6)
Invasive carcinoma	1	1 (100.0)	—	—	1 (100.0)

STL: stemline; WNL: within normal limits; CIN: cervical intraepithelial neoplasia.

Cytologic/Histologic Follow-Up and DNA Aneuploidy

ASC/LSIL and AGC eventually were found to be CIN3 or invasive carcinoma in 18 of 91 patients (19.8%) and 6 of 17 patients (35.3%), respectively (Table 1). Histologic examination was the reference standard in all cases of progressive disease—i.e., no patient with cytologic follow-up of at least 6 months and at least 2 concurring Pap smears had a progressive lesion. The proof of DNA aneuploidy prompted some of the clinicians to seek histologic verification, even in patients with ASC or LSIL. Table 3 describes the correlation between histologic diagnoses and DNA-ICM results on preceding Pap smears. The prevalence of DNA aneuploidy increased from 54% in patients with CIN1 to 64.3% in patients with CIN2 and to 83.3% in patients with ≥ CIN3 (CIN1 and CIN2 vs. ≥ CIN3: chi-square = 3.71; $P < 0.05$). The combination of single-cell aneuploidy and stemline aneuploidy was observed exclusively in patients with CIN3. No aneuploidy was found in 4 patients who had histologically proven CIN3. In

these patients, the median interval between DNA-ICM and conization was 15 months (range, 8–23 months), considerably longer than the corresponding interval in the remaining patients for whom histology was the reference standard. Two of four patients exhibited aneuploidy on repeat DNA-ICM before conization. In two patients who had single-cell aneuploidy, histologic examination did not reveal dysplasia. One of these patients, who had ASC, was diagnosed with follicular cervicitis at biopsy; further cytologic follow-up revealed ASC once again but was negative for the presence of an intraepithelial lesion 14 months after biopsy. In the other patient, single-cell aneuploidy was diagnosed, as DNA-ICM detected 1 cell with a DNA content of 9.2*c*; subsequent investigation disclosed that this patient's specimen was sent to our laboratory for a second opinion due to the discrepancy between abnormal cytology (AGC) and negative histology after the patient underwent dilatation and curettage. No further follow-up data were available. Four of the 7 patients who had previously received irradiation had polyploid histograms with 9cEEs (maximum, 28*c*). All seven histograms were evaluated as being indicative of DNA euploidy, because no stemline aneuploidy was present. Five of these seven patients had lesions that were not progressive; for the remaining two, no reference standard was available.

Diagnostic Accuracy of Cytology and DNA-ICM

The diagnostic accuracy of cytologic and DNA-ICM diagnoses was calculated by comparing initial cytologic/DNA-ICM findings with follow-up findings as evaluated by cytology and/or histology (Tables 1, 2). When CIN2+ was used as the output histologic diagnosis, the positive predictive values (PPVs) for conventional cytology and DNA-ICM were 35.2% and 65.9%, respectively. When CIN3+ was selected as the output histologic diagnosis, the PPVs were 22.2% for cytology and 43.9% for DNA-ICM. The improvement in PPV yielded by DNA-ICM was highly statistically significant for each patient (paired chi-square $\geq$ 29.4; $P < 0.001$). Like PPV, the negative predictive value (NPV) of DNA-ICM was dependent on output criteria (Table 2). Because the initial cytologic diagnosis of all study smears was at least ASC, it was not possible to calculate NPVs for cytologic diagnoses, nor was it possible to calculate sensitivity and specificity for cytology and DNA-ICM.

DISCUSSION

In the current study, we demonstrated that the use of DNA-ICM on Pap smears that yielded diagnoses of ASC, LSIL, or AGC significantly improved the diagnostic accuracy of conventional cytology. When CIN2+ was used as the output histologic diagnosis, the PPVs of conventional cytology and DNA-ICM were 35% and 66%, respectively ($P < 0.001$). In addition, DNA-ICM yielded a relatively high NPV (85%). To our knowledge, the current investigation is the first prospective cohort study of the diagnostic accuracy of DNA-ICM in cervical cytology. Previous studies applied a retrospective design and reported high PPVs (84–100%) for the development of in situ carcinoma or invasive carcinoma from mild/moderate cervical dysplasias with proven DNA aneuploidy.[7–11] However, several factors, such as differences in definitions of input and output criteria, latency periods between Pap smear and histologic diagnosis, sampling error, subjectivity of morphologic diagnoses, acquisition and work-up of biopsy tissues, definitions of reference standards, and DNA-ICM algorithms and procedures, limit our ability to compare these studies with the current one.

Predictive values largely depend on the definition of diagnostic input and output criteria. Cytologic diagnoses of $\geq$ ASC or $\geq$ LSIL represent commonly used input criteria, and histologic diagnoses of $\geq$ CIN2, $\geq$ CIN3, or high-grade squamous intraepithelial lesion (HSIL) commonly serve as output criteria.[1,2,24] In the current study, the use of ASC and LSIL yielded PPVs ranging from 14.3% to 34.5%, values that are in accordance with previous studies on the diagnostic accuracy of conventional cytology that used the same input and output criteria.[1,2,24] Another important confounding variable is the latency period between Pap smear and histologic verification. Because the interval required for a mild or moderate squamous dysplasia cytologically diagnosed on a Pap smear to develop into histologically proven carcinoma may range from months to as long as 10 years,[25] a simultaneously obtained histologic diagnosis may not be adequate for an accurate assessment of the predictive value of a cytologic diagnosis or an adjuvant method. In the current study, the median interval between the initial cytologic/DNA-ICM diagnosis and subsequent biopsy was only 3 months (range, 1–23 months). In contrast, the interval between detection of DNA aneuploidy and histologic follow-up in previous retrospective studies has been reported to be 1–3 years.[7–11] Sudbø et al. demonstrated that although only 10% of all DNA-aneuploid oral squamous dysplasias progressed to invasive carcinoma after 1 year, the progression rate increased to 90% after 5 years.[26]

Sampling errors remain the principal source of discrepancy between cytologic and histologic findings.[27] Biopsies of small, discrete lesions can be performed correctly only under colposcopic visualization. This procedure generally was not performed in the patients investigated in the current study. Koss

pointed out that low-grade intraepithelial lesions may develop peripherally with respect to high-grade lesions or carcinoma in situ or at sites adjacent to invasive malignancies.[28] However, the high-grade lesion, carcinoma in situ, or invasive malignancy in question may not be represented in a given smear, which may only contains cells from the accompanying low-grade intraepithelial lesion; this type of error is known as a *geographic error*. Geographic errors in cell sampling may also explain the observed discrepancies between cytologic/DNA-ICM findings and histologic findings in the current series.

Morphologic criteria for cervical dysplasias in cytology and histology are used inconsistently. Many authors reported poor intraobserver and interobserver reproducibility of histologic and cytologic diagnoses in pathology specimens of the uterine cervix.[2,29,30] A correct cytologic diagnosis may be followed by an incorrect histologic diagnosis, and vice versa. The subjectivity of morphologic diagnoses, therefore, may influence the results of studies on the diagnostic accuracy of cervical cytology and DNA-ICM. In our experience, thorough examination of the entire Feulgen-stained slide (i.e., not only encircled areas) often leads to a dramatic increase in the number of abnormal cells detected. Most of these cells show only minor abnormalities, but cells with major abnormalities also are detected in this way, thereby increasing the detection rate of 9cEEs. A major drawback of the current study was that Pap smears were recruited from 28 different institutions. Consequently, subsequent pathologic work-ups and evaluations of histologic material were not standardized. We attempted to organize such a standardized review but were unable to do so. However, the results of the current study reflect the predictive value of DNA-ICM in a routine setting, rather than its potential validity under experimental conditions.

Before 1995, DNA-ICM results were largely dependent on test procedures and diagnostic algorithms. This made older DNA-ICM studies of cervical dysplasia difficult to interpret. Since 1995, the standardization of DNA-ICM has been promoted by 4 consecutive consensus reports of the ESACP.[3–6] Recently, Nguyen et al. reported a 94% interobserver correlation with respect to the detection of DNA aneuploidy in Pap smears.[31] This correlation is at least 20% greater than the rates reported in the literature for histologic or cytologic diagnoses of cervical dysplasias based on morphology alone.[29,30] Thus, it appears that standardized DNA-ICM represents an objective and a highly reproducible diagnostic procedure.

DNA stemline aneuploidy reflects the clonal expansion of cells with distinct chromosomal aneuploidy. Abnormal stemlines have been reported in 41–85% of invasive cervical carcinomas and have exhibited some degree of correlation with tumor grade and histologic subtype.[32–34] In addition to being a sign of stemline abnormality, *rare events* may indicate DNA aneuploidy. These rare events are likely to be attributable to nonproliferating abnormal cells with different chromosomal aneuploidies and abnormally high numbers of chromosomes.[19] Therefore, rare events may serve as markers of malignant cell transformation, even if they are not relevant to tumor growth. This may explain the considerably higher PPV associated with stemline aneuploidy compared with single-cell aneuploidy (92% vs. 62% for $\geq$ CIN2). It is noteworthy that the highest PPV observed was associated with combined stemline and single-cell aneuploidy (100% for CIN2+).

HPV DNA testing is another adjuvant method that can be used to increase diagnostic accuracy in cervical cancer screening. High-risk HPV detection has a very high NPV, ranging from 98.9% to 99.9% when CIN2+ is used as an output diagnosis.[24,35,36] However, the PPV associated with positive findings of high-risk HPV is low (19.6–29.1%).[24,35,36] Combining high-risk HPV testing, with its high NPV, and DNA-ICM, with its high PPV, may be a major step toward improved diagnostic accuracy in cervical cancer screening.[37] In a related development, the complementary value of direct sequencing of high-risk HPV and laser-scanning cytometry was recently demonstrated.[38,39]

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DOI: 10.7860/JCDR/2021/45836.14478

Review Article

Obstetrics and Gynaecology Section

Diagnostic Efficacy of DNA Ploidy in Liquid Based Cervical Cytology using DNA Cytometry

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ABSTRACT

Worldwide cervical cancer is the fourth most common cancer in women and high incidence is reported from India. Liquid Based Cytology (LBC) provides good morphology for detection of cellular abnormalities. We, therefore, reviewed diagnostic efficacy of conventional Pap staining, flow cytometry and Human Papilloma Virus (HPV) testing in cervical pre cancer and cancer. Narrative review of cervical pre cancer and cancer candidate biomarkers including Pap staining, HPV and flow cytometry from cervical cytology fluids, is based on a detailed review of the literature. Based on the so far conducted studies, a promising conclusion can be drawn, that cytometry when coupled with HPV DNA typing or the conventional cytology gives better results as compared to that of conventional cytology or DNA cytometry alone. Liquid cytology provides a good and stable source of cervical cells to carry out ploidy studies using DNA cytometry. The procedure should be used in conjunction with LBC and HPV detection.

Keywords: Flow cytometry, High grade squamous intraepithelial lesion, Human papilloma virus, Low grade squamous intraepithelial lesion, Squamous cell carcinoma

INTRODUCTION

LBC has been shown to be more effective than the conventional Pap smear for screening of cervical cancer by significantly improving detection of low-grade and high-grade squamous intraepithelial lesions and a significant improvement in specimen adequacy [1]. The greatest advantage of LBC is that ancillary techniques like HPV testing and DNA ploidy can be performed on the remainder left over sample in the vial. DNA ploidy has been effectively performed for diagnostic and prognostic applications of cervical, ovarian and endometrial cancer. Image cytometry is the most commonly used cytometry technique, others include laser scanning cytometry and flow cytometry. Researchers have utilised liquid based preparation for measurement of DNA content of cervical epithelial cells as it provides satisfactory monolayer for DNA measurement. For flow Cytometric DNA analysis, cells in suspension are required which may be prepared from cervical biopsies or LBC sample. Cell cycle analysis on LBC samples provides useful information for selecting women with chance of developing lesion (Aneuploidy or High S phase fraction) [2-4]. This article provides a review of various Cytometric researches aimed at studying DNA ploidy in cervical cytology samples and to evaluate whether LBC proves to be a suitable sample type for ploidy studies.

Cell Cycle and DNA Ploidy

DNA content of a cell is an essential tool to monitor cell proliferation, cell cycle and DNA ploidy. Cell division undergoes through various phases which form the cell cycle with different amount of DNA content in each phase. Before the cell division starts, the cell remains in a resting phase, known as the G0 phase. As soon as the cell receives signal, the cell starts proliferating and enters G1 phase. In this phase, the cells are diploid and the chromosome number is 2N. The cell then enters S phase which is called the synthesis phase and where the DNA replicates. Replication leads to tetraploidy which contains double the amount of DNA content. This is followed by G2 phase when cell prepares for division and enters the mitosis M phase. The cell in a cell cycle has to overcome two checkpoints G1/S and G2/M. At these checkpoints the cells are checked for DNA damage. These checkpoints prevent the cell to enter into S and M phase, respectively until the damage is repaired. In normal steady state conditions and in low grade/ early lesions, 85% cells are in G0/G1 phase and 15% are in G2/M phase. This anomaly can serve as an efficient diagnostic tool to detect cancer in cells at an early stage [5].

Burden of Cervical Cancer

In women, cancer of the cervix is the 4th most common cancer with 528,000 new cases and 266,000 deaths in 2012. This accounted for 7.5% of all female cancer deaths in 2012. Ninety percent of deaths due to cervical cancer occur in developing regions. The occurrence and mortality due to cervical cancer is highest in Africa and Melanesia [6].

It was estimated in 2015 that every year 122,844 women are diagnosed with cancer of cervix and 67,477 deaths are contributed by cervical cancer in India. It is 2nd most common cancer in females of reproductive age group. In general population, 5% women are expected to harbour HPV-16/18 infection, and most of the invasive cervical cancers (83.1%) are attributed to HPV-16/18 [7].

Cervical Cancer Screening and Diagnosis

Invasive squamous carcinoma of the cervix is the result of pre-invasive lesions known as Cervical Intraepithelial Neoplasia (CIN). In histology CIN is graded as mild dysplasia (CIN 1), moderate dysplasia (CIN 2) and severe dysplasia (CIN 3). Out of these CIN 1 and 2 may regress but CIN 3 progresses to the invasive carcinoma [8,9]. The Bethesda system has improved the reporting and classified it as Negative for intraepithelial lesion or malignancy (NILM), Atypical Squamous Cells of Undetermined Significance (ASC-US), Low grade Squamous Intraepithelial Lesions (LSIL), High Grade SIL (HSIL) and Invasive Carcinoma [10].

Cervical cancer is caused by the HPV infection which induces CIN lesions in the cervix [11-13]. Dysregulated viral oncogene expression caused by integration of viral oncogene in affected cells results in chromosomal instability, aneuploidization and progression of the disease [14]. Epigenetic changes and interference of the viral oncogene in the normal cell cycle may also lead to variation in nuclear DNA content [12,15]. There is evidence that chromosomal instability and aneuploidisation precede and favour high risk HPV genome [14]. Studies have shown that the variation in the ploidy content indicates invasive carcinoma or prospective neoplastic development in cervical

dysplasia [16,17]. However, there is no technique which can predict cervical dysplasia clinically with high sensitivity.

Pap test plays important role in screening of cervical carcinoma; however its sensitivity and specificity is limited. It has been reported by Sulik SM et al., (2001) that LBC is more sensitive (90%; 95% CI 77-96%) compared to conventional cytology (79%; 95% CI: 59-91%) for CIN 2 or more severe lesions [18]. LBC has been found to be equivalent or superior to conventional cytology for CIN diagnosis. False positive rate of pre-malignant and malignant lesions by Pap test is approximately 30% and false-negative rate lies between 6-55% [19-23].

Analysis of cervical biopsies has shown that women who develop LSIL have a probability to develop moderate to severe CIN [13]. To diagnose and prevent cervical malignancy a number of diagnostic techniques have been developed. One such technique for the assessment of DNA ploidy to detect cervical dysplasia is DNA cytometry. DNA ploidy has been identified as a prognostic factor for estimation of risk of progression of cervical lesions to invasive cervical carcinoma [24-29]. Aneuploidy aids in identification of dysplasia and provides a predictive value for malignant transformation [30]. Cytometric techniques provide additional information for identification of dysplasia and neoplasia beyond morphology.

Methodology for DNA Ploidy Estimation and Interpretation of Results

DNA image cytometry: Several researchers have utilised the method described below with minor variations to estimate DNA ploidy in LBC. After preparing a second monolayer from the remaining LBC sample, slides are air dried and fixed in buffered formalin for 30 minutes. Following 1-hour acid hydrolysis (5N hydrochloric acid) at room temperature staining with Feulgen (Thionin) is carried out. For calibration of each staining procedure calibration slides are added. Image cytometry is then performed using ploidy measurement software on image cytometer. By and large the interpretation of DNA histogram is similar in all studies with recognition of diploid, polyploid, aneuploid peaks and S Phase fraction. Some researchers have suggested minor modifications in interpretation which are as follows: Auer GU et al., (1980) presented the DNA ploidy value as a "c" for chromosome [31]. The DNA cytometry histogram was classified as normal or suspect; normal corresponding to diploid with low proliferation fraction and polyploid (diploid + tetraploid) histograms without any cells exceeding 5c. All other histograms with any of these were regarded as suspect and patients with suspect results underwent colposcopy.

1. Any cells with DNA content >5c
2. Diploid cells with >10% cells in proliferation fraction
3. Aneuploid cell population

Study of Bollmann M et al., 2006, suggested interpretation of the DNA histogram which is as follows:

1. Diploid as DNA peak between 1.8c and 2.2c.
2. Minimum of 2 stem lines with DNA peaks between 1.8c-2.2c and 3.6-4.4c or around 8c and 16c to be read as polypoid.
3. DNA peaks beyond "diploid" or "Polyploid" peaks and/or presence of single cells with DNA content >9c to be read as aneuploid [32].

Further variation in the histogram interpretation was suggested by Guillaud M et al., 2006 [33]. They defined DNA aneuploidy as a function of three parameters:

1. Total number of counted cells on a slide;
2. A DNA ploidy index, beyond which a cell is called aneuploid; and
3. A cut-off value presenting the number of cells, beyond which a specimen is called aneuploid.

The DNA ploidy index was determined within the range of 2c-9c. The aneuploid cells were determined in the range of 1-50 cells. The sensitivity and specificity was calculated by combining the above definitions to find the best diagnostic accuracy. In a number of studies, 2c DNA content is defined as a diploid cell, 4c as tetraploid cell and 5c as a cut off for aneuploid cell, however Bollmann R et al., Bollmann R et al., and Lorenzeto M et al., suggest 9c [34-36]. Number of cells with DNA exceeding beyond 5c is frequently called the 5c-exceeding rate (5cER) [37].

Flow cytometry for DNA ploidy estimation: Researchers have analysed DNA ploidy by flow cytometry in various solid tumours and LBC samples [38-44]. Single cell suspension was prepared by mechanical or enzymatic disaggregation of the tissue followed by staining with Propidium Iodide (PI) containing Ribonuclease (RNase) for 30-60 minutes at 4°C before analysing on flow cytometer. Gates were set up on FL2W versus FL2A dot plot to exclude doublets and aggregates. FL2A area signals were then used to generate single parameter DNA histograms. Usually two major peaks are observed; one peak is labelled as diploid and another one as an aneuploid (if present). A sample with single G0/G1 peak is defined as diploid, while a sample with two distinct G0/G1 peaks is considered as DNA aneuploid. DNA Index (DI) for aneuploid cells is obtained by dividing the mean channel number of the aneuploid G0/G1 peak by the mean channel number of the diploid G0/G1 peak. For diploid cells DI corresponds to 1 while DI≠1.0 defines aneuploidy. Coefficient of Variation (CV) of G0/G1peak is used to check the quality of DNA histogram. Some studies have also used ModFit software for analysis of DNA histogram.

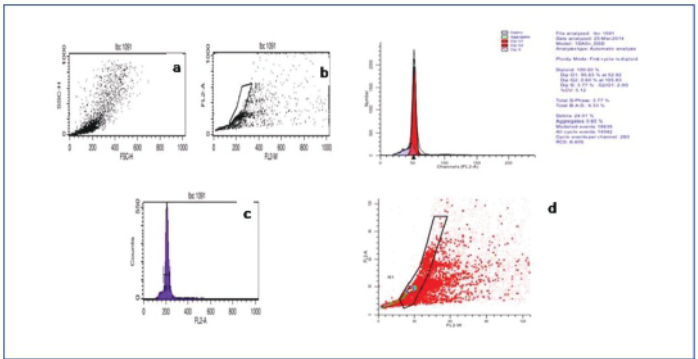
Authors have successfully standardised flow cytometry to assess DNA ploidy in LBC samples of cervical pre cancer and cancer. Cytologically confirmed cases of LSIL, HSIL and SCC along with cases negative for intraepithelial lesion or malignancies (NILM) were used as control for DNA ploidy analysis. Briefly, LBC samples were centrifuged to obtain a cell pellet and washed with equal volume of Phosphate buffer saline (PBS, pH-7.4). Cells were stained with Telford reagent and processed as per Mishra S et al., [45]. Stained cells were acquired using flow cytometer and dot plot and histograms as shown in [Table/Fig-1a,b,2a,b]. Diploid samples were identified by the presence of single G0/G1 peak [Table/Fig-1c,d], while aneuploidy was defined when DI≠1.0 [Table/Fig-2c,d].

Laser Scanning Cytometry

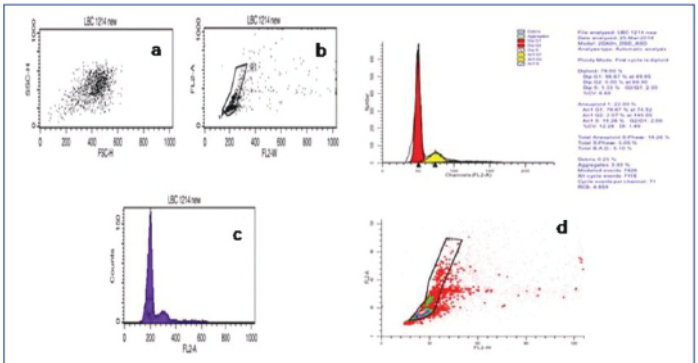
This technique uses the 2nd monolayer slide stained with Propidium iodide and RNase for 1 hour at 37°C. After the incubation slides were mounted in glycerol and covered with glass. Using the laser scanning cytometer, at least 10,000 cells were measured and diploid and aneuploid cells were defined as per the first peak intensity of DNA histogram containing normal leucocytes. The Coefficients Of Variation (CV) were reported in a range between 4.0 and 7.5. Cells with elevated DNA content as stained by PI (>5c and >9c) were individually evaluated. Haroske G et al., defines, isolated cells with non-superficial cell morphology and a DNA content of greater than 9c as "Rare cells" with abnormally high DNA content [30].

Evaluation of Ploidy as a Diagnostic Procedure in Cervical Cancer

DNA ploidy measurement has been established as a prognostic factor and to be of prognostic significance in ovarian and endometrial cancer though in cervical cancer there are conflicting results [46-52]. In few studies, flow cytometric analysis of DNA ploidy in CIN and invasive cervical has been reported to have prognostic significance for estimation of disease progression into more advanced lesion [38]. The published researches aimed to study the value of DNA ploidy by image cytometry as well as flow cytometry on LBC and solid tissue are summarised in the [Table/Fig-3]. According to most of the studies, HPV typing and DNA ploidy measurement helps in the identification of cytologic dysplasia. LBC has proven to be suitable and useful tool for performing DNA ploidy.



[Table/Fig-1]: Shows the acquisition of cervical epithelial cells on flow cytometer, stained with Telford Reagent. (a-c) Shows acquisition of stained cells on FSC vs. SSC, FL2-A vs. FL2-W and FL2-A vs. Count on Cell Quest Pro software (B.D Biosciences, Singapore). (d) Shows the analysis of acquired FCS file on ModFit LT 3.2 (Verity Software House). Based on ModFit analysis case was found to be Diploid with single G0/G1 peak. Histogram statistics showed on top right.
Image courtesy Mishra et al. [45]



[Table/Fig-2]: Shows an Aneuploid case of HSIL on cytomorphology acquired on flow cytometer, stained with Telford Reagent. (a-c) Shows acquisition of stained cells on FSC vs. SSC, FL2-A vs. FL2-W and FL2-A vs. Count on Cell Quest Pro software (B.D Biosciences, Singapore). (d) Shows the analysis of acquired FCS file on ModFit LT 3.2 (Verity Software House). Based on ModFit analysis case was found to be Aneuploid on appearance of second G0/G1 population to the right of first G0/G1 peak with DNA index of 1.49.
Image courtesy Mishra et al. [45]

DISCUSSION

DNA ploidy has proved to be an effective tool in detecting high grade neoplastic lesions which helps in the early screening of cancer. Compared to conventional cytology, DNA Cytometry has better sensitivity and specificity. Among the various kinds of cytometry, image cytometry has been widely used and has given positive results in detecting neoplastic lesions. Although flow cytometry is a common modality for studying DNA ploidy in cell suspension viz., blood cells and body fluids, there are only few studies available for assessment of DNA ploidy by Flow Cytometry in LBC samples [38,39].

[Table/Fig-3] suggests that LBC sample is suitable enough to study DNA ploidy and other ancillary techniques. In a Study by Saxena M et al., sensitivity and specificity for diploid G0/G1 to discriminate the cases from controls was 96.77% and 100%, however total S phase and aneuploidy revealed 100% sensitivity [39]. In contrast to this, Singh M et al., reported aneuploidy in 51.31% mild, 77.77% moderate and 91.66% severe cases. In ASCUS, aneuploidy was found in 14.03% cases and interstingly, in 8.69% of controls [38]. Authors further suggested that cases which were found aneuploid should be followed-up for developing advanced grade lesion.

When both cytometry and conventional cytology tests are considered in combination, the figures rise up to 100% and 91.8%, respectively. Though these additional tests improve the sensitivity and specificity, it increases the cost. DNA ploidy analysis appears to be an attractive technology for established programs [33].

[Table/Fig-4] represents the diagnostic efficacies of various techniques used for the diagnosis of pre cancer/cancer in a cervical sample and suggests a diagnostic algorithm for cervical cancer screening. The LBC is much sensitive and specific as compared to the conventional cytology. Depending on the grade of intraepithelial neoplasia, further workup on HPV testing or DNA ploidy can be carried out. As seen in the [Table/Fig-3] sensitivity of HPV testing is high whereas the specificity of DNA ploidy is high, hence as proposed by various authors DNA cytometry when used in association with HPV testing or conventional cytology gives a better sensitivity and specificity [34-37,53]. Apart from these techniques, some other techniques have also been successfully tried on LBC samples and they are immunocytology using $p16^{INK4a}$ marker and HPV E6/E7 mRNA detection [54]. They can be used along with the other techniques to increase the diagnostic accuracy [55-58].

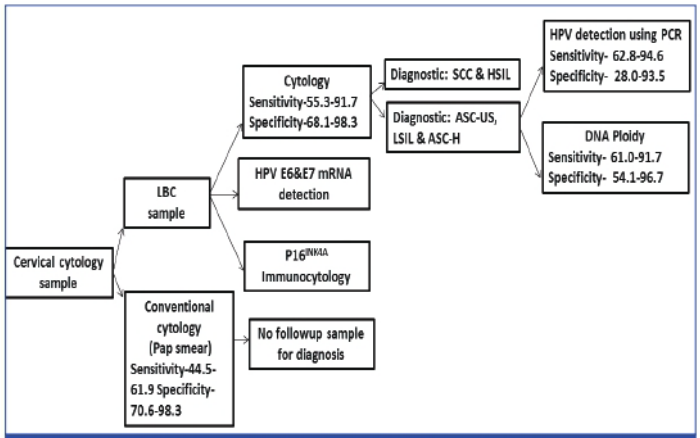
CONCLUSION(S)

Cytometry when coupled with HPV DNA typing or the conventional cytology gives better results as compared to that of conventional cytology or DNA cytometry alone. Thus, LBC media provide a good and stable source of cervical cells to carry out ploidy studies using DNA Cytometry. The procedure should be used in conjunction with LBC and HPV detection. Liquid based preparation allows to measure DNA content of cervical epithelial cells that provides more accurate and sensitive result which can alternatively serve as a marker for early stage diagnosis. Use of LBC sample for measurement of DNA content of cervical epithelial cells in the form of aneuploidy or high S

S. No.	Author (years)	Objective of the study	Number of cases	Result	Conclusion
1.	Lorenzato M et al. (2002) [36]	To study the usefulness of DNA ploidy measurement on LBC smears showing conflicting results between cytology and HR-HPV typing using Image Cytometry	Total 7944 cases out which 984 underwent ploidy	Normal DNA profile predicted clearance of HPV with sensitivity 81.5%, specificity 45.4%, PPV 69% and NPV 62.4% In persistent HR-HPV infection suspected DNA profile PPV increased from 10.8% to 22.7%, for HSIL detection sensitivity was 95.2%	Cytometry should be complemented with HR-HPV test to select women with a high risk for developing a histologic lesion.
2.	Bollmann R et al. (2003) [35]	To determine HPV typing and DNA ploidy of squamous intraepithelial lesions in LBC samples using Laser Scanning Cytometry	112 SIL cases	Out of 112 cases, 110 (98.2%) were HPV+, out of these 95 (84.8%) were HR-HPV+ and 46 out of 95 (48.4%) presented aneuploid squamous cells with >9c DNA content.	Complex analysis of cervical lesions from LBC samples is highly informative HPV typing and DNA ploidy measurement helps in the objectivation of cytologic atypia and both can be performed efficiently from the same LBC sample.
3.	Shirata NK et al. (2003) [55]	To evaluate nuclear DNA content of cervical lesions in LBC specimens using Static Image Cytometry	Total 47 samples out of which CIN1;n=25, CIN2;n=5, CIN3;n=2 and chronic cervicitis=15	Chronic cervicitis All diploid CIN1 44% diploid, 12% tetraploid, 32% aneuploid, 12% polyploid CIN2 60% diploid, 40% aneuploid CIN3 100% aneuploid	LBC proved to be suitable and highly useful for DNA analysis. Discrimination could be made between CIN3 and CIN1,2 but not between CIN1 and CIN2
4.	Guillaud M et al. (2006) [33]	To compare DNA ploidy with HPV-testing and conventional cervical cytology as a primary screening test for HSIL and cancer using Image Cytometry	1555 patients	Cytology Sensitivity 54% Specificity 93% PPV 41% NPV 92% HPV Testing Sensitivity 91% Specificity 80% PPV 70% NPV 90% DNA ploidy Sensitivity 61% Specificity 91% PPV 59% NPV 93%	DNA ploidy shows comparable sensitivity, specificity, PPV and NPV values to conventional cytology and HCII DNA ploidy is semi-automated and can be performed in less than 8 hours.

5.	Yu XR et al., (2011) [56]	To perform cell quantitative analysis of DNA ploidy in cervical cancer screening using Image Cytometry	776 women	Conventional Cytology Sensitivity 61.9% Specificity 98.3% DNA ploidy Sensitivity 83.6% Specificity 96.7%	Automated DNA cytometry may be a useful tool for cervical cancer screening in developed countries and has a competitive sensitivity and specificity compared to conventional cytology.
6.	Tong H et al., (2009) [57]	To perform DNA ploidy cytometry testing for cervical cancer screening in China using Image Cytometry	11,999 women for DNA cytometry testing and 11,994 women for cytologic testing	Diagnosis of cancer: DNA cytometry-40 Cytology-24 Cytometry Sensitivity 91.7% Specificity 54.1% Conventional Cytology Sensitivity 44.5% Specificity 70.6% Cytology and Cytometry Sensitivity 100% Specificity 91.8%	DNA cytometry is more beneficial in mass cervical cancer screening with greater sensitivity and positive predicted value than the conventional cytology testing in the developing countries.
7.	Li Z et al., (2010) [58]	To reduce the false-negative rates of population based cervical screening programs employing conventional cytology in combination with automated DNA Image cytometer	3603 women	Total diagnosis: 51 cases including, 27 CIN2, 16 CIN3 and 8 Invasive cancer cases. Cytology No. of Diagnosis 29 Sensitivity 56.8% Specificity 86.2% DNA Cytometry No. of Diagnosis 38 Sensitivity 74.5% Specificity 81.5% Cytology and Cytometry No. of Diagnosis 42 Sensitivity 82.4% Specificity 81.5%	Screening for high grade neoplastic lesions and cervical cancer by DNA Image cytometer or combination of conventional cytology and DNA Image cytometer is more sensitive than conventional cytology.
8.	Saxena M et al., (2010) [39]	Could addition of DNA content study using flow cytometry improves the detection of cervix cancer	Total of 100 including 38 normal and 62 cancer of cervix cases.	Fraction of Total S phase, Total Aneuploid and G2-M (Diploid) are significantly higher ($p < 0.01$); while G0-G1 (Diploid) and G0-G1 (Aneuploid) are significantly lower ($p < 0.01$) in cancer patients as compared to control. G0-G1 (Diploid) Sensitivity-96.77% Specificity-100% Total S phase or Aneuploid Sensitivity-100% Specificity 100%	G0-G1 (Diploid) may help in the diagnosis of carcinoma of the cervix which correlates well with histologically confirmed varied grading of cervical cancer as well as patient survival.
9.	Singh M et al., (2008) [38]	Study the DNA content by flow cytometry and to compare it with the cytological findings.	184 Cytologically diagnosed cases of mild (79),moderate (36), and severe (12) dysplasia along with 57 cases of ASCUS and 69 controls	Aneuploidy was found in 39/79 of mild, 28/36 of moderate, 11/12 of severe dysplasia, 8/57 of ASCUS and in 6/69 controls.	DNA flow cytometry can detect progressive lesions with the greatest possible sensitivity and specificity.
10.	Melsheimer P et al., (2004) [13]	DNA Aneuploidy and Integration of Human Papillomavirus Type 16 E6/E7 Oncogenes in Intraepithelial Neoplasia and Invasive Squamous Cell Carcinoma of the Cervix Uteri	Total 85 samples out of which CIN1/2 n=20, CIN3 n=50, Cacx=15	DNA aneuploidy CIN1/2= 4/20 CIN3= 16/50 Cacx= 12/15 HPV E6/E7 integration CIN1/2=1/20 CIN3=7/50 Cacx=12/15	Aneuploidization precedes integration of HR-HPV genomes in the progression of cervical dysplasia.
11.	Mishra S et al., (2017) [45]	Flow cytometric Analysis of DNA Ploidy in Liquid Based Cytology of Cervical Pre-cancer and Cancer	50 Cytologically diagnosed cases of Cervical cancer including 10 LSIL, 20 HSIL, 20 SCC and 31NILM cases as control	Mean diploid G1 values lowered significantly ($p<0.0$) while diploid S values were significantly ($p<0.01$) higher in both HSIL and SCC as compared to control	Diploid G1 and diploid S phase analysis do not appear to increase the overall sensitivity and specificity of detection.

[Table/Fig-3]: Summary of the studies assessing DNA Ploidy in LBC samples of cervical cytology and solid tissues.



[Table/Fig-4]: Suggested diagnostic algorithm for cervical cancer screening.

phase fraction provides an objective method to prognosticate and select women who may be developing lesions.

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PLAGIARISM CHECKING METHODS: [Jain H et al.]
- Plagiarism X-checker: Jul 11, 2020
- Manual Googling: Oct 31, 2020
- iThenticate Software: Dec 28, 2020 (10%)

ETYMOLOGY: Author Origin

AUTHOR DECLARATION:
- Financial or Other Competing Interests: None
- Was informed consent obtained from the subjects involved in the study? NA
- For any images presented appropriate consent has been obtained from the subjects. NA

Date of Submission: **Jul 10, 2020**
Date of Peer Review: **Oct 06, 2020**
Date of Acceptance: **Nov 22, 2020**
Date of Publishing: **Feb 01, 2021**

cancers

MDPI

Article

Evaluation of Static DNA Ploidy Analysis Using Conventional Brush Biopsy-Based Cytology Samples as an Adjuvant Diagnostic Tool for the Detection of a Malignant Transformation in Potentially Oral Malignant Diseases: A Prospective Study

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Simple Summary: Oral cancer is one of the most common malignant diseases worldwide. What is particularly challenging is not the visual detection of a change in the oral cavity, since this is a clearly visible area, but rather the classification between benign and malignant lesions. With brush biopsies and the evaluation of the DNA content of cells using image cytometry, an examination method was found that is non-invasive and offers a patient-friendly possibility to clarify changes in the oral mucosa. The application of the method is controversial for various reasons. With a cohort of 602 cases, we have examined the effectiveness of this examination method on different oral potentially malignant disorders as well as on a large number of confirmed squamous cell carcinomas. In our study, we confirm the efficiency of DNA ploidy analysis to improve the diagnostic accuracy of conventional cytology.

Abstract: Background: The accuracy of DNA image cytometry as an investigation method for potentially malignant disorders of the oral cavity is currently still a subject of controversy, due to inconsistently applied definitions of DNA aneuploidy, small cohorts and different application techniques of the method. The aim of this study was to examine the accuracy of the method as a supplementary diagnostic tool in addition to the cytological examination using internationally consented definitions for DNA aneuploidy. Methods: A total of 602 samples from 467 patients with various oral lesions were included in this prospective study. Brush biopsies from each patient were first cytologically examined and categorized by a pathologist, second evaluated using DNA image cytometry, and finally compared to either histological biopsy result or clinical outcome. Results: Using the standard definition of DNA aneuploidy, we achieved a sensitivity of 93.5%, a positive predictive value for the detection of malignant cells of 98.0%, and an area under the curve of 0.96 of DNA ploidy analysis for the detection of severe oral epithelial dysplasia, carcinoma in situ or oral squamous cell carcinoma. Importantly, using logistic regression and a two-step model, we were able to describe the increased association between DNA-ICM and the detection of malignant cells (OR = 201.6) as a secondary predictor in addition to cytology (OR = 11.90). Conclusion: In summary, this study has shown that DNA ploidy analysis based on conventional specimens of oral brush biopsies is a highly sensitive, non-invasive, patient-friendly method that should be considered as an additional diagnostic tool for detecting malignant changes in the oral cavity.

Keywords: aneuploidy; oral cancer; DNA image cytometry; oral potentially malignant disorders; brush biopsy; mass screening tool

Citation: Bechstedt, N.; Pomjanski, N.; Schramm, M.; Remmerbach, T.W. Evaluation of Static DNA Ploidy Analysis Using Conventional Brush Biopsy-Based Cytology Samples as an Adjuvant Diagnostic Tool for the Detection of a Malignant Transformation in Potentially Oral Malignant Diseases: A Prospective Study. *Cancers* **2022**, *14*, 5828. https://doi.org/10.3390/cancers14235828

Academic Editor: David Wong

Received: 30 October 2022
Accepted: 22 November 2022
Published: 26 November 2022

1. Introduction

Cancer of the lip and oral cavity is one of the most common malignancies worldwide. In 2020, the World Health Organization recorded 377,713 new cases and 177,757 new deaths for lip and oral cavity cancer [1]. In total, 65.8% of these cases are recorded in Asia, with the highest estimated incidence rates in Papua New Guinea, India, Pakistan and Bangladesh [1].

Histologically, the most common is oral squamous cell carcinoma (OSCC), and main risk factors include tobacco use, chewing betel nut, and alcohol consumption [2].

Oral potentially malignant disorders (OPMD), including mainly leukoplakia, erythroplakia and oral lichen planus, are mucosal diseases that have a risk of malignancy being present at the time of initial diagnosis or a future date [3]. The majority of these OPMDs may not progress to OSCC, yet they provide a field of abnormalities in which possible cancer development is more likely than in patients without such disorders [4]. Overall, oral lesions can be easily detected visually during dental screening, however, classification between benign lesion, OPMD and OSCC is challenging.

At an early stage of diagnosis, the 5-year survival rate of OSCC is 85%; however, only 28% of oral cancers are diagnosed at this point. Almost 50% of cases are not recognized until the tumor is in an advanced stage and has infiltrated the locoregional lymph nodes. In this case, the 5-year survival rate drops to 68%. Furthermore, 18% of the tumors are not recognized until metastases spread throughout distant parts, causing the 5-year survival rate to drop to 40% [5].

In various cytogenetic carcinogenesis theories of preneoplastic and neoplastic cells, the transition from stable diploid to unstable aneuploid cells is discussed as one of the main causes. The progression of malignant cells arises from cancer-specific primary, secondary, and tertiary chromosomal changes. Secondary aberrations can be detected by collecting cells, through non-invasive brush biopsies, whose DNA content is measured by DNA image cytometry (DNA-ICM). DNA aneuploidy is assumed to be the quantitative equivalent of chromosomal aneuploidy [6]. The detection of DNA stemline aneuploidy corresponds to the detection of malignant cells [7,8], a technique that can be performed automatically and objectively [9].

Studies have shown that identifying DNA aneuploidy in squamous epithelium can lead to an earlier detection and diagnosis of OSCC by up to two years [8]. Since this non-invasive procedure is well tolerated by patients, OSCC could be detected at an earlier stage, which could significantly increase the chance of recovery and thereby reduce the burden on the healthcare system.

Although DNA aneuploidy is an accepted biomarker for malignancy [10], the effectiveness of the procedure is still controversially discussed [11,12]. Considering various studies, pooled sensitivities and specificities of 55–100%, differently applied methods of DNA measurement (image cytometry vs. flow cytometry), small cohort sizes <200 and inconsistently applied definitions of aneuploidy resulted in the fact that there is limited evidence [12,13]. Therefore, a uniform implementation of the procedure, clearly defined parameters and statistical power are necessary to obtain an objective statement on the accuracy of the procedure, as an additional tool.

PICO statement: This paper presents a prospective study examining outcomes from November 1997 to August 2010 at our oral medicine unit. Parts of the cytological examination and DNA ploidy measurement data used in this article were preliminary published in another study [14].

The outcome parameters were defined as follows: In view of the aforementioned limitations, the aim of this study, thus, was to statistically verify the diagnostic accuracy of DNA-ICM as an adjunctive tool to oral brush biopsy-based cytology samples to detect severe oral epithelial dysplasia, carcinoma in situ or OSCC in a large series of various clinical lesions in 602 cases. In addition, we tested, by logistic regression, whether DNA-ICM is an independent discriminator for the detection of malignancy.

2. Methods and Material

2.1. Patients and Cell Collecting Procedure

This study was approved by the ethical committee of the University Hospital Leipzig, Germany (no. 1272002), and all participating subjects were informed and signed consent. In total, 467 patients with clinical aspects of oral lesions visiting Leipzig University Hospital during 1997–2010 were enrolled in this study. Each lesion was first inspected visually, palpated and classified into a clinical diagnosis (Table 1). Staining of the specimens, cytological diagnoses and DNA-ICM were performed at the Institute of Cytopathology, Heinrich Heine University, Düsseldorf, Germany, as part of patient care.

Table 1. Distribution of the most frequently occurring clinical diagnoses according to the gender of the subjects.

Clinical Diagnoses		Gender		Total
		Female	Male	
Oral Leukoplakia	number of cases	68	44	112
	%	60.7	39.3	
Oral Lichen planus	number of cases	8	27	35
	%	22.9	77.1	
Lichen erosivus	number of cases	5	19	24
	%	20.8	79.2	
OSCC *	number of cases	228	73	301
	%	75.7	24.3	
Ulceration	number of cases	31	18	49
	%	63.3	36.7	
Other lesions **	number of cases	40	41	81
	%	49.4	50.6	

* oral squamous cell carcinoma. ** for example: pemphigus vulgaris, canker sores, candidiasis, smokers keratosis, herpes simplex, etc.

For the examination, cell material from the suspected lesion was first removed in a rotating manner using brush biopsy (Cytobrush GT). The cells and cell clusters obtained were smeared and fixed with alcohol spray on a glass slide for conventional cytology. This obtainment procedure was repeated up to four times. Following the alcoholic fixation of the preparations, a special cytological routine staining using Papanicolaou (PAP) was carried out. Tissue was biopsied from the same location of the brushing, fixed with formalin, embedded in paraffin and subjected to histopathological examination.

In our cross-sectional study, approximately five swabs from each lesion were cytologically examined by experienced cytopathologists, and the specimens were classified as negative (1), doubtful (2), suspicious (3), and positive (4) for tumor cells. The DNA content of each sample suspected or indicative of malignancy (only group 2–4) was determined on the identical slide by DNA ploidy analysis with DNA-ICM and compared to either the histological results of scalpel biopsy or clinical follow-up.

The inclusion criteria were as follows:

(a) Primary clinical diagnosis of OPMD or verified diagnosis of OSCC;
(b) Both brushing and biopsy samples were obtained;
(c) DNA content analysis was completed by DNA-ICM;
(d) Histopathological examination by pathologist, or for the cytologically negative controls, clinical follow-up on average of 60 months by experienced oral surgeons.

All oral lesions of the period of investigation with not negative cytological diagnoses (categories 2–4) and available DNA ploidy results were included. In addition, 204 randomly

All oral lesions of the period of investigation with not negative cytological diagnoses (categories 2–4) and available DNA ploidy results were included. In addition, 204 randomly selected samples with no evidence of malignancy in the clinical follow-up, which were cytologically assessed as negative, were included and examined as negative controls by DNA-ICM (Figure 1).

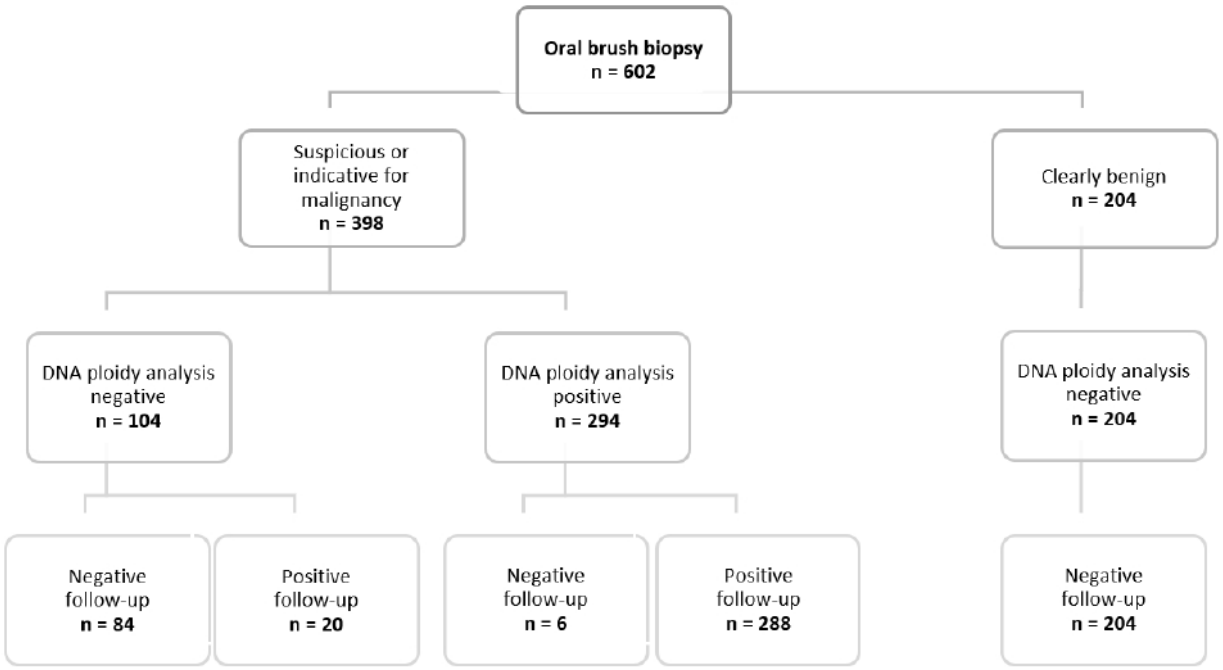

Figure 1. Presentation of the results of the oral brush biopsy, the subsequent DNA ploidy analysis and their follow-up outcome.

The results of cytology and DNA-ICM were compared to the histological and/or clinical follow-up. Severe oral epithelial dysplasia, cytological diagnosis, evidence of DNA aneuploidy with a consistent clinical course (i.e., OSCC therapy, definitive imaging, or palliative care), or histologically confirmed carcinoma in situ and OSCC was defined as positive follow-up. Histology of low-grade/intermediate oral epithelial dysplasia, benign histology, or a negative clinical follow-up of 2 years in the same oral region was defined as negative follow-up.

2.2. Technical Approach of DNA-ICM

Slides prestained with PAP were examined microscopically; cells of interest were marked with a felt pen on the coverslip and photocopied to ensure marked cell clusters could be found after uncovering during the Feulgen staining process. Feulgen staining was used for quantitative staining of nuclear DNA. The QUIC-DNA system (Tripath, Burlington, NC, USA) was used in combination with a conventional light microscope Axioplan 2 (Zeiss, Jena, Germany) for photometric analysis of the integrated optical density (IOD) of the cell nuclei.

The mean DNA content of >30 cytologically normal epithelial cells or lymphocytes was measured as an internal reference. Around 300 randomly selected, atypical cells from the cell population examined were measured. The reference absorbance was normalized to the 2c value of the reference cells. Coefficients of variation of reference cells were below 5%. The European Society for Analytical Cellular Pathology (ESACP) standards and guidelines for DNA-ICM were followed [6].

2.3. Criteria of Aneuploidy

Most human tumors are characterized by a numerical and/or structural chromosome aberration. The DNA content of 2c (c = content) of physiological epithelia is determined by

measuring around 30 reference cells (normal squamous epithelial cells) using their average IOD value.

The DNA histogram of a normal proliferating cell population shows a first peak at 2c and a second at 4c (Figure 2).

If the following criteria were met, the result was interpreted as aneuploid.

According to the ESCAP [6], DNA aneuploidy is recognized either in a deviation of the DNA stemlines or in appearance of so-called "rare events".

1. DNA stemline aneuploidy: The usual precision of recent DNA image cytometric measurements should at least allow DNA stemlines to be identified as abnormal (or aneuploid) if they deviate more than 10% from the diploid (2c) or tetraploid region (4c), i.e., if the modal values of DNA stemlines are outside 2c ± 0.2c or 4c ± 0.4c (examples: Figures 3 and 4).
2. DNA single cell aneuploidy: Rare events in DNA histograms are abnormal cells often called 5c or 9c exceeding events, having a nuclear DNA content higher than the quadruplicate quadruplicate region of normal G1/G0 phase population, i.e., not belonging to the G2M phase. Accordingly, we defined "single cell aneuploidy" as the occurrence of at least one cell with a DNA content of >9c (example: Figures 3 and 4).

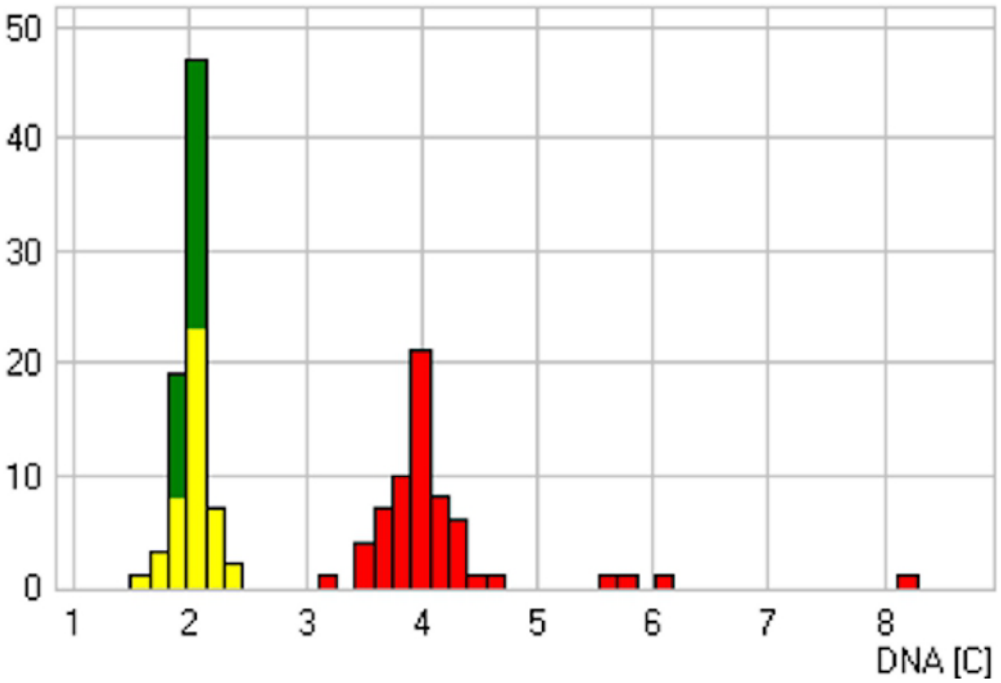

Figure 2. Sample with a doubtful cytological diagnosis and euploid–polyploid DNA histogram. Two euploid DNA stemlines at 2c and 4c and single cells with DNA contents up to 8.3c are DNA content in c-units (x-axis), number of cells (y-axis), reference cells (green), analysis cells (red), biggest stemline (yellow). In this patient case, a 66-year-old female patient showed an ulceration on the right edge of the tongue, which was histologically diagnosed as granulation tissue and healed after 6 weeks without irritation.

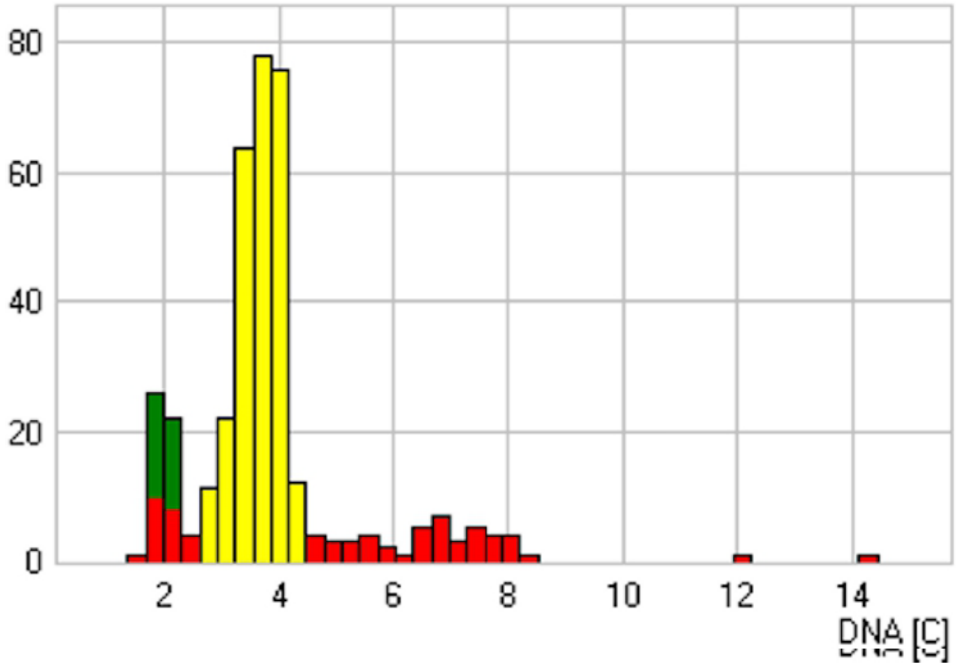

Figure 3. Sample with a suspicious cytological diagnosis and presence of DNA aneuploidy as a sign of malignant transformation. An atypical stemline at 3.52c is shown with an associated doublet peak and two cells with a DNA content >8c. DNA content in c-units (x-axis), number of cells (y-axis), reference cells (green), analysis cells (red), biggest stemline (yellow). In the follow-up of this sample, a second carcinoma of the floor of the mouth was diagnosed in an 86-year-old male patient.

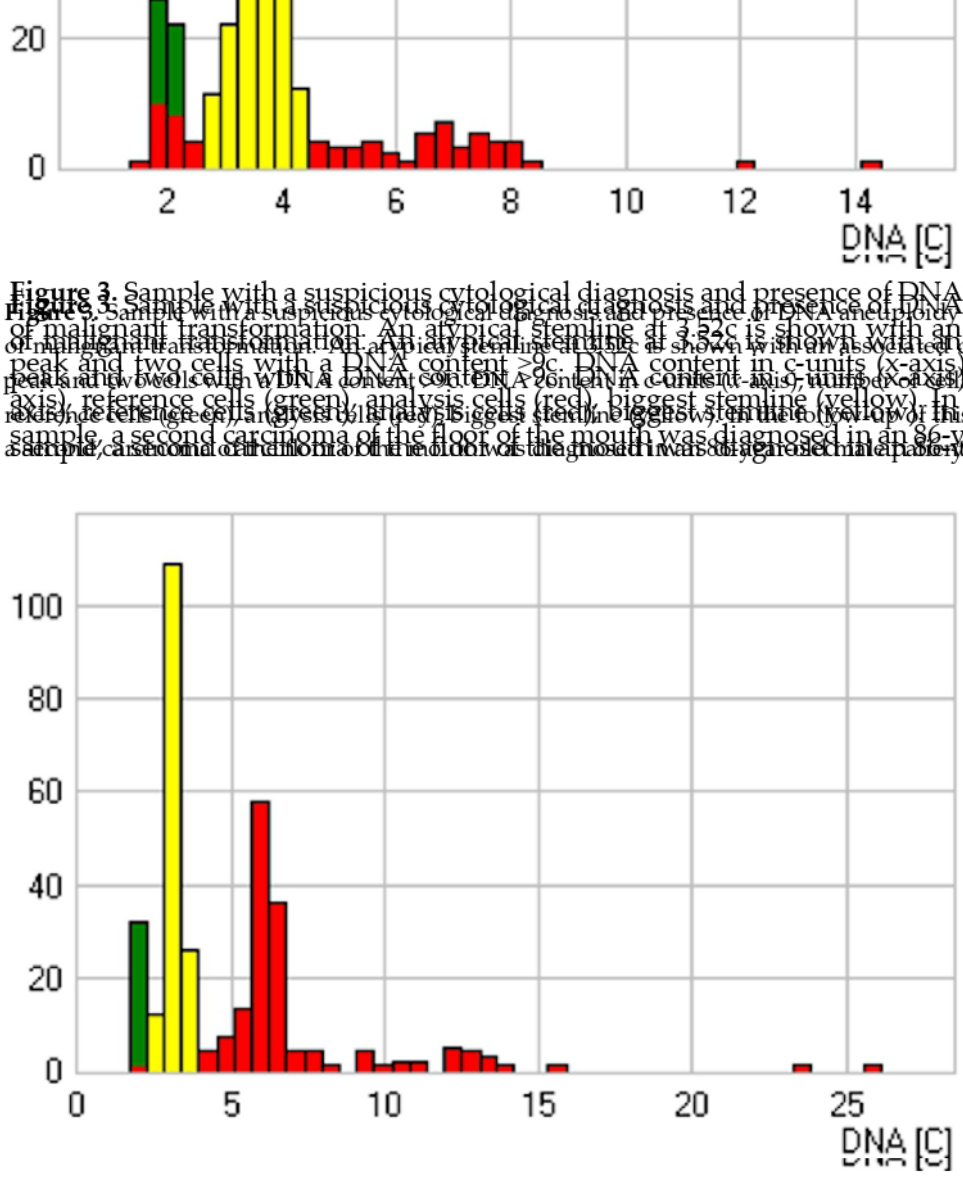

Figure 4. Sample with a positive cytological diagnosis and DNA aneuploidy as a sign of malignant transformation. Atypical stemline distribution at 3.24c and 6.5c. In addition, several cells with DNA content >9c are shown. DNA content in c-units (x-axis), number of cells (y-axis), reference (green), analysis cells (red), biggest stemline (yellow). This sample is a carcinoma of the soft palate in a 68-year-old male patient.

2. DNA single cell aneuploidy: Rare events in DNA histograms are abnormal cells called 5c or 9c exceeding events, having a nuclear DNA content higher than the duplicate or quadruplicate region of a normal G1/G0 phase population, i.e., not belonging to the G2M phase. Accordingly, we defined "single cell aneuploidy" as the occurrence of at least one cell with a DNA content of >9c (example: Figures 3 and

2.4. Statistical Analysis

Grouping of cytopathology diagnoses was performed for the application of logistic regression. A negative diagnosis was declared as 1 = negative, and the above-mentioned cytopathological diagnostic categories 2–4 have been combined as 2 = positive. For DNA-ICM, a case was scored as "negative" if no DNA aneuploidy could be detected and as "positive" for evidence of DNA aneuploidy. Then, 95% confidence intervals were given for the sensitivity, specificity and AUC measurements. All analyses were performed with SPSS (Version 26; IBM Corporation, Armonk, NY, USA) and STATA (Version 16., StataCorp LLC, College Station, TX, USA) for Windows.

3. Results

3.1. Enrolled Patients

A total of 602 samples of 467 patients were included in this study: 375 samples of the study population were women (62.3%), and 227 samples were men (37.7%) (Table 1). At the time of sampling, the mean age for women was 57.73 years and for men 63.65 years.

Upon final histopathological examination, 308 patients were diagnosed with OSCC and 90 patients with varying degrees of epithelial dysplasia. Independently, an additional 204 samples with no indication of malignancy were examined, which also proved to be negative in the clinical follow-up. OSCC and epithelial dysplasia occurred mainly on tongue (30.3%), floor of mouth (21.54%), and buccal mucosa (17.7%). The mean age of the total cohort for OSCC was 59.05 years (SD 12.17) and 60.93 years (SD 13.63) for epithelial dysplasia. These values are two-tailed (0.075) by *t* test with Satterthwaite correction for heterogeneous data. From 308 samples diagnosed as OSCC (Table 2), 76.20% were seen in women, mean age 57.82, and 23.8% were seen in males, mean age 63.07. The age correlation between the male and female cohort was significant (Chi^2 N-1-Test).

Table 2. Overview of the classification of the investigated OSCC in their TNM classification, regarding tumor extent, lymph node involvement and presence of distant metastases.

T-Status	(*n*)	N-Status	(*n*)	M-Status	(*n*)
Tx	55	Nx	55	Mx	55
Tis *	1	N0	139	M0	186
T1	84	N1	43	M1	71
T2	85	N2a	14		
T3	37	N2b	42		
T4a	46	N2c	12		
T4b	0	N3	3		

* Including severe dysplasia and carcinoma in situ.

3.2. Cytological Examination

In a cytological examination, the specimen is examined by a pathologist under the light microscope for abnormalities in cell structure, especially of the nucleus. A diagnosis is made of negative, doubtful, suspicious and positive for tumor cells. For further statistical calculations, a dichotomization into positive and negative was made (see Statistical Analysis). In comparison to the histological biopsy/clinical follow-up of the patients with lesions suspicious or indicative for malignancy, 308 cases were identified as true positive, 204 cases as true negative, 90 cases as false positive and no cases as false negative. This results in a sensitivity of 100% (95% CI 98.8–100.0%), specificity of 69.4% (95% CI 63.8–74.6%), PPV = 77.4% (95% CI 73.0–81.4%) and NPV = 100% (95% CI 98.2–100%) (Table 3). The area under the curve was 0.85 (95% CI 0.82–0.87) (Table 3).

Table 3. Sensitivity, specificity, positive and negative predictive values (PPV and NPV) of cytology and DNA aneuploidy for oral potentially malignant disorders (OPMD) and OSCC.

Method	Specificity	Sensitivity	PPV	NPV	AUC	95% CI (AUC)
Conventional Cytology	69.4%	100.0%	77.4%	100%	0.85	0.82–0.87
DNA-ICM	98.0%	93.5%	98.0%	93.5%	0.96	0.94–0.97

3.3. DNA Image Cytometry

In the field of diagnostic studies, the AUC serves as an overall measure of a diagnostic test's accuracy (Figure 5). With the criteria for DNA aneuploidy mentioned above, the >10% deviation of DNA stemline from physiological values and/or cells >9c, the sensitivity for detecting severe epithelial dysplasia or carcinoma within OPMD due to DNA ICM was 93.5% (95% CI 90.1–96.0%), and specificity was 98.0% (95% CI 95.6–99.2%). The recorded positive predictive value was 98.0%, and the negative predictive value was 93.5% (Table 3). A total of 20 cases with positive follow-up were missed by DNA-ICM, and 6 cases of OPMD were false positives. Using post hoc power analysis, it can be evaluated that the cohort size of 602 cases is statistically significant at the 5% level (alpha level = 0.05). A comparison of cytology and DNA ploidy analysis of the 602 specimens is shown in Table 4. The frequencies of aspects of DNA ploidy in relation to the biological behavior of the lesion are given in Table 5.

Table 4. Overview of the results of the cytological examination and the DNA-ICM in comparison.

Cytological Examination	DNA Ploidy Analysis with DNA-ICM	
	DNA Aneuploidy	No DNA Aneuploidy
positive	222	6
suspicious	49	14
doubtful	23	84
negative	0	204

Table 5. Frequency of met criteria for DNA ploidy in relation to the biological behavior of lesions.

Aspects of DNA-Ploidy	Biological Behavior of the Lesion	
	Malignant	Benign
Normal stemline $[1.8 < c < 2.2]^{x}$ x = 1,2,3	20	288
Atypical stemline $[1.8 > c > 2.2]^{x}$ x = 1,2,3	227	5
1	78	3
2	118	2
>2	31	
Cells > 9c	301	4
1 to 3	112	3
4 to 10	86	1
>10	103	0
Atypical stemline and cells > 9c	222	3

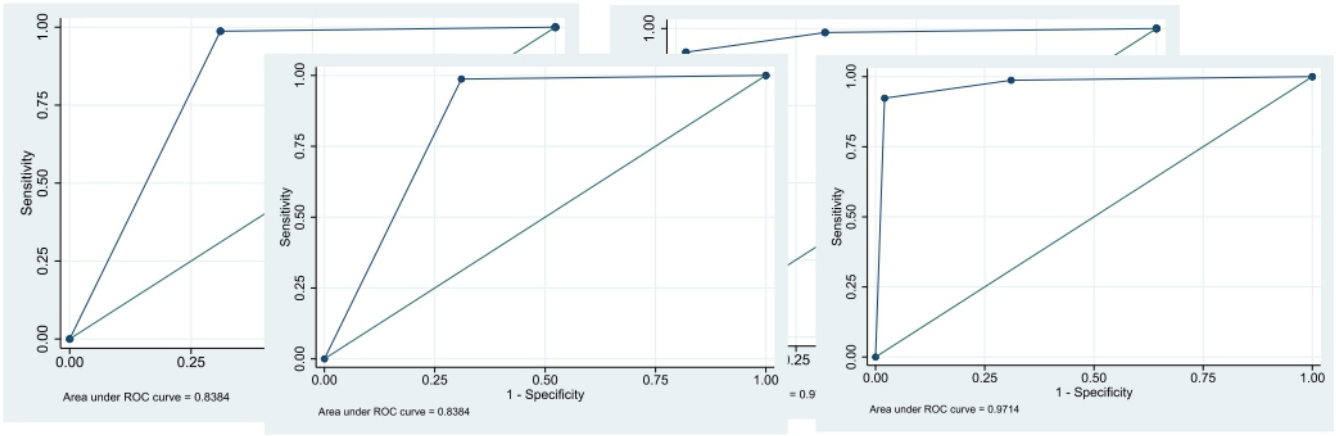

Figure 5. ROC (receiver operating characteristics) curves for cytological examination (**left**) of the cells and DNA image cytometry (**right**).

3.4. Logistic Regression

Logistic regression was applied to evaluate odds ratio (OR) and the association between binary target variables. First, we analyzed the association among cytological examination and histological biopsy using bivariate analysis. Odds ratio for the cytological examination as a sole predictor was 171.11 (95% CI 66.74–438.70) (Figure 6). Using subsequent multiple logistic regression, we included DNA image cytometry and obtained odds ratio (cytology) = 41.90 (95% CI 3.94–35.88) and OR (DNA-ICM) = 201.6 (95% CI 78.42–518.22, $p = 0.001$). Cytological examination predicted AUC of 0.85 (95% CI 0.82–0.87) and AUC (DNA-ICM) = 0.96 (95% CI 0.94–0.97), respectively (Figure 7). The areas under the curve are significantly different ($Chi^2(1) = 112$; $p < 0.001$).

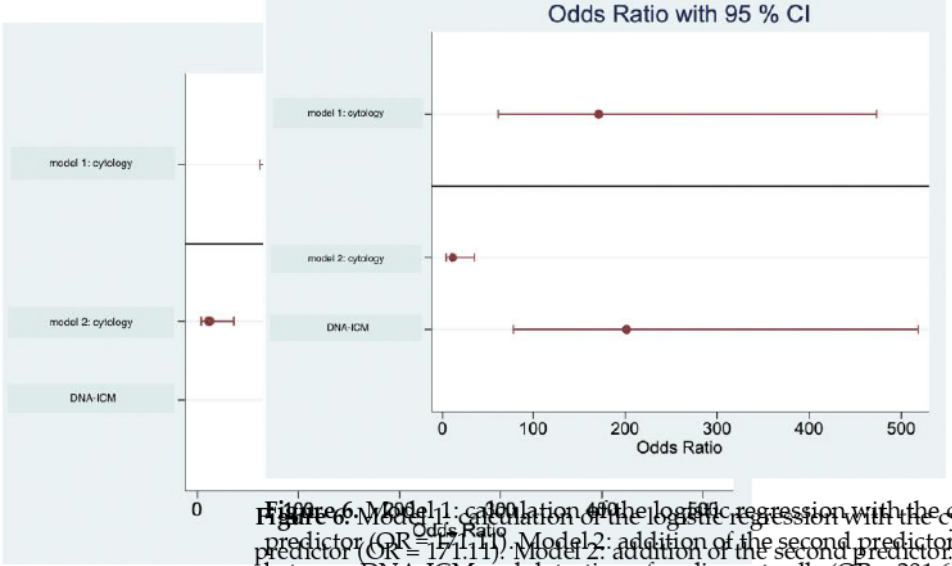

Figure 6. Model 1: calculation of the logistic regression with the cytological examination as the sole predictor (OR = 171.11). Model 2: addition of the second predictor. DNA-ICM: increased association between DNA-ICM and detection of malignant cells (OR = 201.6) in addition to decreased association for cytological examination (OR = 11.90).

4. Discussion

In our study, the value of DNA ploidy analysis using DNA-ICM as an additional diagnostic screening tool for the detection of severe oral epithelial dysplasia and OSCC in clinically suspect oral lesions including OPMD was examined and rated with a high accuracy. Our prospective study was carefully designed to eliminate other possible variables that have led to controversial literature statements in the past, such as lack of heterogeneity of included clinical lesions, small study population, and inconsistent definition of aneuploidy by following the guidelines according to the ESACP consensus reports [6].

geneity of included clinical lesions, small study population, and inconsistent definition of aneuploidy by following the guidelines according to the ESACP consensus reports [6].

Aneuploidy is a meaningful, recognized biomarker that indicates numerical chromosome changes and thus a developmental anomaly, which in addition is often used in cancer diagnostics [15–17]. The cytometric equivalent of chromosomal aneuploidy, determined by DNA-ICM, is DNA aneuploidy [6].

Although the oral cavity is a highly visible area of the human body, too little visual attention is paid to it in practice. Taking brush biopsies offers the dentist the opportunity to have questionable changes in the oral mucosa examined in an uncomplicated way. This procedure takes only a few minutes during treatment, and the cost of materials is vanishingly small compared to the cost of therapy for OSCC in advanced stages. By examining by means of brush biopsies, the treating dentist can help to minimize the secondary time loss of tumor patients until adequate therapy is available and can significantly improve the prognosis [18]. Furthermore, brush biopsies make it possible to examine larger altered areas, whereas with conventional biopsies, the treating dentist has to decide on a limited area. By using brush biopsies, the referral hurdle to an oral surgeon is eliminated and possible bleeding, wound healing disorders or the consideration of anticoagulants in the patient can be neglected with brush biopsy.

There are several already communicated advantages of brush biopsy-based oral cytology. Gupta et al. has found that it is a way of early detection, and as a result, the time between diagnosis and treatment can be significantly reduced [18]. Furthermore, it is a painless, non-invasive technique that is well accepted by patients.

There are various indications for the additional use of brush biopsies. The noninvasive, painless use of brush biopsies results in high patient compliance in contrast to conventional biopsy, which is why they are useful for monitoring oral lesions in general [19], for patients with allergies to local anesthesia and thus limited compliance for biopsy [20], and for patients who require frequent follow-up, such as in Fanconi anemia [21].

In various studies investigating the use of DNA ploidy analysis or DNA-ICM of the last decades, there is a wide range for sensitivity (16–100%) and specificity (66–100%). One of the main reasons is the inconsistent definition of DNA aneuploidy across studies. In the study by Pektas et. al, which indicates a sensitivity of 16%, DNA aneuploidy was defined if the peaks in the histogram were at 3c, 5c, 7c and 9c or if the number of nuclei with a DNA content of more than 5c or 9c was over 1% [22]. With this definition, only 2 out of 12 OSCC could be detected, resulting in low sensitivity. In contrast, the study by Maraki et al. reached a sensitivity of 100%. The definition used corresponds to the definition from our study design [23].

In terms of specificity of our study, DNA-ICM was 92.3% (Table 3), which was much higher than the specificity of conventional cytology, which was 69%. Early detection of dysplastic squamous cells is highly desirable, and accordingly, the doubtful and suspicious cytology in this study was assigned to the statistical category of "positive," which allowed for maximum sensitivity but at the expense of lower specificity. With this assignment of doubtful and suspicious cytology, the sensitivity of conventional cytology is 0.8% higher than that of DNA-ICM. However, this is only a statistical effect, as the determination of DNA ploidy provides a more definitive result for a clinical measure than does doubtful or suspicious cytology. In general, the probability of malignancy is considered to be approximately 30% and 70% for equivocal and suspicious cytology, respectively [2–4,24].

In our study, the positive predictive value of DNA-ICM was 98.0% and the negative predictive value was 93.5%. A total of 20 cases of severe epithelial dysplasia or OSCC were assessed as false negative. One of the reasons could be the number of cells examined. On average, at least 300 cells should be measured [25]. In 16 of the 20 cases above, only 57–186 cells were examined instead of the required minimum of 300 cells. Furthermore, a possible misjudgment can be attributed to the fact that cell overlaps can occur, which make it difficult to measure their DNA content [19].

Inflammatory exudate and necrotic deposits on the lesion can lead to an insufficient number of evaluable cells to be obtained for examination, thus increasing the false-negative rate. Therefore, in this clinical condition, cell harvesting should be avoided, and a brush biopsy should be performed only after this condition has passed [19].

Finally, using logistic regression, a clear effect for the sole predictor of cytological examination as an examination method could be demonstrated. However, with the addition of ploidy examination as a second predictor, the effect of cytology is reduced, whereas the effect of DNA-ICM is very clearly recognizable.

Especially for cancer screening, the sample size is essential in order to be able to achieve statistical power. To the best of our knowledge, the sample size ($n = 602$) was the largest-scale series evaluating DNA-ICM following the definitions of DNA aneuploidy consented by the ESACP [6].

We are aware of the limitations of the study design and recognize that handling brushing devices as well as interpreting DNA-ICM results require a certain amount of skill. The proportional distribution of the examined clinical lesions that are enriched for (pre)malignant ones is inconsistent with the frequency in daily practice. This is due to the fact that manual DNA-ICM is mostly performed subsequent non-negative cytology. A majority of the lesions examined were OSCC, leukoplakia and oral lichen, resulting in statistically underpowered results for minor subtypes of OPMD. For statistical calculation of the diagnostic accuracy of the DNA-ICM, the DNA ploidy of 204 randomly selected samples of the cytological classification "negative" (1) was examined. Their clinical follow-up of an average of 60 months proved to be "definitely negative". In conclusion, it can be confirmed that DNA aneuploidy is a marker for malignancy in epithelial oral cells.

Nevertheless, our study design is a cross-sectional study. A longitudinal study would have to be carried out for a valid examination regarding the use of brush biopsies as a follow-up screening tool and therefore the impact on disease progression.

Recently, liquid biopsy, circulating biomarkers, and noninvasive sampling have attracted great interest from the scientific community. Regarding the use of brush biopsies as a noninvasive diagnostic method, one study has shown that liquid biopsies provide a faster result with the same sensitivity and specificity because only one sampling is required [26]. Another research focus is the determination of biomarkers as a diagnostic tool for the detection of malignant changes in the oral cavity. To date, however, no sole, unique biomarker has been identified, but the use of brush biopsies to quantify cancer-specific methylation characteristics has been shown to be helpful in establishing risk stratification schemes for OSCC [27–29]. In another study, brush biopsies were used to screen patients' saliva for the presence of *p*-53 codon 63 mutations, which could serve as predictors of OSCC [30]. In conclusion, by means of our study, DNA image cytometry was shown to be a highly sensitive, noninvasive, and patient-friendly method in addition to conventional cytology for detecting potentially malignant oral lesions with malignant transformation requiring clinical intervention. Widespread use as a routine screening method could improve early detection of suspicious lesions, increase OSCC survival, and thus reduce the burden on the healthcare system.

5. Conclusions

In summary, this study has shown that DNA ploidy analysis based on conventional specimens of oral brush biopsies is a highly sensitive, non-invasive, patient-friendly method that should be considered as an additional diagnostic tool for detecting malignant changes in the oral cavity.

Author Contributions: Conceptualization, T.W.R.; methodology, T.W.R. and M.S. and N.B.; software, N.B.; validation, T.W.R., M.S. and N.B.; formal analysis, N.B.; investigation, N.B. and N.P.; resources, T.W.R.; data curation, N.B.; writing—original draft preparation, N.B.; writing—review and editing, T.W.R. and M.S.; visualization, T.W.R. and M.S.; supervision, T.W.R.; M.S.; project administration, T.W.R.; All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: The study was conducted in accordance with the guidelines of the Declaration of Helsinki and was approved by the Ethics Committee of the University Hospital Leipzig (127-2002; 7 August 2002).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data presented in this study are available upon request from the corresponding author. Data are not publicly available for privacy reasons.

Conflicts of Interest: The authors declare no conflict of interest.

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Analytical Cellular Pathology 25 (2003) 159–166
IOS Press

Case report

Earliest detection of oral cancer using non-invasive brush biopsy including DNA-image-cytometry: Report on four cases

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Received 6 February 2003
Accepted 20 May 2003

Abstract. *Objective:* We describe four patients presenting early oral cancers, detected cytologically on non-invasive brush biopsies including DNA-image cytometry as an adjunctive method before histology on scalpel biopsies confirmed the evidence of malignancy.

Methods: Brush biopsies were performed and smears thereof investigated cytologically. After Feulgen restaining, DNA-measurements were performed using a DNA-Image-Cytometer.

Case reports: Oral squamous cell carcinomas were diagnosed cytologically in macroscopically suspicious lesions and malignancy confirmed by DNA-cytometry. The initially performed scalpel biopsies did neither supply evidence of oral cancer nor of severe dysplasia. After at least one to 15 months the occurrence of cancer was finally proven histologically on a second scalpel biopsy each (three microinvasive and one *in situ* carcinoma).

Conclusion: Non-invasive brush biopsies are a suitable instrument for early cytologic detection of cancer of the mouth. DNA-image-cytometry, as an adjunctive method, can be used to confirm the cytologic diagnosis or suspicion of cancer in patients with doubtful lesions (dysplasias). DNA-aneuploidy is a marker for (prospective) malignancy in smears of the oral cavity, which may detect malignancy months prior to histology. In future this method could be used as a mass screening tool in dentists practise.

Colour figures can be viewed on http://www.esacp.org/acp/2003/25-4/remmerbach.htm.

Keywords: Brush biopsy, oral cancer, DNA-image-cytometry, mass screening tool

1. Introduction

Squamous cell carcinomas of the oral cavity (OSCCs) are among the ten most common cancers in the world, accounting for 3–5% of all malignancies [16]. In 1993 in Germany, approximately about 3100 new cases in males and 1000 in females were encountered [13]. The prognosis for many of these patients is devastating. Up to 50% of the patients will die in the first five years after the cancer has been diagnosed [8]. For patients, lack of early and correct identification

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of carcinomas in the oral cavity may result in unnecessary inadequate further examinations and decreased survival probability. Poor education of dentists in this field often delays necessary therapy with inevitable progression of the tumour. Not only clinicians fail diagnosing cancer in early stages, but also histopathologists sometimes miss preinvasive oral cancer evaluating scalpel biopsies and state dysplasias or reactive lesions instead [5].

2. Material and methods

Alcohol-fixed smears from oral brushings were stained according to Papanicolaou (Pap). DNA-cytometry was performed secondarily on Pap-stained routine slides after restaining according to Feulgen. After postfixation with 10% buffered formalin and staining with pararosaniline, measurements were performed using an AutoCyte QUIC pathology workstation (TriPath, Burlington NC, USA and Carl Zeiss, Jena, Germany). The normal 2c reference value was established measuring 30 normal epithelial cells as an internal reference (mean IOD value). Three hundred abnormal or atypical epithelial cells were measured at random. We assumed aneuploidy either in the presence of an abnormal stemline (modal value ⟨1.80c⟩ 2.20c or ⟨3.60⟩ 4.40 or ⟨7.20⟩ 8.80c), or if cells >9cEE (9c Exceeding Events) occurred. The performance standards of the ESACP consensus reports on diagnostic DNA image cytometry were obeyed [3,4,6,7].

3. Presentation of cases

3.1. Case A

A 47-year-old white male presented a painful, softish ulcer (4 mm in diameter) with white speckled border on the right side of the tongue (Fig. 1). The cytologic examination of a brush biopsy unequivocally revealed cells from a squamous cell carcinoma (Fig. 2). DNA-cytometry showed a stemline at 4.31c and seven cells greater 9c (Fig. 3). Two scalpel biopsies with histological examination resulted in the diagnosis of a florid ulcer with pseudoepithelimatous hyperplasia without any signs of malignancy. Seven months later, the patient was in close meshed recall, total excision of the lesion confirmed the presence of a microinvasive squamous cell carcinoma of the tongue. Thus DNA-

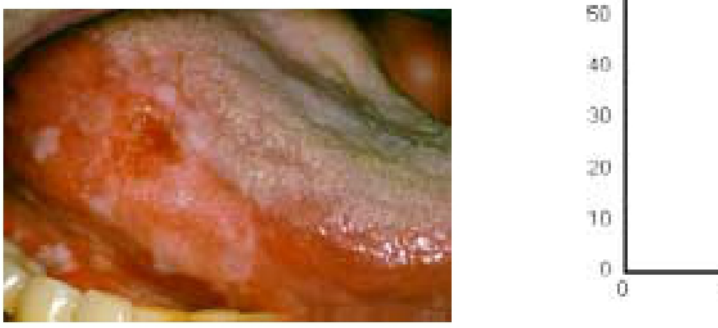

Fig. 1. Clinical view of the tongue from patient A at his first visit in our clinic. This figure can be viewed on http://www.esacp.org/acp/2003/25-4/remmerbach.htm.

Fig. 2. Cluster of exfoliated coherent cells from an early squamous cell carcinoma of the tongue revealing hyperchromatic small but polymorphic nuclei with enlarged nuclear–cytoplasmic ratio and prominent nucleoli. Patient A. Primary magnification 630×.

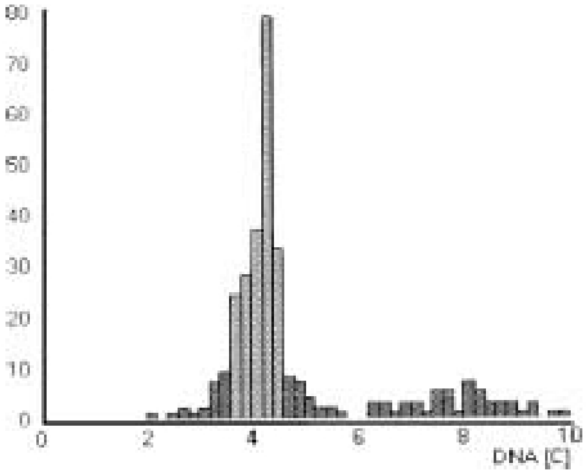

Fig. 3. DNA-histogram of a smear from a squamous cell carcinoma of the tongue (patient A). Different aspects of DNA-aneuploidy can be detected: abnormal stemline 4.31c and 7 cells greater 9c (9cEE).

Table 1
Case A

Date	Cytologic diagnosis	DNA-cytometry	Histologic diagnosis of scalpel biopsy	Final histological diagnosis
21/ Dec/ 98	positive for tumor cells	aneuploid (abnormal stemline at 4.31c and 7 cells greater 9cEE)		
6/ Jan/ 99			floride ulcus, no signs of malignancy	
23/ Jul/ 99				oral squamous cell carcinoma (G2)
Elapsed time between first cytologic/DNA-cytometric cancer diagnosis and histological verification: 7 months				

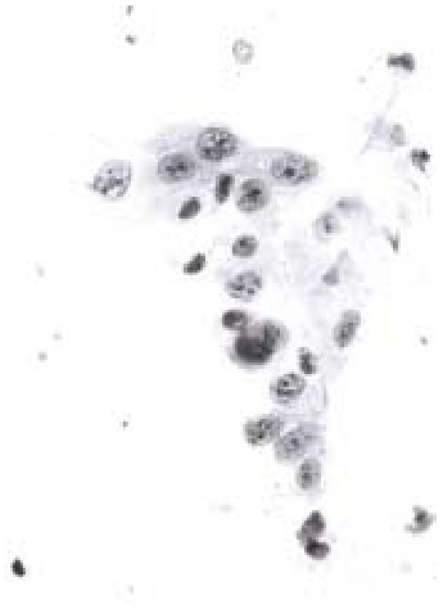

Fig. 4. Atypical oral musoca cells, suspicious for squamous cell carcinoma with enlarged and slightly polymorphic nuclei containing prominent nucleoli and coarse chromatin. Patient B. Primary magnification 630×.

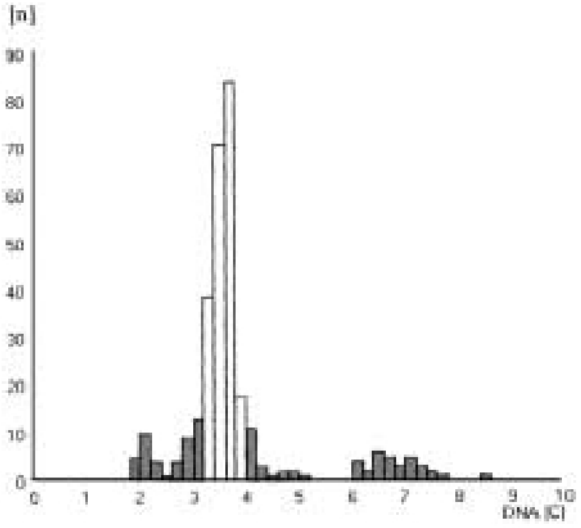

Fig. 5. DNA-histogram of a smear from a squamous cell carcinoma of the alveolar ridge from patient B. One aspect of DNA-aneuploidy can be detected: abnormal stemlines at 3.6c and 7.2c.

image cytometry (DNA-ICM) established the diagnosis of malignancy seven months prior to biopsy histology (Table 1).

3.2. Case B

A 74-year-old male diagnosed in 1997 with oral squamous cell carcinoma (OSCC), the altered area was the left mandible in region 33–36. This was removed by resection. Follow up reviews were performed at regular intervals. In May 2000 a leukoplakia occurred in this area and was checked cytologically on a brush biopsy. The smears were suspicious for malignant cells. DNA-cytometry could not be performed, because of insufficient number of cells. Scalpel biopsies carried out three times showed pseudoepithelimatous hyperplasias. Three months later, the brush biopsy was repeated. The cytological diagnosis was doubtful for tumor cells (Fig. 4), but DNA-cytometry revealed aneuploidy with abnormal stemlines at 3.6c and 7.2c (Fig. 5). Total excision of this area with histologic examination definitely confirmed microinvasive OSCC, five months after first performed brush biopsy with strong suspicion for tumor cells. The delay from first brush biopsy until histological confirmation of an OSCC thus was five months (Table 2).

3.3. Case C

A 67-year-old male, diagnosed with oral cancer in 1994. After total excision of the tumor including a suprahyoid lymph node dissection, the patient was in

Table 2
Case B

Date	Cytologic diagnosis	DNA-cytometry	Histologic diagnosis of scalpel biopsy	Final histological diagnosis
26/ May/ 00	suspicious for tumor cells			
6/ Jun/ 00			pseudoepitheliomatous hyperplasia	
4/ Sep/ 00	doubtful for tumor cells	aneuploid (abnormal stemline at 3.6c and 7.2c cells)		
24/ Oct/ 00				microinvasive oral squamous cell carcinoma (G2)

Elapsed time between first cytologic/DNA-cytometric cancer diagnosis and histological verification: 5 months

Fig. 6. Abnormal cells from an oral squamous cell carcinoma revealing nuclear polymorphism, chromatin clumping, nucleoli and increased nuclear-cytoplasmic ratio. Patient C. Original magnification 630×.

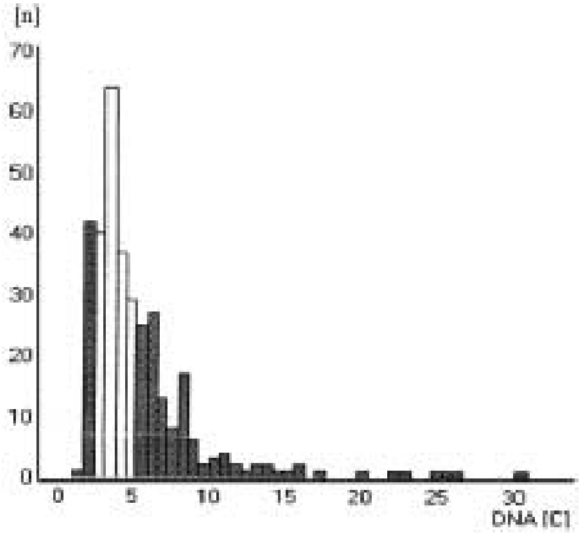

Fig. 7. DNA-histogram of a smear from a squamous cell carcinoma of the arcus palatoglossus (patient C). Different aspects of DNA-aneuploidy can be detected: abnormal stemline at 3.1c and 24 cells greater 9c (9cEE).

closed meshed recall. In January 1999 a small, soft and white-patched homogenous area of 3 mm in diameter appeared on the arcus palatoglossus of the right side. The performed brush biopsy unequivocally showed tumor cells of an OSCC (Fig. 6), DNA-cytometry revealed an aneuploid stemline at 3.1c and 24 cells greater 9c (Fig. 7). At the same time an ostentatious supraclavicular lymph node on the right side appeared and a conventional neck dissection was performed (histologically a metastasis of an OSCC was proven). The clinicans started from the principle, that the suspicious area in the mouth was not the reason for this metastasis and from clinical view seemingly in back formation. Two months later scalpel biopsy from the above described area with histological examination showed no atypia or dysplasia. Five months later a second brush biopsy was suspicious for tumour cells but the smears were technically insufficient for DNA measurement. Four months later the third brush biopsy was doubtful for tumor cells and DNA-cytometry revealed DNA-aneuploidy with an abnormal stemline at 3.34c and four cells greater 9c. After six weeks (15 month after first performed brush biopsy) total excision of this area revealed a leukoplakia with severe dysplasia. Three months later, the patient was diagnosed with a second squamous cell carcinoma of the oesophagus with metastases to the lung. Thus DNA-ICM established the diagnosis of malignancy five months prior to histology diagnosis (Table 3).

3.4. Case D

An 82-year-old white male presented a widespread verrucous leukoplakia of the sinistral pouch (Fig. 8)

Table 3
Case C

Date	Cytologic diagnosis	DNA-cytometry	Histologic diagnosis of scalpel biopsy	Final histological diagnosis
18/ Jan/ 99	positive for tumor cells	aneuploid (abnormal stemline at 3.1c and 24 cells greater 9cEE)		
26/ May/ 99			supraclavicular lymph node metastasis of a an oral squamous cell carcinoma	
20/ Jul/ 99			no dysplasia, no atypia, no signs of malignancy	
1/ Oct/ 99	suspicios for tumor cells	not enough cells for measurement		
25/ Feb/ 00	doubtful for tumor cells, c.i.s.	aneuploid (abnormal stemline at 3.34c and 4 cells greater 9cEE)		
4/ Apr/ 00				leukoplakia with severe dysplasia
28/ Jul/ 00				*squamous cell carcinoma (G3) oesophagus*
Elapsed time between first cytologic/DNA-cytometric cancer diagnosis and histological verification: 15 months				

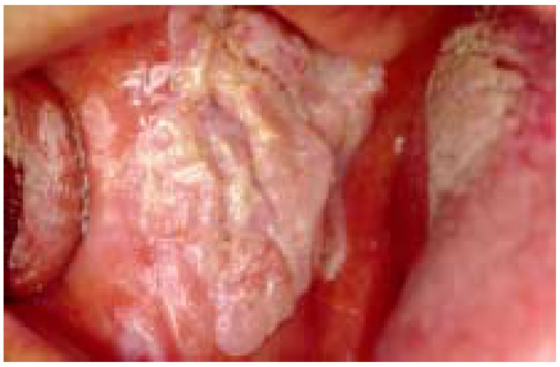

Fig. 8. Clinical view of a verrucous leukoplakia of the sinister pouch from patient D at his first visit in our clinic. This figure can be viewed on http://www.esacp.org/acp/2003/25-4/remmerbach.htm.

with an unknown progression and medical history. A brush biopsy was performed. No abnormal or atypical cells were detected; the total excision with histological examination confirmed the cytological result. After five months, a leukoplakia reappeared and two brush biopsies were performed: one in the proximal part and one in the distal part of the left pouch. The outcome of the cytological examination of the proximal smears was suspicious for cancer cells, the distal part was positive for tumour cells of an OSCC (Fig. 9). DNA-image cytometry showed aneuploidy with multiple abnormal stemlines at 2.5c, 3.38c, 3c, 4c and 5c (Fig. 10). The outcome of thorough examination of scalpel biopsies revealed no evidence of tumour cells.

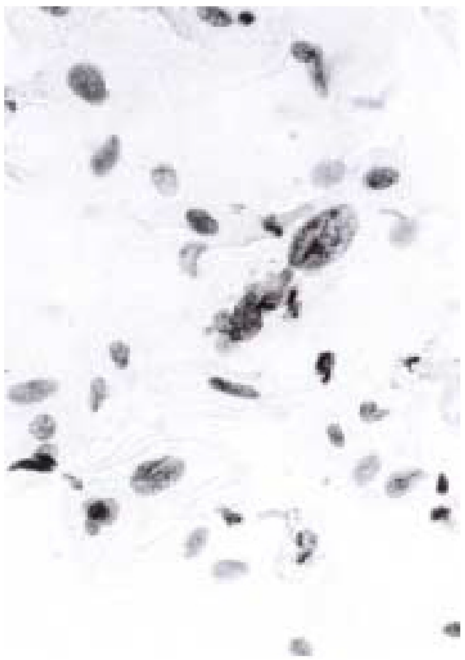

Fig. 9. Few abnormal cells from a squamous cell carcinoma showing coarse chromatin and prominent nucleoli in polymorphic nuclei of variable size. Patient D. Original magnification 630×.

After one month the scalpel biopsy was repeated and the occurrence of a microinvasive squamous cell carcinoma was proven histologically. Between the first brush biopsy and the histological conformation of an OSCC seven weeks had elapsed (Table 4).

Table 4
Case D

Date	Cytologic diagnosis	DNA-cytometry	Histologic diagnosis of scalpel biopsy	Final histological diagnosis
7/ Dec/ 99	negative for tumor cells			
8/ Dec/ 99			leukoplakia with mild dysplasia	
10/ Jan/ 00				keratosis, pachydermia
25/ Apr/ 00	positive for tumor cells	aneuploid (abnormal stemlines at 3.38c, 2.5c, 3c, 4c, 5c)		
11/ May/ 00			no signs of malignancy, chronic inflammation	
14/ Jun/ 00				microinvasive carcinoma (G2)

Elapsed time between first cytologic/DNA-cytometric cancer diagnosis and histological verification: 7 weeks

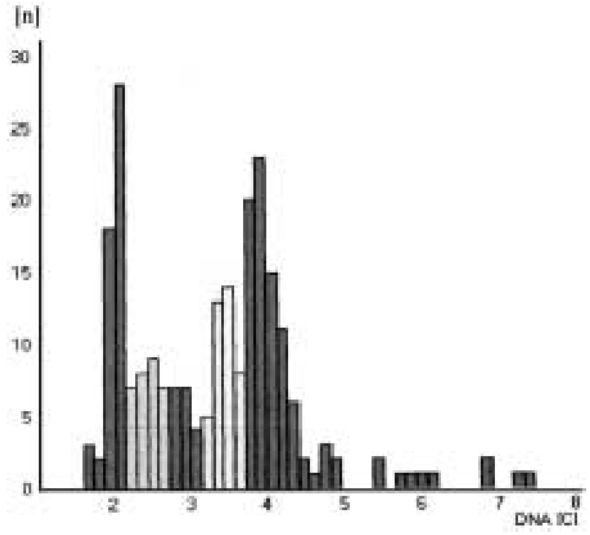

Fig. 10. DNA-histogram of a smear from a squamous cell carcinoma of the buccal mucosa (patient D). One aspect of DNA-aneuploidy can be detected: abnormal stemlines at 2.5c, 3.38c, 3c and 4c.

4. Discussion

Colour figures can be viewed on http://www.esacp.org/acp/2003/25-4/remmerbach.htm.

There is a need to promote the early diagnosis of oral cancers in order to reduce its unacceptably high morbidity and mortality. Sudbø et al. (2001) [15] have shown in a retrospective study that the DNA content in cells of oral dysplasias histologically diagnosed on scalpel biopsies was able to predict the occurrence of oral carcinoma later up to five years before their clinical appearance. Of 150 patients, 25 (17 percent) had DNA aneuploid lesions at the time of the initial diagnosis. Disease-free survival was assessed in relation to DNA-histogram type and histologic grade or dysplasia. Carcinomas developed in 21 of the 25 patients with DNA-aneuploid lesions within five years, yielding a positive predictive value of 84 percent. Our four cases reported here represent the clinical equivalent to this study.

Our group has demonstrated in a previous prospective study, that exfoliative cytology performed on oral brushings can help dentists to decide, if a given oral lesion is malignant or not. Sensitivity of our cytological diagnosis on oral smears for the detection of cancer cells was 94.6%, specificity 99.5%, positive predictive value 98.1% and negative predictive value 98.5%. DNA-aneuploidy was assumed if abnormal DNA-stemlines or cells with DNA-content greater 9c were observed. On this basis the prevalence of DNA-aneuploidy in smears of oral squamous cell carcinomas *in situ* or invasive carcinomas was 96.4%. Sensitivity of DNA-aneuploidy in oral smears for the detection of cancer cells was 96.4%, specificity 100%, positive predictive value 100% and negative 99.0%. The combination of both techniques increased the sensitivity to 98.2%, specificity to 100%, positive predictive value to 100% and negative to 99.5% [12].

In the cases reported here, cytology alone detected carcinoma cells in three of four cases. DNA cytometry revealed aneuploidy as a marker for neoplasia in all four cases of early cancer. Yet the clinicians doubted these diagnoses, as they were not consistent with the results of the histological examination of the scalpel biopsies up to this point. False negative results occasionally occur in histological examinations, but they are very rarely reported in the literature [5].

False negative cancer diagnoses have similar reasons in histology and cytology: (1) Sampling errors. Surgical biopsies may be taken at non-representative sites for histology, as well as brush biopsies and mean

for cytology. (2) Screening errors: Fixed and paraffin-embedded tissues may not sufficiently be worked up as serial sections in histology and smears may not be screened thoroughly by cytologists. (3) Interpretation errors: Due to subjectivity of interpretation of microscopical images of tissues and cells interobserver variations in histological and cytological diagnoses occur. (4) Furthermore epithelial dysplasias and borderline lesions represent morphological alterations which are suspicious for malignancy but do not represent sufficient evidence for its definite diagnosis. The latter situation offers the opportunity for adjuvant methods to identify (prospective) malignancy earlier than subjective interpretation of histological or cytological images. This is the case for DNA-cytometry and AgNOR-analysis [10,11].

Furthermore data from the literature show insufficient inter- and intra-individual reproducibility of histological grading epithelial dysplasias (e.g., oral cavity [1,2], squamous intraepithelial lesions [17] and cervical abnormalities [14]) and problems in unequivocally identifying carcinoma *in situ*.

DNA-image-cytometry was able to confirm the malignant nature of suspicious abnormal epithelial cells. Thus, in one case the initial cytological diagnosis "doubtful" could be changed to "definitely malignant, consistent with a squamous cell carcinoma". In three cases DNA cytometry confirmed the cytological report stating unequivocal squamous malignant cells. Thus this method should be applied for the purpose of quality assurance of cytologically suspicious or doubtful oral squamous lesions.

In most cases clinicans are responsible for false negative cancer diagnosis (sampling error), due to non-representable acquisition of cells or tissues. In gynaecological and pulmonary cytology this error accounts for about 80% of false negative diagnoses. Errors due to microscopically overlooked tumour cells by cytopathologists are less frequent than due to sampling errors [9]. Most likely, in two of our cases (C and D) geographic errors occurred performing scalpel biopsies while brush biopsies revealed cancer diagnoses despite this source of error. This may be explained by the larger area from which brushings sample cells compared with a limited mucosal area from which a biopsy is taken. Mostly oral surgeons do not excise suspicious lesions in toto to establish its nature at first. Thus, they have to decide, which part of the lesion represents the area of strong suspicion of malignancy. This disadvantage is minimized using brush biopsy, because it is possible to brush all areas of the relevant lesions.

We believe that the initial tumour negative histological diagnoses of case A and B might have been errors in microscopical interpretation although it still has to be proven that pseudoepitheliomatous hyperplasia without signs of malignancy in other cases does not reveal DNA-aneuploidy.

The malignant transformation at the beginning of carcinogenesis affects only few cells long before small parts of the tissue were involved. Thus cytologic examination should be a suitable method to elucidate the dignity of suspicious oral lesions earlier than histology. In cases of early cancer diagnosis at least some hundred abnormal cells are required, so that, for example, DNA-image-cytometry may identify signs of aneuploidy to support the cytological cancer diagnosis of cancer.

We conclude that the application of DNA-image cytometry increases the diagnostic accuracy of brush biopsies of the oral cavity. This adjuvant method is able to identify malignant cells in brush biopsies via detection of abnormal DNA stemlines or atypically high single DNA-values (>9c). The advantages of brush biopsies are obvious: brushings of all visible oral lesions, even if they are clinically not considered as suspicious for cancer, are an easily practicable, cheap, non-invasive, largely painless, safe and accurate screening method for detection of oral precancerous lesions (dysplasias), carcinomas *in situ* or invasive squamous cell carcinoma in all stages. We conclude that DNA-image cytometry is a very sensitive, highly specific and objective adjuvant tool for the earliest identification of neoplastic epithelial cells in oral smears.

Acknowledgements

We thank Dr. C. Huppa, King's College Hospital, London, for his critical review of the manuscript.

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Fourth updated ESACP consensus report on diagnostic DNA image cytometry

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A task force of experts in the field of diagnostic DNA image cytometry, invited by the ESACP, and further scientists or physicians revealing experience in that diagnostic procedure (names are given in Addendum A), agreed upon the following 4th updated Consensus Report on Standardised Diagnostic DNA Image Cytometry during the 7th International Congress of that society in Caen, 2001. This report is based on the three preceding ones [6,14,17]. It deals with the following items:

- Critical review and update of the definitions given in the 1997 Consensus Update;
- Review and detailed description of basic terms, principles and algorithms for diagnostic interpretation;
- Recommendations concerning diagnostic or prognostic applications in specific fields of tumour pathology.

This update is not aimed to substitute the 1997 consensus, but to make necessary addenda and give more detailed descriptions of those items not unequivocally to interpret by potential users of the methodology.

1. Introduction

Cytogenetics have opened new sights in the understanding of tumour pathology during the last decade [1, 11,12,21,23]. It could be confirmed that chromosomal aneuploidy, characterised by numerical and/or structural chromosomal aberrations, is an early key event in tumorigenesis caused by genetic instability [4,13].

- The cytometric equivalent of chromosomal aneuploidy, DNA aneuploidy, serves as a marker of neoplasia by assessing large-scale genomic alterations resulting from genetic instability [24,28].
- DNA cytometry is furthermore able to monitor the effect of cytogenetic tumour progression on nuclear DNA content. Quantitation of DNA aneuploidy may therefore serve as a prognostic marker [2,7,8,11,29].
- Changes in DNA ploidy may indicate therapeutic effects [20].

As an example, recently an "International Consensus Conference on the Fight against Cervical Cancer" agreed that DNA image cytometry is indicated for the identification of prospectively malignant cells in squamous intraepithelial lesions and ASCUS, because chromosomal and DNA aneuploidy is consistent with high grade squamous intraepithelial lesions and cervical carcinoma. The finding of aneuploidy qualifies SIL therefore as high grade, needing further clinical management [15].

Having this in mind, the task force has critically reviewed the 1997 consensus reports as to check whether the definitions, algorithms and recommendations agreed upon, are still appropriate to be applied in tumour diagnostics and prognostication, and to allow exchange and comparison of results obtained by different laboratories.

2. Background and aims of DNA image cytometry

Quantitation of nuclear DNA content by cytometry has become practice for assistance in the diagnosis and grading of malignant tumours for some years. The DNA content cannot be measured directly by cytometry. After quantitative DNA-staining, the nuclear IOD

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Analytical Cellular Pathology 23 (2001) 89–95
ISSN 0921-8912 / $8.00

(Integrated Optical Density) is the cytometric equivalent of its DNA content. Therefore the DNA content is expressed in a "c" scale in which 1c is half the mean nuclear DNA content of cells from a normal (non-pathological) diploid population in G0/G1 cell cycle phase.

For practical reasons as a term being accepted and used throughout the literature "DNA ploidy" will be further used. However, we want to point out that in practice the cytometric evaluation of nuclear DNA content is often improperly called "DNA ploidy" which is assumed to be the quantitative cytometric equivalent of "chromosomal ploidy". Both terms are not identical. Whereas "chromosomal ploidy" is theoretically detectable by cytogenetic methods in each single cell, its DNA content cannot be equated with a certain chromosomal outfit [25,26,30], but the term "DNA ploidy" is therefore the expression of the typical large-scale genomic status of a cell population. It can be the equivalent of that status also in single cells.

Indeed, the quantity of nuclear DNA may be influenced by the following mechanisms: replication, polyploidization, gain or deletion. Each affects the size or the number of chromatids. Viral infections may change the nuclear DNA content detectable by flow and image cytometry. Among others, the unspecific effects of cytostatic or radiation therapy, vitamin B12 deficiency, apoptosis, autolysis and necrosis on nuclear DNA content also play a role [3,9,24,27,31,32]. Furthermore, the DNA content of a cell is regularly changed throughout the cell cycle.

All these effects have to be taken into consideration when a diagnostic interpretation of DNA histograms is performed.

At present the basic aim of diagnostic DNA cytometry is to identify DNA stemlines outside the normal (euploid) regions as abnormal (or aneuploid) at a defined statistic level of significance. Furthermore DNA image cytometry should give information about

- Number of abnormal (sive aneuploid) DNA stemlines;
- Polyploidization of euploid or aneuploid DNA stemlines;
- Occurrence of rare cells with an abnormally high DNA content, most likely resulting from genomic alterations;
- Cell cycle fractions.

During the past few years a huge body of methodological experience has been gathered allowing ICM-DNA users to perform their DNA measurements at a high level of quality. Recommendations for the entire process of preparation and measurement have been given previously [17].

3. Principles of the method

Because DNA image cytometry results in nuclear IOD values in arbitrary units, equivalent but not identical with nuclear DNA content, the quantitation of nuclear DNA requires a rescaling of IOD values by comparison with those from cells with known DNA content, so-called reference cells. By means of reference cells the arbitrary unit scale (AU) will be transformed in a reference unit scale (2c, 4c, 8c, for example) [10,25]. In general, there are two types of reference cell systems: external and internal ones, respectively. Whereas the external reference cells are very easily to identify by the investigator, but often not to prepare in parallel with the clinical sample, the internal reference cells have the advantage of sharing all preparatory steps with the analysis cells in the clinical specimens.

The nuclear IOD values of reference cells own the same methodological limitations in terms of precision of the measurements as the appropriate IOD values of the analysis cells.

The mean ratio between the modal IOD values of the non-pathologic cells of the tissue under study and the reference cells used is called corrective factor. This corrective factor must be applied to DNA measurements from the clinical sample before any DNA histogram interpretation [25]. Due to the methodological variability, mentioned above, that ratio is not constant. The accuracy of each diagnostic DNA evaluation depends decisively on the standard error of the corrective factor used during the rescaling procedure [16].

Because most of the interpretations of DNA measurements are population-based, the results are usually displayed as DNA histograms. The bin size of such histograms should be adapted to the precision of the actual measurements, i.e., the lower the variability in the reference cell peak, the smaller the bin size of histogram classes could be.

The grammalogues "ICM-DNA" (image cytometric DNA) and "FCM-DNA" (flow cytometric DNA) are good descriptors used to designate the type of nuclear DNA measurement.

4. Basic performance standards

The usual precision of recent DNA image cytometric measurements should at least allow DNA stemlines to be identified as abnormal (or aneuploid), if they deviate more than 10% from the diploid (2c) or tetraploid region (4c), i.e., if they are outside $2c \pm 0.2c$ or $4c \pm 0.4c$.

To achieve this goal with an error probability $p < 0.05$ the test statistics [16] require a measurement performance described by:

- the cv of the ratios between modal IOD-values of reference cells and non-pathologic G0/1 cells in a series of measurements is <5%;
- the relative standard error (rSEM $= \mathrm{cv}/\sqrt{n}$) of reference cells in each sample is <1.5%.

Furthermore, a DNA-stemline should be identified as polyploid within the duplication position of a G0/1-phase-fraction $\pm 0.2c$ (at 4c), and $\pm 0.4c$ (at 8c), respectively, with an error probability $p < 0.05$ if

- the cv of the ratios between modal IOD-values of non-pathologic G0/1- and G2/M-phase-fractions in a series of measurements is <2.5%.

Every scientist and physician who applies DNA image cytometry is free to choose his appropriate methodological specification, if he only meets the performance standards agreed above.

The different aspects of the measuring process and of the interpretation should be regularly subjected to quality control measures in order to warrant a steadily high level of quality of the diagnostic procedure. Appropriate protocols for such a quality assurance guide have been described previously [14].

These protocols have also been implemented into the EUROQUANT quantitation server [18,19], adopted by the ESACP.

5. Definitions of basic terms of DNA image cytometry

DNA histogram

means a frequency distribution of IOD values obtained by cytometric measurements of cells stained stoichiometrically for their DNA and rescaled by IOD values from reference cells in "c" units.

DNA histogram peak

means a statistically significant local maximum in a DNA histogram. A recommended principle of finding and describing a peak by objective methods is given in the addendum.

Modal value of a DNA histogram peak

means the most frequent value in the peak, i.e. the mean value of that histogram class containing the highest number of nuclei. This is close or equal to the mean value of a fitted Gaussian curve according to the principle mentioned above.

DNA stemline

A stemline means a proliferating cell population with a unique chromosomal outfit.

A DNA-stemline is the G0/G1cell-phase fraction of a proliferating cell population (with a first peak and a second doubling one, or nuclei in the doubling region).

DNA euploidy

means that type of DNA distributions which cannot be differentiated from those of normal (resting, proliferating, or polyploidizing) cell populations.

DNA aneuploidy

means those types of DNA distributions which are different at a statistical significant level from those of normal (resting, proliferating, or polyploidizing) cell populations. DNA aneuploidy can either be seen as DNA stemline aneuploidy or can be indicated by "rare events" (see below).

DNA diploidy

Means that type of euploid DNA histograms which is the cytometric equivalent of a resting or proliferating cell population with a diploid chromosomal set.

Polyploidisation

Means the (repeated) doubling of a chromosomal set.

Euploid DNA polyploidisation means the occurrence of peaks in the duplication ($\times 2$, $\times 4$, $\times 8$, ...) regions of euploid stemlines. In human tissues usually the highest peak is at 2c.

Aneuploid polyploidisation means the occurrence of peaks in the duplication region of aneuploid stemlines.

Rare events in DNA histograms

are abnormal cells often called 5c or 9c exceeding events, having *a nuclear* DNA content higher than the duplicate or quadruplicate region of a normal G1/G0 phase population, i.e., not belonging to G2M phase. They likely represent non-proliferating abnormal cells with different chromosomal aneuploidies and abnormally high numbers of chromosomes.

DNA histogram typing

Histogram types are the result of a diagnostically and/or prognostically valid (i.e., statistically proven) classification of DNA distributions. It results in specific histogram types or classes (e.g., diploid, tetraploid, hypodiploid, aneuploid, etc., see also below).

DNA histogram grading in case of malignancy

The terminology for grading is only applicable to neoplasias, either proven by morphological investigations or in case of DNA aneuploidy. The prognostic interpretation of grading has principally to be tumour type specific. Grading can be performed as "histogram typing" using specific histogram types or by complex grading algorithms (see below).

The following *DNA histogram types* are helpful in the prognostication and monitoring of solid tumours:

- A peridiploid DNA histogram is supposed to with a stemline between 1.8c and 2.2c.
- A peritetraploid DNA histogram is supposed to exist in case of proven neoplasia or DNA aneuploidy and a stemline between 3.6c and 4.4c.

(The setting of the thresholds depends on the performance of the specific measurement, i.e., instrumentation and specimen preparation. The thresholds given above are therefore around 2c and 4c, respectively, $\pm 2 \cdot$ cv of the corrective factor (see below) of the system. They are around 2c and 4c, respectively, $\pm 10\%$ according the *minimal* performance standards, see above.)

- An x-ploid DNA histogram is supposed to exist in case of proven neoplasm and a stemline, alone or additional to a peridiploid/peritetraploid one, outside the thresholds mentioned above. "x" should be substituted by the DNA ploidy value of that stemline (e.g., triploid, hypertetraploid, or 2.6 ploid, etc.).
- A multiploid DNA histogram is supposed to exist in case of proven neoplasia and more than one stemline at positions outside the thresholds mentioned.

For each of those DNA histogram types the exact position of the stemline should be given. However, one has to take into consideration that the prognostic relevance of these classes may be different among the various tumour types.

6. Recommendations for clinical reporting on diagnostic DNA image cytometry

6.1. Identification of neoplasia

- Repetition of histological/cytological diagnosis;
- Indication for DNA-cytometry;
- Type of investigated material:
  - preparation,
  - type and number of reference cells,
  - type and number of analysis cells;
- Short description of the DNA-histogram (DNA histogram type);
- Interpretation of the DNA-histogram concerning the occurrence of DNA-aneuploidy and/or the histogram type;
- Summarised morphologic/cytometric diagnosis;
- Enclosures (DNA-histogram, relevant listing of indices of DNA-distribution, applied algorithms).

Remark. Changes of diagnoses in one-dimensional, unspecific nomenclatures referring only to the probability of presence of tumour cells are allowed (doubtful for malignancy → strong suspicion for malignancy → unequivocal malignancy).

Changes of morphological diagnoses in multidimensional, specific nomenclatures as the Bethesda system for reporting cervical smear diagnoses are not allowed as the given entities are only morphologically defined.

6.2. Grading of tumour malignancy

- Repetition of histologic/cytologic diagnosis;
- Indication for DNA-cytometry;
- Type of material:
  - preparation,
  - type and number of reference cells,
  - type and number of analysis cells;
- Short description of DNA-histogram;
- Prognostic interpretation of DNA-histogram;
- Summarised morphologic/cytometric diagnosis;
- Enclosures (DNA-histogram, relevant indices of DNA-distributions, applied algorithms).

Remark. Specific histologic or cytologic grades of malignancy (e.g., Bloom & Richardson for breast cancer, Gleason for prostatic cancer) should not be changed by discrepant DNA-results. Morphologic grades remain the same, despite deviating DNA-grades of malignancy.

Yet, the biological interpretation concerning the occurrence of malignant cells, or the malignant potential of tumour cells may be changed in a retrospective synopsis of morphologic and cytometric results.

Addendum A

The following scientists participated at the Consensus meeting during the 7th Congress on Analytical Cellular Pathology:
J.B.A. Baak, Stavanger, J. Belien, Amsterdam, A. Böcking, Düsseldorf, M.G.W. Bol, Alkmaar, A. Buhmeida, Turku, D. Chiu, Vancouver, Y. Collan, Turku, H.E. Danielsen, Oslo, J. Dufer, Reims, A. Elzagheid, Turku, J. Erenpreisa, Riga, N. Friedrichs, Düsseldorf, L. Giacomelli, Padova, F. Giroud, Grenoble, A. Gschwendtner, Innsbruck, T. Hanselaar, Nijmegen, G. Haroske, Dresden, M. Hubler, Basel, E. Janssen, Alkmaar, G. Jenkinson, Cambridge, P. Kiehl, Hannover, A.J. Kruse, Alkmaar, J. Lavrencak, Ljubljana, J. van Marsdyn, Amersfort, W. Meyer, Dresden, A. Reith, Oslo, F. Theissig, Dresden, J. van der Laak, Nijmegen, M. Vogelbruch, Hannover.

Addendum B

Algorithmic principles for understanding and following the definitions given above:

B.1. Finding a histogram peak [18,19]

(a) sort all measured IOD values in increasing order;
(b) count all measurement values inside a window of IOD ± 5%;
(c) move that window along the ranked measurement values, look for the window position with the highest number of values;
(d) test that maximal number Mnumb for significance:
    If (Mnumb · 1.5 > 6 · ($\log_{10}$(total number) $-1/\log_{10}$(total number)) + 8)
    Then a peak was found;
(e) a peak was found:
    fit parameters of a Gaussian curve for
    – peak position
    – peak width
    – peak height
    to the measurement values inside the window, use the integral of the Gaussian curve (error function erf) for avoiding digitalisation of a histogram;
(f) subtract from the measurement values the values fitted to the peak;
(g) for searching the next peak go to (b)
    end if
    no further peaks found.

B.2. Coefficient of variation

The coefficient of variation is the the quotient of the standard deviation divided by the mean, given in percent:

$$cv = \frac{SD}{m} \cdot 100.$$

B.3. DNA-index

The modal value of a DNA stemline (or the mean of its fitted Gaussian curve) divided by the modal value of G0/1 peak of the reference cells.

B.4. DNA stemline ploidy [18,19]

The modal value of a DNA stemline (or the mean of its fitted Gaussian curve) in "c" units.

B.5. DNA stemline abnormality (aneuploidy) [16,18,19]

An error probability p for belonging a peak modal value to the population of non-pathological peaks is given by:

$$p(t) = 2 \cdot \frac{1}{\sqrt{2\pi}} \cdot \int_t^\infty \mathrm{e}^{-x^2/2} \cdot \mathrm{d}x.$$

The test value t is given by:

$$t = \frac{|1 - M_\mathrm{A}/(M_\mathrm{R} \cdot cf)|}{\sqrt{cv_\mathrm{R}^2/n_\mathrm{R} + cv_\mathrm{A}^2/n_\mathrm{A} + cv_\mathrm{cf}^2}},$$

where:

cf, corrective factor;
M_A, modal value of analysis cell peak;
M_R, modal value of reference cell peak;
cv_A, cv of analysis cell peak;
cv_R, cv of reference cell peak;
n_A, number of analysis cells in the peak;
n_R, number of analysis cells in the peak;

cv_{cf}, cv of the corrective factor.

If p is below a given threshold (e.g., 0.05, 0.01, 0.001) the classification of a peak as aneuploid (abnormal) is done with a false positive rate (FPR) of 5%, 1% or 0.1%, respectively.

B.6. Rare events [18,19]

The threshold above that the cells do not belong to a G2/M phase with an error probability of 0.15% is given by:

$$M_{A(d)} \cdot (1 + 3 \cdot cv_{A(d)}) \cdot cf_{poly} \cdot (1 + 2 \cdot cv_{cfpoly}),$$

where:

$M_{A(d)}$, modal value of the analysis cell peak classified as diploid(see above),
$cv_{A(d)}$, cv of the (diploid) analysis cell peak,
cf_{poly}, duplication factor of polyploidising populations, i.e., $M_{A(4c)}/M_{A(2c)}$,
cv_{cfpoly}, cv of the duplication factor.

B.7. Complex grading algorithms

The 2c Deviation Index (2cDI) [5]:

$$2cDI = \sum_{i=1}^{n} \frac{(c_i - 2c)^2}{n}$$

is the DNA content of a single nucleus, rescaled by the mean corrective factor of the tissue type under investigation.

The ploidy balance (PB) [22]:

$$PB = \frac{(n_{eu} - n_{an})}{N} \cdot 100,$$

n_{eu} is the number of all cells in euploid regions of the DNA histogram rescaled by the mean corrective factor of the tissue type under investigation (1.8c–2.2c; 3.6c–4.4c; 7.2c–8.8c); n_{an} is the number of all cells outside the euploid regions of rescaled DNA histogram; N is the total number of cells.

B.8. Finding optical disturbances (glare and diffraction effects) [16,18,19]

If the amount of the coefficient of correlation between nuclear area and IOD (DNA) in a single peak is greater than 0.40, then optical disturbances outside tolerable limits are assumed.

The coefficient r is calculated from all objects around the peak modal value (see 1.) $\pm 2 \cdot 5\%$.

B.9. Finding sampling inhomogeneities [18,19]

The proof of sampling inhomogeneities requires an unsorted sequence of IOD (DNA) values, originating from a stochastic sampling approach.

A sorting concerning time of acquisition (order of aquisition, resp.) allows conclusions for temporal or spatial inhomogeneity, a sorting concerning xy-coordinates leads to the same conclusions.

The evaluation of the entire sample gives hints for the distribution of reference cells throughout the sampling, the evaluation of peaks shows temporal and spatial inhomogeneities.

For each type of evaluation the sample (peak) is divided in 2 to 10 equal parts. In each part the distribution of IOD (DNA) values is tested non-parametrically (U-test) for being equal to the distributions of all other parts. After Bonferroni adjustage the error probability for being equally distributed is computed.

B.10. Rescaling of DNA values [18,19]

The rescaling is aimed at correcting systematic deviations from the theoretical 2c; 4c; 8c ratio of euploid cell populations. It allows the intercomparison of DNA values obtained under different technological conditions.

The actual ratios for the peak positions in a given technological condition should be determined by measurement of at least 36 non-pathological samples, comprising all peak regions of interest (usually 2c and 4c, sometimes 8c, too).

For each cell or peak a rescaling factor (corrective factor)

$$cf = \frac{2^i}{\overline{m}_i} + \frac{x - \overline{m}_i}{\overline{m}_{i+1} - \overline{m}_i} \cdot \left(\frac{2^{i+1}}{\overline{m}_{i+1}} - \frac{2^i}{\overline{m}_i} \right)$$

with $\overline{m}_i \leqslant x < \overline{m}_{i+1}$, where $\overline{m}_i$ is mean modal DNA value of the euploid peak$_i$ and $i = 1, 2, 3, \ldots$ is computed.

Measurement values around a peak$_i$ are all values greater than the geometrical mean between peak$_{i-1}$ and peak$_i$ as well as all values smaller than the geometrical mean between peak$_i$ and peak$_{i+1}$.

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