

Figure 1 Four-year event-free survival of neuroblastoma infants detected by mass screening based on MYCN amplification. The curve was generated with the Kaplan and Meier product limit method. The 4-year OS rate was 99% for patients without MNA, 89% for patients with amplification from three to nine copies, and 53% for patients with more than 10 copies ($P < 0.001$).

MNA, it is necessary to establish and perform the appropriate treatment, including not only surgical resection but also chemotherapy.

MYCN amplification was strongly and inversely correlated with the prognosis in infantile cases, although the frequency of MNA in the cases discovered through the MSPN was small (2.2%). Prediction of the presence of MNA in the tumour based on urinary levels of HVA and VMA and stage of the tumour was difficult in the cases we encountered. Our results demonstrate that evaluation of MNA is important for the selection of appropriate treatment for infantile NB.

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Appendix A1

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Pretreatment and one-shot separating analysis of whole catecholamine metabolites in plasma by using LC/MS

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Abstract Catecholamines are biogenic amines that play an important role in the nervous system. Some catecholamines have been used as tumor makers of pheochromocytoma, paraganglioma and neuroblastoma. The analysis of total catecholamine metabolites should be useful for one-shot screening of multiple aspects of diseases; however, it is difficult to do this, because the catecholamine metabolites are divided into three groups: five amines, one amino acid and three carbonic acids. Catecholamines and small molecules were separated from plasma proteins by an internal-surface reversed-phase column (protein-coated octadecylsilica column) and were analyzed by liquid chromatography (LC)/mass spectrometry (MS) using electrospray ionization time-of-flight MS. Using a reversed-phase column and hydrophilic mobile phases, we succeeded in the separation of nine catecholamines, all of which had similar structures. These nine substances were eluted in the following order: norepinephrine, epinephrine, normetanephrine, dopamine, metanephrine, 3,4-dihydroxyphenylalanine, vanillomandelic acid, 3,4-dihydroxyphenylacetic acid and homovanillic acid. The reproducibility of this method was acceptable. The highest coefficient of variation was 7.4%. In addition, various types of compounds were separated from and detected in plasma proteins by applying LC/MS. The plasma direct injection method, which uses an internal-surface reversed-phase column and an ion-pair reagent, allowed us to separate small molecules from plasma proteins. MS detected some compounds that high-performance LC could not succeed in separating and detecting with UV detection. We think that the method can be applied to find new markers in neuroblastoma, by comparing the plasma of patients with that of normal infants. The method can be also used to help in making a diagnosis of other diseases and finding their new makers.

Keywords Neuroblastoma · Catecholamine · Internal-surface reversed-phase column · Liquid chromatography/mass spectrometry

Abbreviations BSA: Bovine serum albumin · DOPA: 3,4-Dihydroxyphenylalanine · DOPAC: 3,4-Dihydroxyphenylacetic acid · ESI: Electrospray ionization · HPLC: High-performance liquid chromatography · HVA: Homovanillic acid · LC: Liquid chromatography · MS: Mass spectrometry · ODS: Octadecylsilica · VMA: Vanillomandelic acid

Introduction

Neuroblastoma is a malignant tumor especially found in infants. The frequency of the incidence is the second highest after that of leukemia. Because of its high frequency and the possibility of curing the disease with early detection, a nationwide neonatal mass screening at 6 months of age was conducted in Japan between 1985 and 2004. Infants with neuroblastoma produce an excess of catecholamines compared with normal infants [1]. Therefore, a considerable amount of vanillomandelic acid (VMA) and homovanillic acid (HVA), the final metabolites of catecholamines, is excreted in the urine [2–7]. The present mass screening observed an increase of the final metabolites by high-performance liquid chromatography (HPLC) [8, 9]; however, this screening method had problems with both the accuracy of metabolite measurement and the determination of a definitive cutoff value. The committee of the Ministry of Health, Labour and Welfare in Japan reached the conclusion that the screening has no effect on mortality from neuroblastoma, and thus it was suspended [10, 11]. Contrary to the negative opinions, some believe that the mass screening actually helped reduce mortality due to neuroblastoma. This disease is still a convalescent unsatisfactory disease and, therefore, mass screening is vital to a reduction of the mortality due to infantile cancers [12–14]. In order to restart the mass screening, the current method, which targets only the final

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substances (VMA and HVA) and considers metabolic substances of catecholamine to be insignificant, needed to be reexamined. We analyzed all metabolic substances of catecholamine, including metabolic by-products, and set up an analytical method using liquid chromatography (LC)/mass spectrometry (MS). Urine is the correct sample for the screening, posing the least burden and risk to infants and their parents; however, urine does not contain all of the compounds of the human body and there is also a problem with the stability of components generated by contamination. Blood, on the other hand, contains all of the compounds of the human body; thus, we established a test method for analysis of plasma.

In the plasma, small molecules unite with plasma proteins, especially albumin. For the analysis of small molecules in the plasma, these small molecules needed to be separated from plasma proteins. For the separation, we employed a direct plasma injection analysis, which uses an internal-surface reversed-phase column. Figure 1 shows the characteristics of the column. The outside surface of porous resins was coated with bovine serum albumin (BSA), so that the column did not adsorb the plasma proteins, but still had the reversed-phase characteristics for small molecules. The internal-surface reversed-phase column was selected as a precolumn for the deproteinization and trapping of small molecules [15, 16].

In the human body, catecholamines are formed by the following sequence of reactions: tyrosine→3,4-dihydroxyphenylalanine (DOPA)→dopamine→norepinephrine→epinephrine. They all have physiological activity. After they have performed their roles, DOPA is metabolized and becomes HVA via 3,4-dihydroxyphenylacetic acid (DOPAC). Dopamine also changes into HVA. Norepinephrine transforms into normetanephrine, and epinephrine becomes metanephrine. They then both transform into VMA. These reactions are catalyzed by the following enzymes: tyrosine hydroxylase, DOPA decarboxylase, dopamine- β -hydroxylase, phenylethanolamine-*N*-methyltransferase, catechol-*O*-methyltransferase, and monoamine oxidase. Finally HVA and VMA are excreted in the urine (Fig. 2).

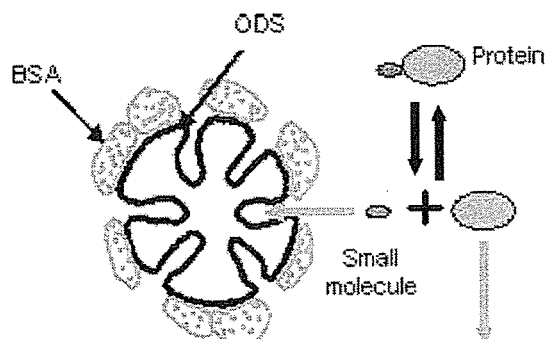


Fig. 1 The characteristics of the internal-surface reversed-phase column. This column was selected as a precolumn for the deproteinization and trapping of small molecules. BSA bovine serum albumin, ODS octadecylsilica

Materials and methods

Chemicals

DOPA, dopamine, DOPAC, epinephrine, normetanephrine, metanephrine, HVA, VMA and formic acid were obtained from Sigma. Norepinephrine was from Fluka. Methanol, acetonitrile, ammonia solution, ammonium hydrogencarbonate and pentadecafluorooctanoic acid [$\text{CF}_3(\text{CF}_2)_6\text{COOH}$] were purchased from Kanto Chemical. Trifluoroacetic acid (CF_3COOH), pentafluoropropionic acid ($\text{CF}_2\text{CF}_2\text{COOH}$) and heptafluorobutyric acid [$\text{CF}_3(\text{CF}_2)_2\text{COOH}$] were obtained from Aldrich. Nonfluorovaleric acid [$\text{CF}_3(\text{CF}_2)_3\text{COOH}$] was from Tokyo Kasei Kogyo. Methanol and acetonitrile were HPLC grade, and all other reagents were analytical grade. Water was deionized and purified by a Milli-Q Academic A10 purification system from Millipore.

Sample preparation

Catecholamine metabolites were dissolved in water at a concentration of 1 mg/mL and mixed in one solution to a final concentration of 100 $\mu\text{g/mL}$ to form a standard solution. The standard solution was diluted with fetal bovine serum or human plasma to 10 $\mu\text{g/mL}$ and was used as sample-spiked plasma.

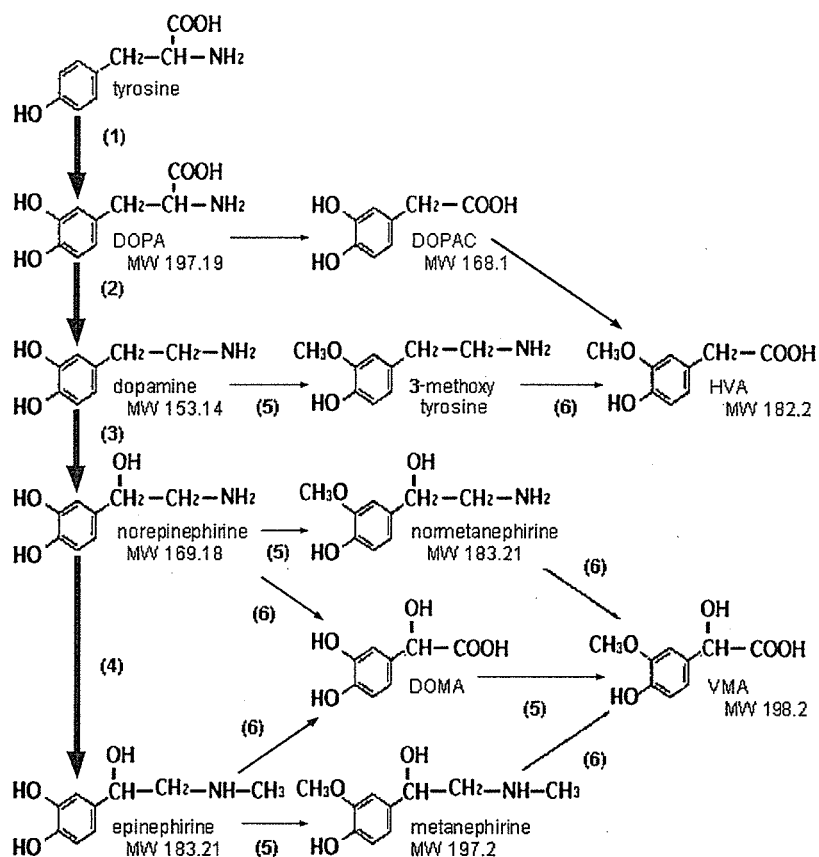
Instrumentation

The HPLC-UV system was a Gulliver series system from JASCO, consisting of a DG-980-50 3 line degasser, an LG-1580-02 ternary gradient unit, a PU-980 Intelligent HPLC pump and a UV-970 Intelligent UV/vis detector. Pretreatment was performed by using the internal-surface reversed-phase column (TSK precolumn BSA-ODS:TOSOH) and a column-packed anion-exchange resin (TOYOPEARL QAE-550:TOSOH). The analysis column was an L-column ODS (150 mm \times 4.6 mm, 5 μm) from CERI. MS analysis was performed using a Mariner electrospray ionization (ESI) time-of-flight mass spectrometer from Applied Biosystems.

Chromatographic conditions

Mobile phases for the pretreatment column were water containing 1% of formic acid (solvent A) and 200 mM ammonium hydrogencarbonate buffer/acetonitrile (5:95 v/v) (solvent B). The internal-surface reversed-phase column was equilibrated with solvent A at a flow rate of 0.5 mL/min, and samples were injected into the HPLC system. Following the removal of plasma proteins and other hydrophilic compounds, the mobile phase was changed to solvent B by switching a six-port valve. Catecholamine metabolites and hydrophobic compounds were eluted, and the fractions were collected. Then, the fractions were evaporated. The residue

Fig. 2 The pathway of synthesis and metabolites of catecholamines: 1 tyrosine hydroxylase, 2 3,4-dihydroxyphenylalanine (DOPA) decarboxylase, 3 dopamine- β -hydroxylase, 4 phenylethanolamine-*N*-methyltransferase, 5 catechol-*O*-methyltransferase, 6 monoamine oxidase. DOPAC 3,4-dihydroxyphenylacetic acid, HVA homovanillic acid, VMA vanillomandelic acid



was dissolved in 100 μ L of the mobile phase, and 20 μ L was injected into the LC/MS system. The two mobile phases used for the analysis of these treated samples were solvent A and methanol containing 1% of formic acid (solvent C). Separation was conducted at a flow rate of 0.2 mL/min. For the separation of amines, the concentration of solvent C was 5% for the first 18 min. In order to elute VMA, DOPAC and HVA the concentration of solvent C was raised to 45% for the next 12 min. The UV detector was set to monitor 280 nm. The mass spectrometer was used in the positive-ion ESI mode.

Results and discussion

Separation of catecholamine metabolites

We developed a method for the HPLC separation of catecholamine metabolites. The catecholamine metabolites were divided into two groups: five amines and one amino acid, and three carbonic acids. In order to separate the five amines and one amino acid, the structures of which were similar, the hydrophilic mobile phase needed to be used. In this case, we used 1% of formic acid/methanol (5:95 v/v) and achieved a good separation. Figure 3 shows the LC and MS chromatograms. The elution order was norepinephrine, epinephrine, normetanephrine, dopamine, metanephrine and DOPA. The peaks of norepinephrine were at m/z 152 ($[M+H-H_2O]^+$) and m/z 170 ($[M+H]^+$). Epinephrine was

detected at m/z 184 ($[M+H]^+$), normetanephrine was detected at m/z 166 ($[M+H-H_2O]^+$) and m/z 184 ($[M+H]^+$), dopamine was detected at m/z 154 ($[M+H]^+$), and metanephrine and DOPA were detected at m/z 180 ($[M+H-H_2O]^+$) and m/z 198 ($[M+H]^+$).

DOPAC, HVA and VMA have a strong affinity with ODS. To accelerate the elution of these three substances, the concentration of the organic mobile phase was raised from 5 to 45%. As a result, all procedures were carried out within 60 min. The elution order of carbonic acids was VMA, DOPAC and HVA (Fig. 4). The peaks of DOPAC were at m/z 169 ($[M+H]^+$), m/z 186 ($[M+NH_4]^+$) and m/z 191 ($[M+Na]^+$). HVA was detected at m/z 183 ($[M+H]^+$), m/z 200 ($[M+NH_4]^+$) and m/z 205 ($[M+Na]^+$). No peak was found for VMA. It is suspected that because VMA has a hydroxyl group close to the carboxyl group, the pK_a is lower compared with that of other carbonic acids. Thus VMA could not be ionized well with the positive ESI mode.

By trapping the catecholamine metabolites on the internal-surface reversed-phase column, we separated catecholamine metabolites and small molecules from plasma proteins. Plasma proteins ran through the column without being trapped, because of the size exclusion and the coated BSA. We set the elution time of proteins by the HPLC-UV and Lowry method (absorbance of 700 nm). At a flow rate of 0.5 mL/min and 100 μ L of sample volume, plasma proteins were eluted from the column within 3 min (Fig. 4a,b). Under these conditions the internal-surface ODS did not hold catecholamine metabolites well enough,

Fig. 3 **a** The liquid chromatography (LC) chromatogram of the elution order was norepinephrine, epinephrine, normetanephrine, dopamine, metanephrine, DOPA, VMA, DOPAC and HVA. Flow rate 0.2 mL/min, sample concentration 10 µg/mL, sample volume 20 µL, detection 280 nm. **b** Mass spectrometry (MS) total ion chromatogram (TIC) (electrospray ionization time-of-flight, ESI-TOF, MS; positive mode). **c** Norepinephrine *m/z* 152 ([M+H-H₂O]⁺), *m/z* 170 ([M+H]⁺). **d** Epinephrine *m/z* 184 ([M+H]⁺). **e** Normetanephrine *m/z* 166 ([M+H-H₂O]⁺), *m/z* 184 ([M+H]⁺). **f** Dopamine *m/z* 154 ([M+H]⁺). **g** Metanephrine, DOPA *m/z* 180 ([M+H-H₂O]⁺), *m/z* 198 ([M+H]⁺). **h** DOPAC *m/z* 169 [M+H]⁺, *m/z* 186 [M+NH₄]⁺, *m/z* 191 [M+Na]⁺. **i** HVA *m/z* 183 [M+H]⁺, *m/z* 200 [M+NH₄]⁺, *m/z* 205 [M+Na]⁺

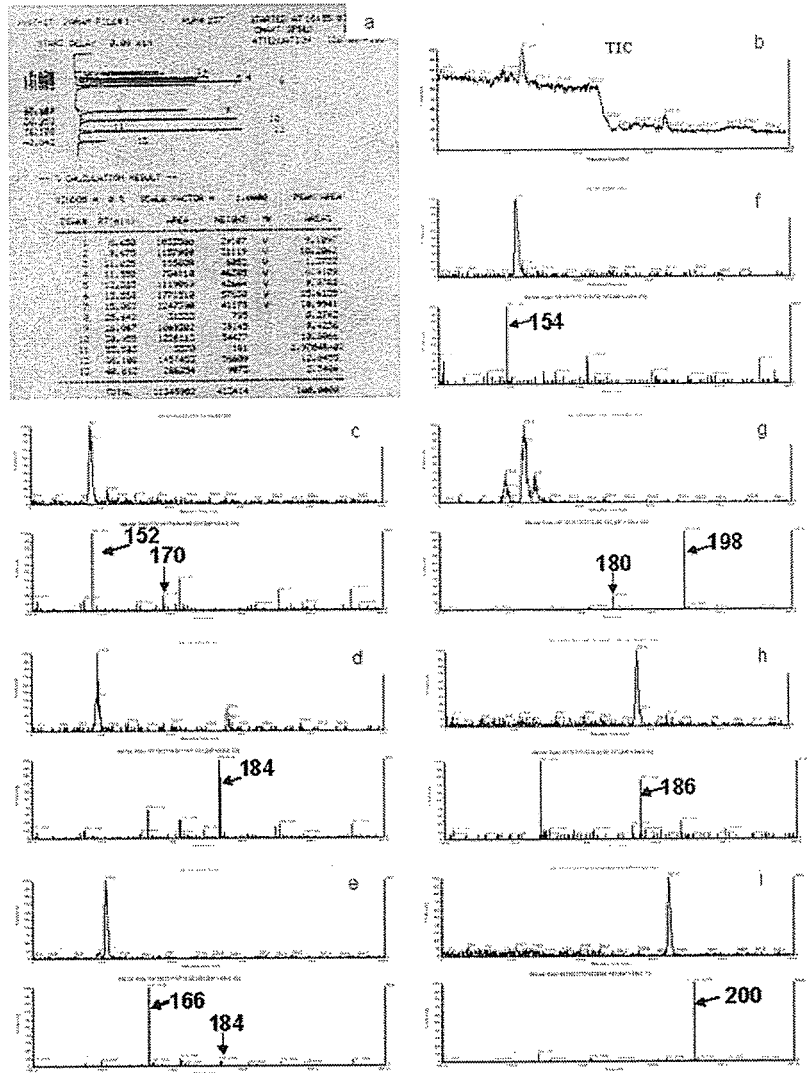


Fig. 4 **a** LC chromatogram of sample-spiked fetal calf serum (FCS). ODS did not hold catecholamine metabolites. **b** Change of protein concentration. Plasma proteins ran down within 3 min. **c** The effect of the ion-pair reagent: 0.1 mM pentadecafluorooctanoic acid/1% formic acid gave good separation. **d** A Injection→3 min; B after column switching

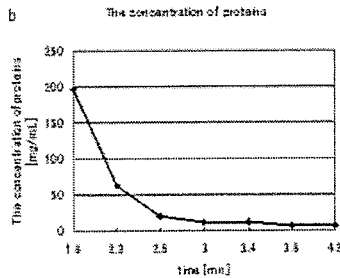
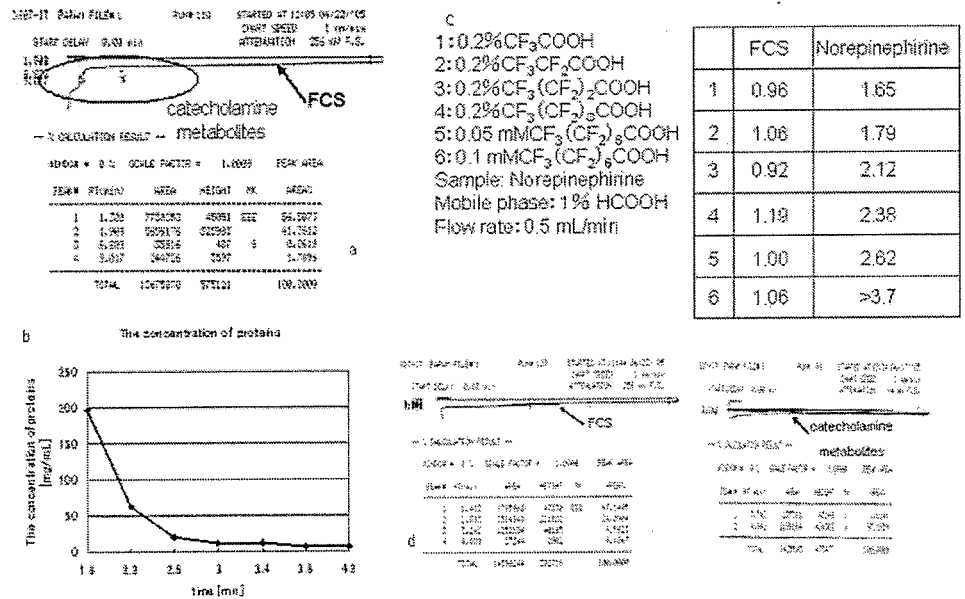
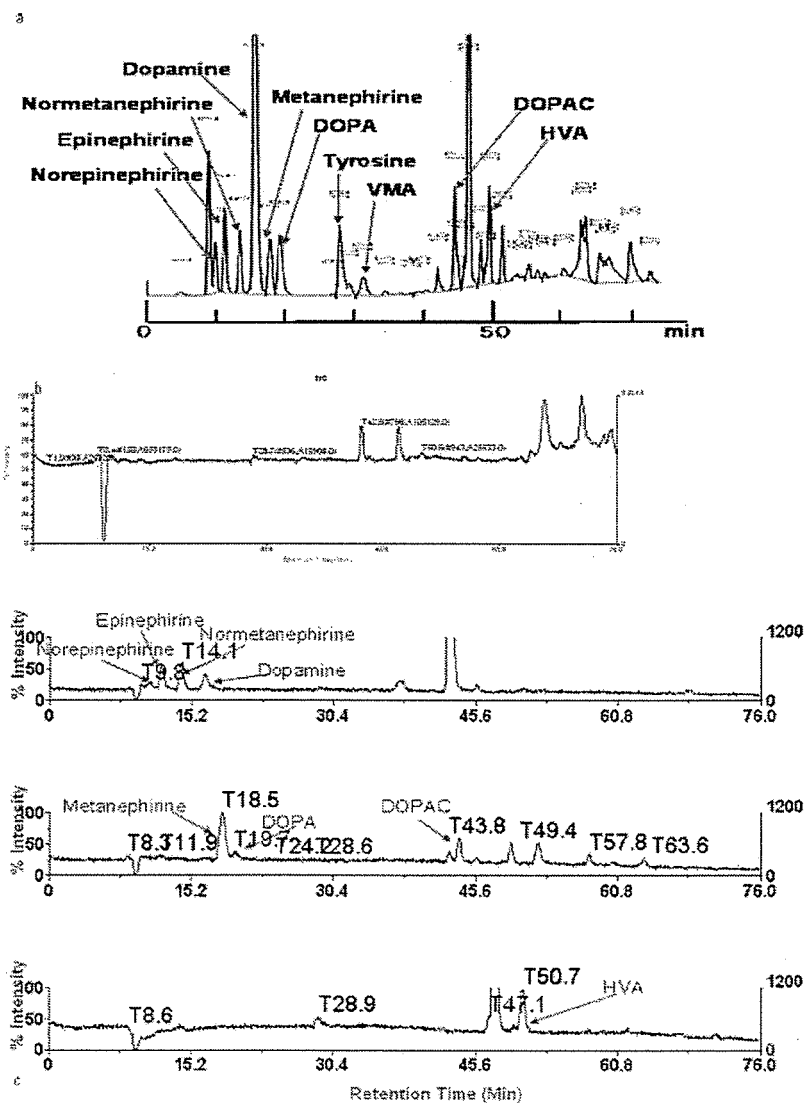


Table 1 The evaluation of reproducibility, recovery and detection limit ($n=5$)

	Recovery (%)	Mean	Standard deviation	Coefficient of variation (%)	Detection limit: signal-to-noise ratio of 3 ($\mu\text{g/mL}$)
Norepinephrine	94.1	305,000	21,400	7.0	1
Epinephrine	94.1	363,000	27,700	7.6	1
Normetanephrine	97.9	294,000	7,900	2.7	1
Dopamine	93.3	341,000	12,300	3.6	1
Metanephrine	93.3	357,000	18,400	5.2	1
3,4-Dihydroxyphenylalanine	94.0	323,000	7,300	2.6	1.5
Tyrosine	97.4	207,000	7,200	3.5	1
Vanillic acid	87.0	145,000	11,000	7.4	—
3,4-Dihydroxyphenylacetic acid	97.7	350,000	11,000	3.1	1.5
Homovanillic acid	98.0	435,000	26,000	6.0	1

Fig. 5 a The LC chromatogram of sample-spiked plasma. We investigated a variety of compounds in plasma and achieved a clear separation by high-performance LC-UV detection for standard compounds. b MS-TIC (ESI-TOF/MS; positive mode). c The MS chromatogram. Standard compounds were detected by their peaks



time	m/z						time	m/z				time	m/z					
8.5	147	149	163	167	172	189	44.8	256			58.1	239						
8.8	177						45.7	167	184		57.8	183	180					
8.9	268	267	268				45.8	196			59	190						
9.6	271						46.1	181			60.1	191						
9.7	266	267					46.7	283	300	301	60.9	297	298					
9.8	172						47	239			63.3	182						
9.9	205	234	264	279	290		47.6	168	205	208	207	63.8	191					
10.1	175						49.4	180	181	195	63.7	208						
10.2	162	183	227	226	228		49.5	285			64.8	223	224	241	242	263		
14	204						49.7	222			65.2	288						
15	235						50.4	222			65.5	264	249	250	251	271	272	273
16	137						50.7	183	200	201	66.7	266	267	288				
17.5	150	151					50.8	282			67.6	283	287					
28.8	193						52.3	165	187	277	68.1	294						
37.5	168						52.8	153			68.8	269	270					
41.9	195						53	256			69.9	280						
42.8	165	167	168	169			53.2	172			70.1	299						
43.2	181						53.3	160			70.8	284						
43.8	285						55.1	181			71.3	279	280	281				
43.9	193	180	262				56	291			72.3	252						

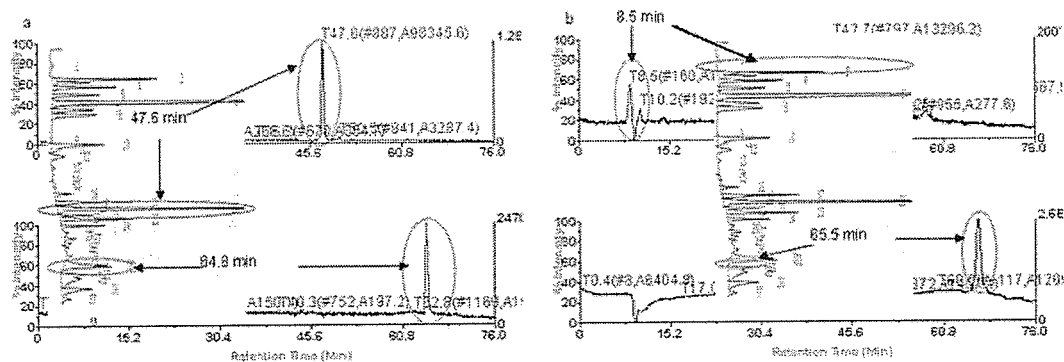


Fig. 6 The ion peaks without catecholamine metabolites detected by ESI-TOF/MS; positive mode. **a** Two peaks detected by both HPLC-UV and MS. **b** Two peaks detected only by MS

because they were ionized. In order to increase the affinity of ODS, we used a volatile ion-pair reagent [17, 18]. With the reagent, trifluoroacetic acid, pentafluoropropionic acid, heptafluorobutyric acid, nonafluorovaleric acid and pentadecafluorooctanoic acid were tested (Fig. 4c). We accomplished a good separation using 0.1 mM pentadecafluorooctanoic acid/1% formic acid (Fig. 4d). With this mobile phase, the ion-pair reagent was not removed by evaporation. We placed a column-packed strong anion-exchange resin between the switching six-port valve and the UV detector. As a result, the ion-pair reagent was adsorbed to anion-exchange resin and removed.

Recovery, reproducibility and detection limit

The recovery, reproducibility and detection limit of this method are summarized in Table 1. The sample concentration was 10 $\mu\text{g/mL}$ and the volume was 100 μL . The evaluation was done based on the peak area of the chromatogram. The recovery for VMA was rather low owing to weak retention, but for the other compounds it was more than 90%. The reproducibility for norepinephrine, epinephrine and VMA, which are retained weakly in the pretreatment column, were rather high, but for the other compounds it was within an acceptable level of less than

6% for the coefficient of variation. Since the retention times of these compounds fluctuate in the pretreatment process depending on the presaturated ion-pair concentration, the dispersion of the reproducibility for the compounds seemed to be caused by the concentration of the ion-pair reagent in the internal-surface reversed-phase column. The detection limit at a signal-to-noise ratio of 3 was around 1–1.5 $\mu\text{g/mL}$. In order to improve the sensitivity, micro-LC/MS and Quadruple mass filter detection can be adopted.

Analysis of the total catecholamine metabolites in plasma

A 100- μL aliquot of sample-spiked plasma was analyzed by the present method. Figure 5 shows the results. We investigated a variety of compounds in plasma and achieved a clear separation by HPLC as shown by UV and MS detection. Figure 6 shows the ion peaks without catecholamine metabolites. Some of the peaks were identified by both HPLC-UV and MS (Fig. 6a), and the rest of them were detected only by MS, indicating that they had no significant UV absorption (Fig. 6b).

Conclusion

For analysis of the metabolic process of catecholamines released from cells such as nerve cells, we established an analytical method for all catecholamine metabolites using a preconcentration column and LC/MS. We succeeded in separating catecholamine metabolites and hydrophobic compounds from plasma proteins in a single separation process by using the internal-surface reversed-phase column and an ion-pair reagent in the pretreatment process. In addition, MS was able to identify some compounds that could not be separated or detected by HPLC. The recovery was almost 90%, the reproducibility had a coefficient of variation of less than 7% and the detection limit at a signal-to-noise ratio of 3 was around 1–1.5 µg/mL. This method can be applied to help find new markers in neuroblastoma, by comparing the plasma of patients with that of normal infants. The method can also be used to make a diagnosis of other diseases or to find their new markers.

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Videovisualization of dynamic cell responses and its molecular analysis for nanomedicine

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This report proposes and reviews a new approach that provides a more straightforward methodology for visualizing and determining molecular mechanisms as they occur within cells. A direct observation of the dynamic behavior of cells using a video microscope shows unexpected but very rational behavior that challenges us to elucidate its molecular mechanism. Since mass spectrometry is a rapid and sensitive tool for molecular analysis, single-cell matrix-assisted laser desorption-ionization time of flight mass spectrometry is useful and a morphological and molecular analysis combined method called video-mass-scope is also proposed. For analysis of the function of new molecules, single molecular imaging should be straightforward and the dynamic image of molecular movement or transport, called nanokinetics, is also necessary for the application to nanomedicine. Various research examples, based mainly on biological self-defense or secretion processes, are reviewed. The combination of these analytical techniques will enable us to understand the dynamic molecular mechanisms of cells and this knowledge could be applied to nanomedicine in the future.

For the development of molecular-based medicine, molecular analyses of biological systems are essential. Conventional biochemical molecular analyses require a large number of cells. Therefore, the conclusions are, in fact, an average of all the cells involved. However, when we observed cell behavior over time, we learned that even cells of the same type do not have identical responses. Cells are independent to some extent and their behaviors are task-oriented and unexpected. Direct observation can be more valuable than mere textbook descriptions. Therefore, we started to consider more direct and synchronized methods of molecular analysis with time sequence observations of cell behavior. In this report, we show how dynamic the cell response is when the cell is observed using a video microscope and how the resultant video analysis can be applied to molecular analysis. We also demonstrate a direct approach of analyzing molecular mechanisms underlying cell behavior, using our experiences as examples.

Rather than observing cells directly, one can employ optical microscopes [1] and, currently, a 2D video device can be used to detect the behavior of cells objectively. This can be recorded by video media as digital data of images at limited magnification [1,2]. Optical microscopic observation has a limit of resolution at approximately 200 nm [2,3] and can

improve the apparent clarity of the images by video-enhanced contrast (VEC) image processing [2]. Recently, this resolution was improved by using far-field optical systems at 60 nm [4]. The digitized image data can be image processed by a computer in order to extract the essential aspects of the living mechanisms. These results triggered our desire to elucidate the underlying molecular mechanisms. However, current biochemical analyses must homogenize cells whose responses are different and not synchronized. In order to analyze each individual cell, very sensitive molecular analysis needs to be developed. Molecular dynamics or kinetics, even in minute regions, such as micro-organs, must also be clarified. Nanomedicine should be established with these clear conceptual but real dynamic images of biomedical phenomena from the nano- to macro-scale. In this report, We review the research examples according to a flow-chart of new combinations of analytical approaches (Figure 1).

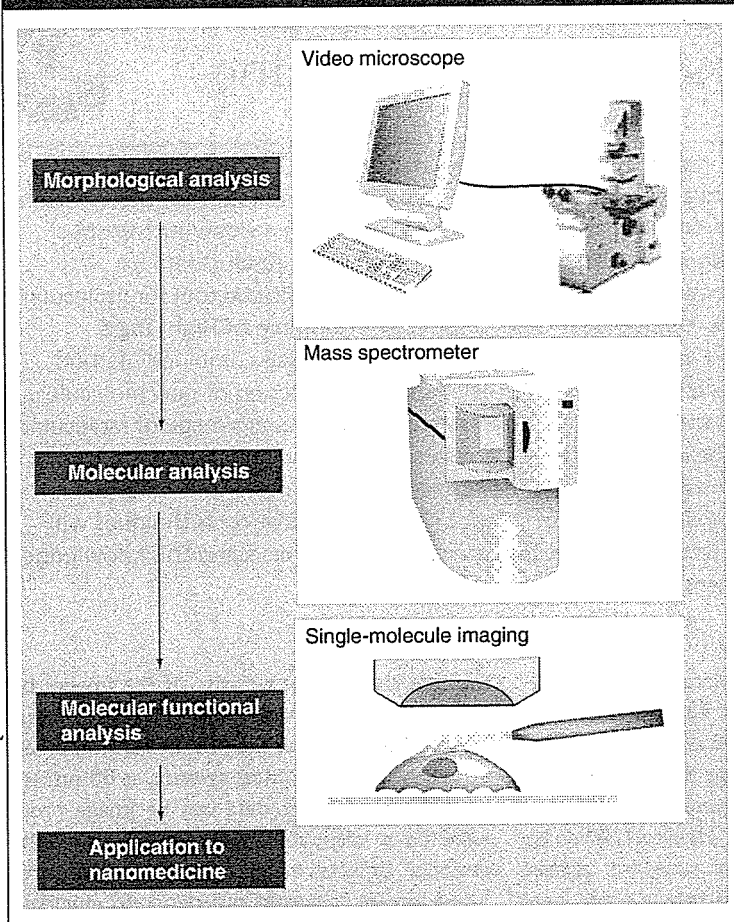
Microvisualization of cell dynamism by video microscopes as a morphological approach

Science starts with the direct observation of objects. Video detection, unlike direct observation, which is 3D, is only an artificial 2D detector. However, video detection has advantages: it can be more sensitive at wider wavelengths; the

Keywords: image analysis, imaging, mass spectrometry, single cell dynamism, single molecular imaging, video-mass-scope, video microscope

future
Medicine

Figure 1. A new strategy for more straightforward analytical methodology for future medicine.

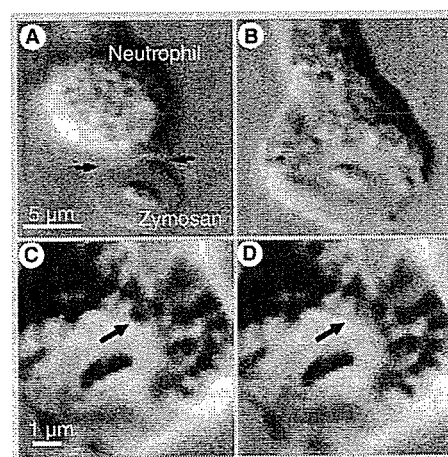


intensities at each point (pixel) of the detector (e.g., charge-coupled device) can be digitized to produce quantitative data; the huge amount of digitized data can be manipulated by computers for image analyses or image processing for our aims; and these data can be recorded precisely on data medias, such as video tape or DVD, with accurate time sequences. This methodology is now called 'bioimaging' [5,6].

One example of microvisualization of cell dynamism by video microscopes is shown in Figure 2. Neutrophils trap and digest invading microorganisms, a process called phagocytosis. Under the video microscope, the whole sequence of phagocytosis can be observed clearly over time [7]. We used an inverse-type fluorescence microscope (Axiovert 135-TV; Carl Zeiss, Germany) with a differential interference contrast (DIC) illumination system and the images were processed by VEC to produce clear images at limited magnification.

Neutrophils moved slowly on the bottom of the culture dishes in various directions. When we observed this behavior for 1 day using a time-lapse video recorder, the cells spread and retracted pseudopodia to contact the surrounding substances, as if checking whether they are invading outsiders or safe insiders. We know that neutrophils guard our body from invaders; however, various dynamic aspects of the guarding behavior were impressive when we saw the video with a shortened time axis. When a neutrophil approached an invader, such as a zymosan (a boiled and processed yeast cell) [8], it formed a contact after migration (Figure 2A). After a lag time following the capture of a zymosan, the neutrophils extended pseudopodia around the zymosan (Figure 2B) and engulfed it into an intracellular space, called a phagosome (Figure 2C). The average time ($n = 30$) from pseudopodia attachment was approximately 34 s and the complete closure of phagosomes ($n = 30$) takes approximately 36 s. During the phagosome formation, rapid transport of microgranules to the phagosomes was observed and some granules in the peripheral area of the phagosome finally burst (Figure 2D) [7]. The photon-counting supersensitive video imaging for luminol-dependent chemiluminescence showed that the oxygen radicals were only released inside the phagosome [7]. Harmful molecules,

Figure 2. Sequence of phagocytosis (A & B) and exocytosis (C & D) of a neutrophil.

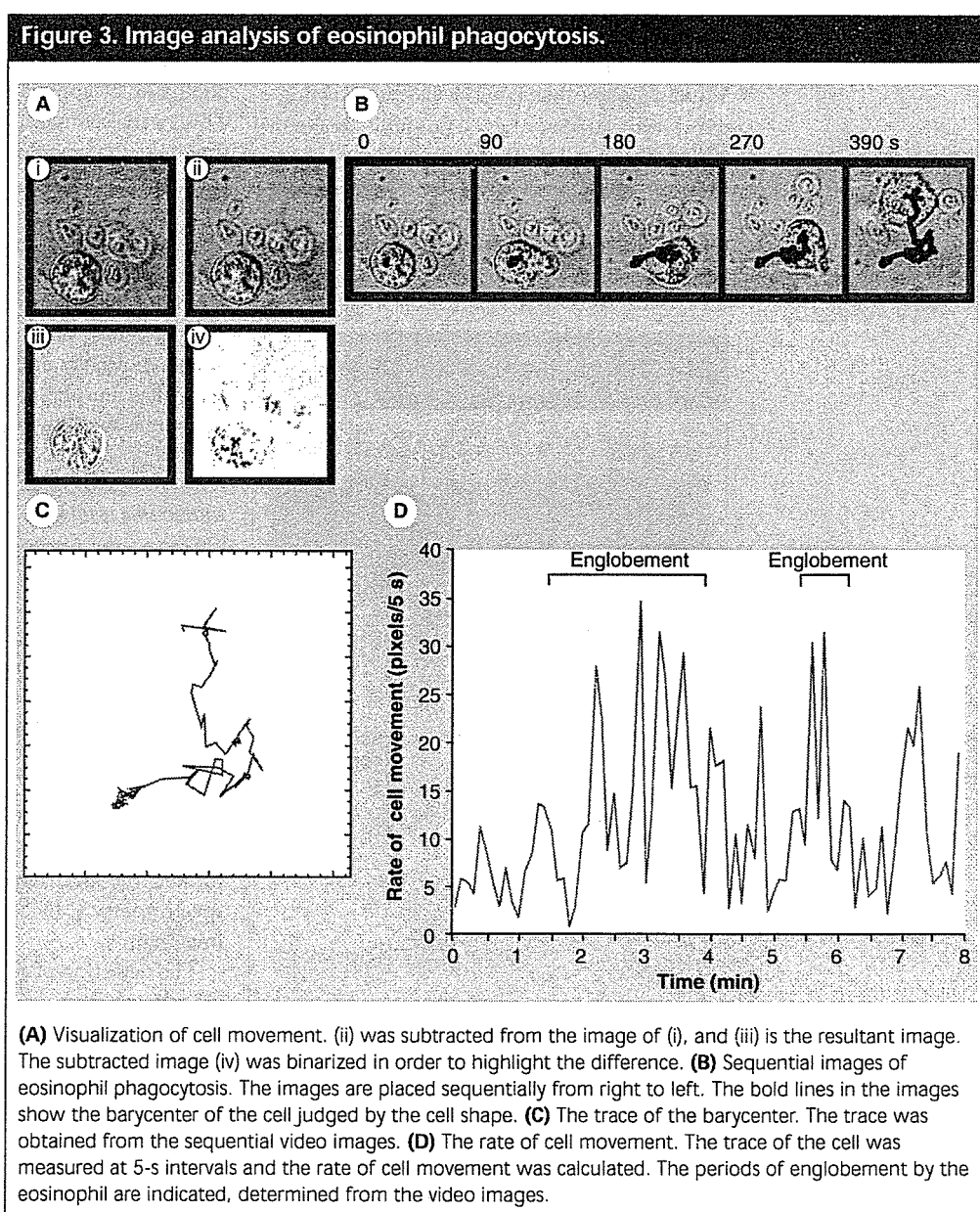


Arrowheads in (A) indicate extending pseudopodia. Arrows in (C) and (D) show the points where the popping of a microgranule (so called exocytosis) occurred.

such as superoxide species, are localized spatially inside of the phagosome only to protect neighboring cell bodies from attack. These tactics are very effective for neutrophils to attack invading micro-organisms. Since these programmed behaviors can be presumed to be the normal time course of the process, the period and shape of some medicinal treatments by molecules, such as drug candidates, various other factors and inhibitors, will show that changes in cellular behavior and shape will reflect the function of the factor.

However, video image analysis can be tedious and is not adequate for routine analyses, such as screening methods for new medicinal

molecules. In Figure 3, we show the automating trial of imaging analysis for the potential application to mass screening for new medicinal molecules [9]. Eosinophils are another type of leukocyte that also perform phagocytosis. The automated sequential subtraction imaging method is used to extract the difference between two adjacent images (Figure 3Ai & ii). Figure 3Aiii depicts the result of the subtracted image that shows only moved parts of a cell, and this image was finally contrasted by binarization as Figure 3Aiv. Figure 3B shows the sequential images at 30-s intervals when an eosinophil located foreign particles. The curves in Figure 3B



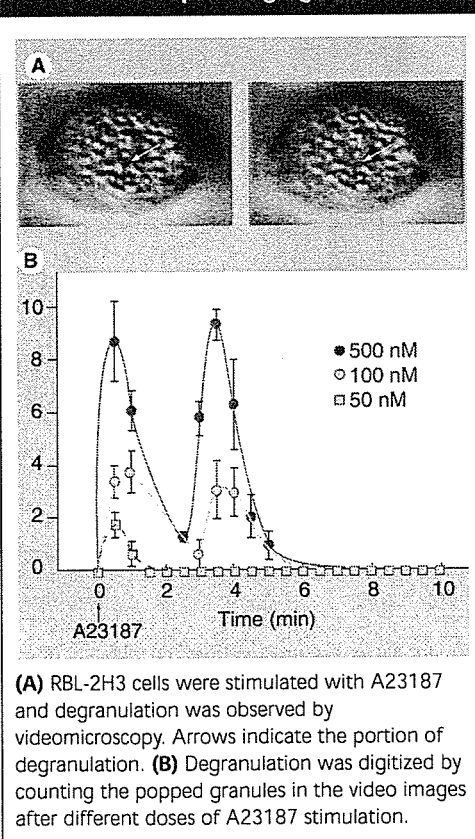
& 3C show the traces using the center of gravity of an eosinophil subtracted from the starting point. The drawn trace shows the cell's migration trajectory. Rate analysis of this trace in Figures 3B & 3C shows that cell movement is faster when it is attaching to a zymosan, as shown in Figure 3D.

This is an example of the automation of image analysis. Presently, various aimed automated morphological analyses using image processing can be performed using free software, such as the NIH image used here [101].

From morphological to molecular analysis: video & mass spectrometry combined analysis

Morphological aspect of the allergy response
Visualizing another type of exocytosis using a mast cell model, rat basophilic leukemia (RBL)-2H3 cells, a tumor analog of rat mucosal mast cells [10], has been studied (Figure 4) [11]. Here, we can again see the popping of microgranules at the moment of stimulation by an antibody or calcium ionophore,

Figure 4. Degranulation of mast cells by video microscopic imaging.



A23187 (Figure 4A). In order to apply these imaging results to molecular mechanisms, we counted the amount of popping during the time course. Two distinct peaks representing the number of microgranules that popped after A23187 stimulation, depending on its concentration (Figure 4B), were found. Thus, we concluded that there are two signaling pathways involved in the popping process triggered by calcium ion influx. Time-base counting of these exocytosis events in combination with the use of various inhibitors of cell signal transduction pathways revealed the sequential order of the molecular interplay in the signaling process. One process finishes fast (within 2 min), in which protein kinase C is involved, and another starts after the first process, with phospholipase D appearing to be involved [11,12]. This is an example of how we have used this time-based morphological analysis in molecular analysis, and further examples are cited in review articles [13].

As seen in our analysis, we need a known molecular mechanism or network in order to use the inhibitors that are known to block this process. Video analysis may show the roles of known molecules or the effect of some unknown molecules morphologically; however, it will not determine an unknown molecular mechanism.

From morphological observation to molecular analysis

Owing to the finding of two Nobel prize winners, Fenn and Tanaka, we are now in the age when molecules in solution, even if they have high molecular weight, can be detected by mass spectrometry [14,15]. Matrix-assisted laser desorption-ionization time of flight (MALDI-TOF) mass spectrometry (MS) is very sensitive and is suited to the direct detection of molecules (e.g., peptides and proteins, in cells or tissues). By scanning the laser beam for ionization, molecular mapping can be performed [16] and dynamic secondary ion mass spectrometry (SIMS) has also been applied to imaging [17].

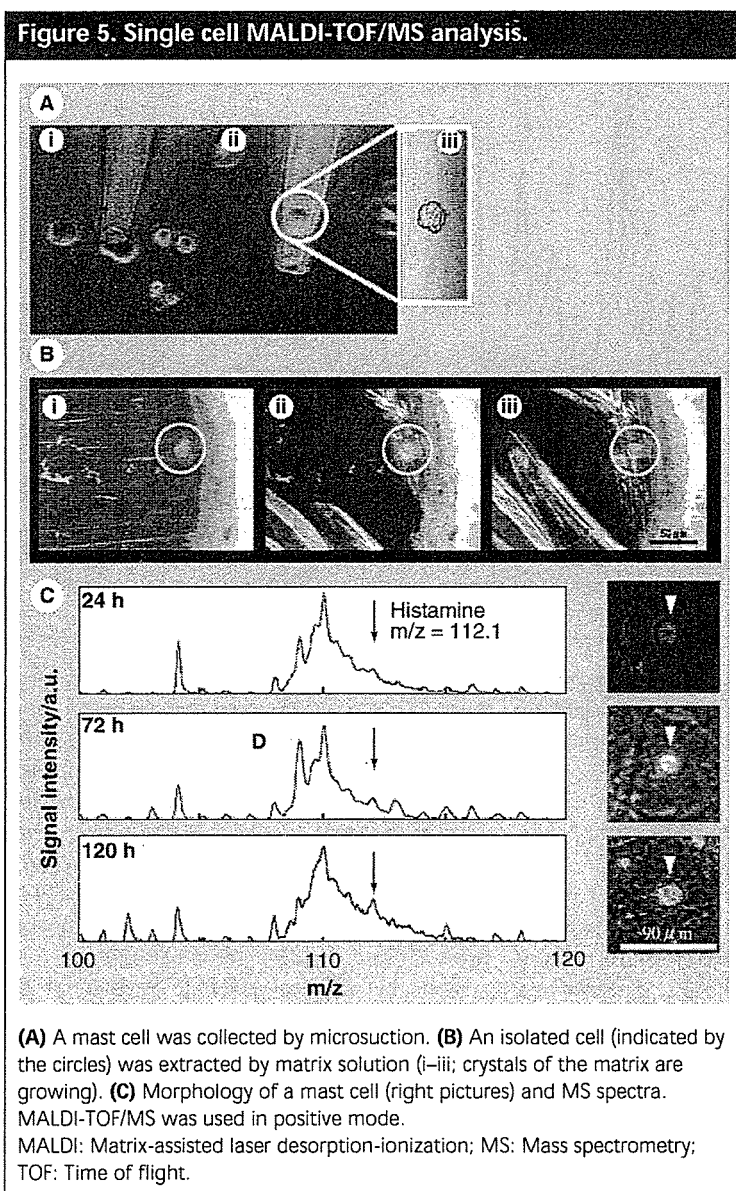
The detection of secreting molecules in a single cell was examined using MALDI-TOF/MS, which is called single-cell MALDI-TOF/MS [18,19]. Many attempts using single-cell MALDI-TOF/MS have been published previously [20-22]. For large cells, such as neurons, single-cell handling is easy, but regular-sized cells (~10 μm) are too small to trace their positions in the matrix crystallizing process.

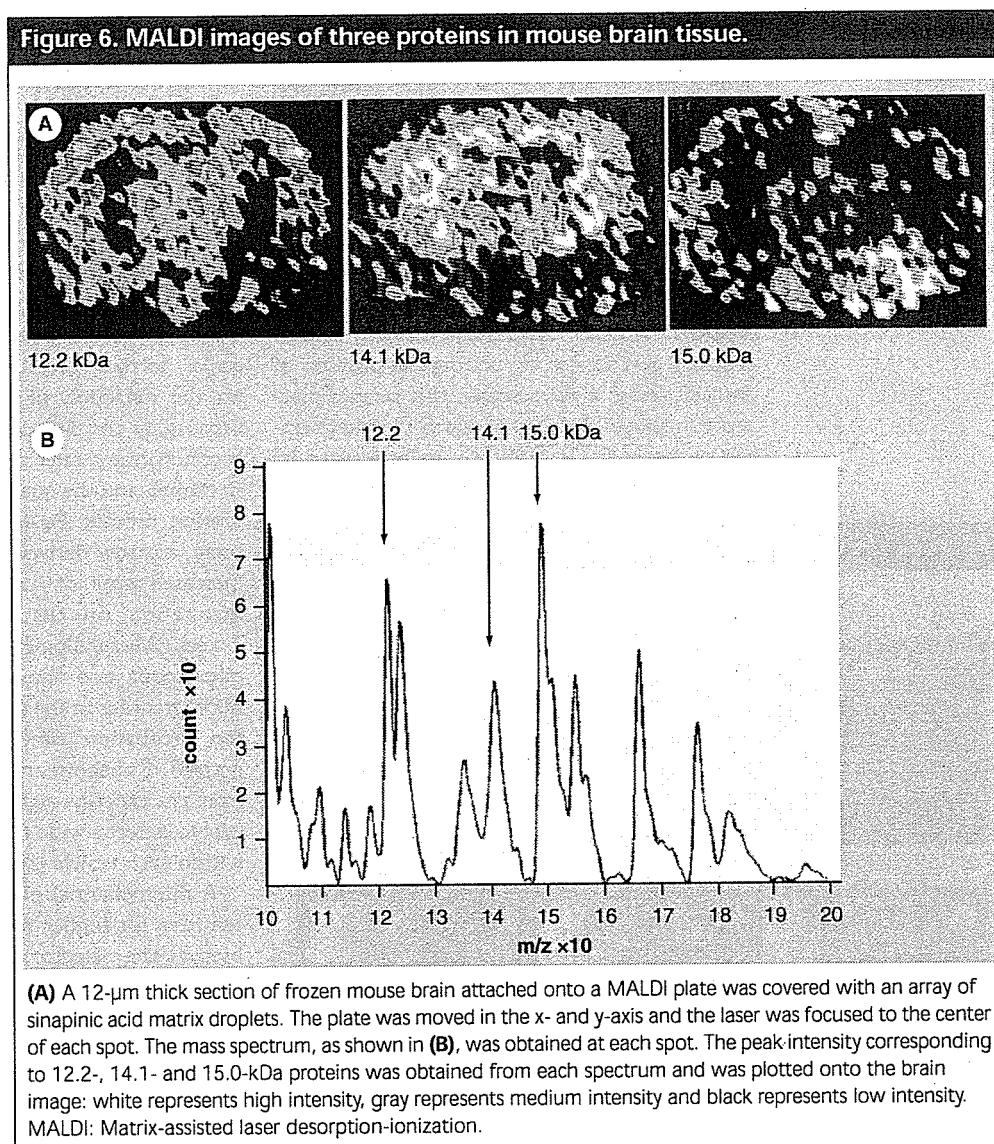
Figure 5A shows an experimental process for microsucking of a single cell into a capillary pipette to transport the cell from a culture dish to the sample plate of a MALDI-TOF/MS. A droplet of the matrix solution (in this case, 2,5-dihydroxybenzoic acid [DHB]-saturated solution) was dropped onto the single cell on a sample plate. As shown in Figure 5B, matrix DHB started to crystallize owing to vaporization of a tiny amount of solvent and the cell was surrounded by these microcrystals of water-soluble matrix (Figure 5Bi-iii). By tracing the position of the cell under a microscope, this sample plate was introduced into the vacuum sample chamber of the MALDI-TOF/MS, to be ionized by spot irradiation from a nitrogen-pulse laser.

Many histone-related basic proteins and basic molecules were detected in positive mode [9]. The molecular species in a cell should be numerous; however, there were fewer mass peaks detected than expected. Several possibilities could have accounted for this: the presence of salt in the buffer solution for cells appears to prohibit the ionization of various molecules; when the easily ionized molecule is dominant in a cell, the molecule(s) dominate the total ionization and mask the detection of other target molecules. In these detected molecules, we focused on the detection of histamine ($m/z = 112.1$) because, as the chemical mediator of allergy, it is concentrated selectively in the microgranules in the cell and the quantity reflects the cell maturation process. Figure 5C demonstrates that a bone marrow-derived mast cell showed an increased peak of histamine after accelerated maturation on the fibroblast layer during 1 week. Morphological observation by a video microscope also showed that the number of microgranules in the cell increased according to the incubation time (Figure 5C) (see photographs). Rubakhin and colleagues used vesicles from the exocrine atrial gland of *Aplysia californica* to detect a wide range of bioactive peptides within each vesicle [23].

A microchannel plate (MCP), which is not sensitive for higher mass molecules, is usually used as the detector of MALDI-TOF/MS. In place of MCP, a new cryogenic detection has been developed that has mass-independent sensitivity [24]. Using this new detector, MS imaging for proteins in a mouse brain was performed (Figure 6). Three dominant proteins at 12.2, 14.1 and 15.0 kDa were mapped to show that the 14.1-kDa molecules originated predominantly from the white matter of the brain and the 15.0-kDa molecules originated from the gray matter, while the 12.2-kDa proteins were distributed more evenly [24].

The MALDI-TOF/MS method has a distinct advantage in analyzing the whole molecular weight of components with less fragmentation at higher sensitivity, but a molecular collision tool and a method to identify the molecule is not so common, unless a new MALDI-TOF/TOF is used. The concept of single-cell MALDI-TOF/MS analysis combined with videovisualization appeared to provide a basis for a probable future method; however, we should be careful because the ionization process of target molecules in a cell body may include easily ionizing molecules



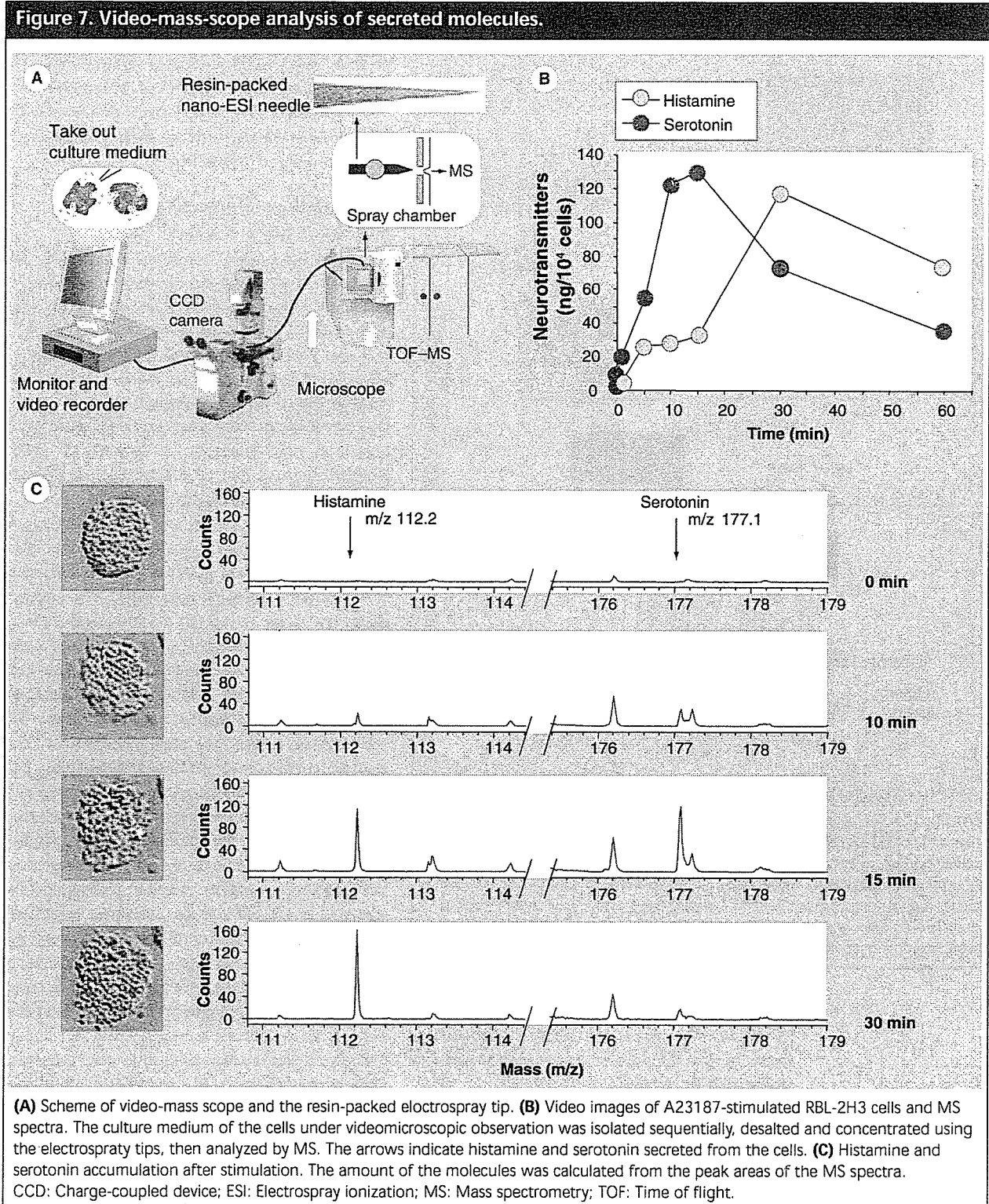


that could monopolize the ionization, while lipid molecules of cell membranes and salts in the cytosol may prohibit the ionization of target molecules.

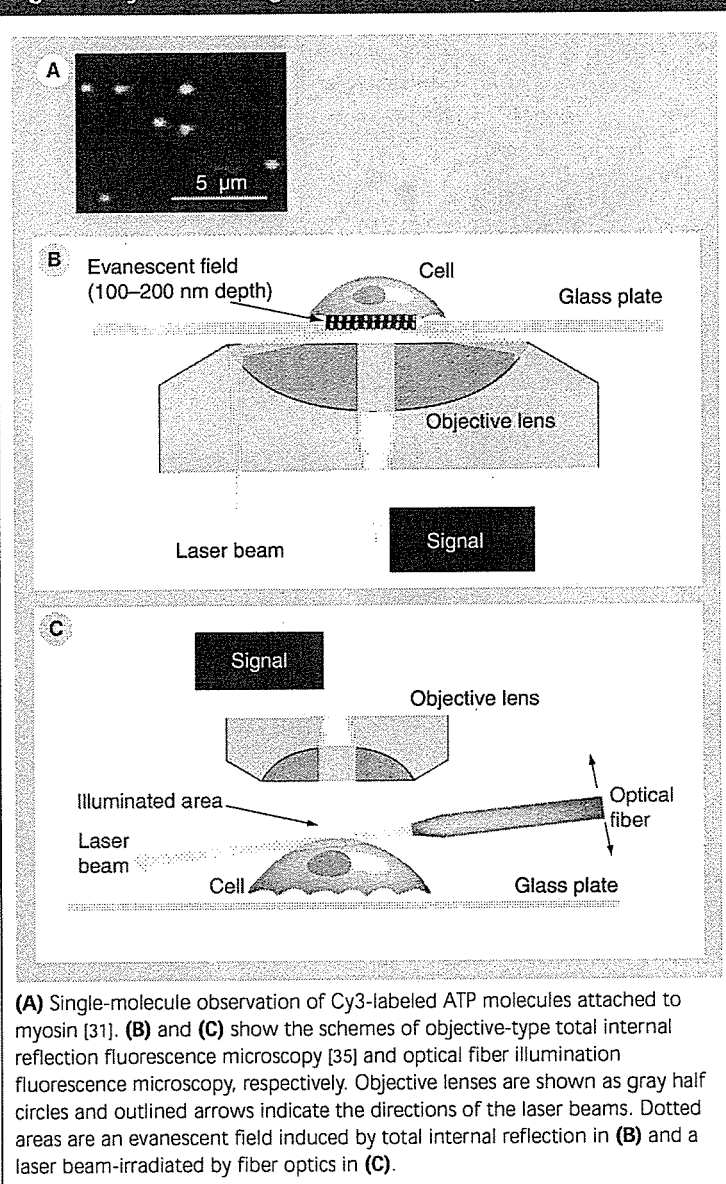
Simultaneous analysis of molecules & morphology: video-mass-scope

In order to obtain steady ionization of target molecules and analytical versatility, we are now developing a more direct way to analyze cellular contents using MS. Figure 5A shows one experimental setting for our concept of 'video-mass-scope' [9], by which simultaneous video microscopic observation and mass spectrometric analyses are conducted [25]. Since the level of molecules secreted from cells is very low, we need to innovate a preconcentration

(with desalting) and deproteinization procedure. Many trials have resulted in preconcentrating tips for biological fluids [26–30]. We developed gold-coated grass capillary nanoelectrospray tips, which were packed originally with high porosity reversed-phase resin and mixed mode (cation exchange and reversed-phase) resin (Figure 7A) [25]. These tips make it possible to desalt and concentrate a few microliters of sample and the sample is enforced into analysis by elution, which is carried out by nanospraying. By observing the cell dynamism by video microscopes, secreted molecules from cells in 1 μl of culture buffer were sucked into the tips and analyzed simultaneously by a mass spectrometer at a sensitivity of 0.25 $\text{pg}/\mu\text{l}$. Figure 7B shows the total results when we



applied this video-mass-scope to the analysis of the allergy response of a mast cell model, using rat basophilic leukemia RBL-2H3 cells [25]. After stimulation with a calcium ionophore, A23187, the cell began exocytosis, as seen in the video images of the left row of Figure 7C [25]. By simultaneous mass spectrometric detection of molecules in the cell

Figure 8. System of single molecular imaging.

medium, histamine and serotonin were detected, showing their secretion following stimulation [25]. These molecules were also identified by MS/MS.

In accordance with these images of exocytosis, the time course of mass peaks of histamine and serotonin showed increasing trends at 30 and 10 min, respectively (Figure 7B). The accumulation curves also indicate that the mechanisms of histamine and serotonin release were different, as histamine showed a stepwise increase in accordance with the two-peak popping time course (as seen in the video observation in Figure 4B) [11], in contrast to serotonin [25]. These results suggest

that the pathways and mechanisms of secretion and release of histamine are different. Therefore, we now have a more direct and versatile method to analyze molecular mechanisms, with a simultaneous morphological observation. At this stage, video-mass-scope remains as such and further developments are progressing.

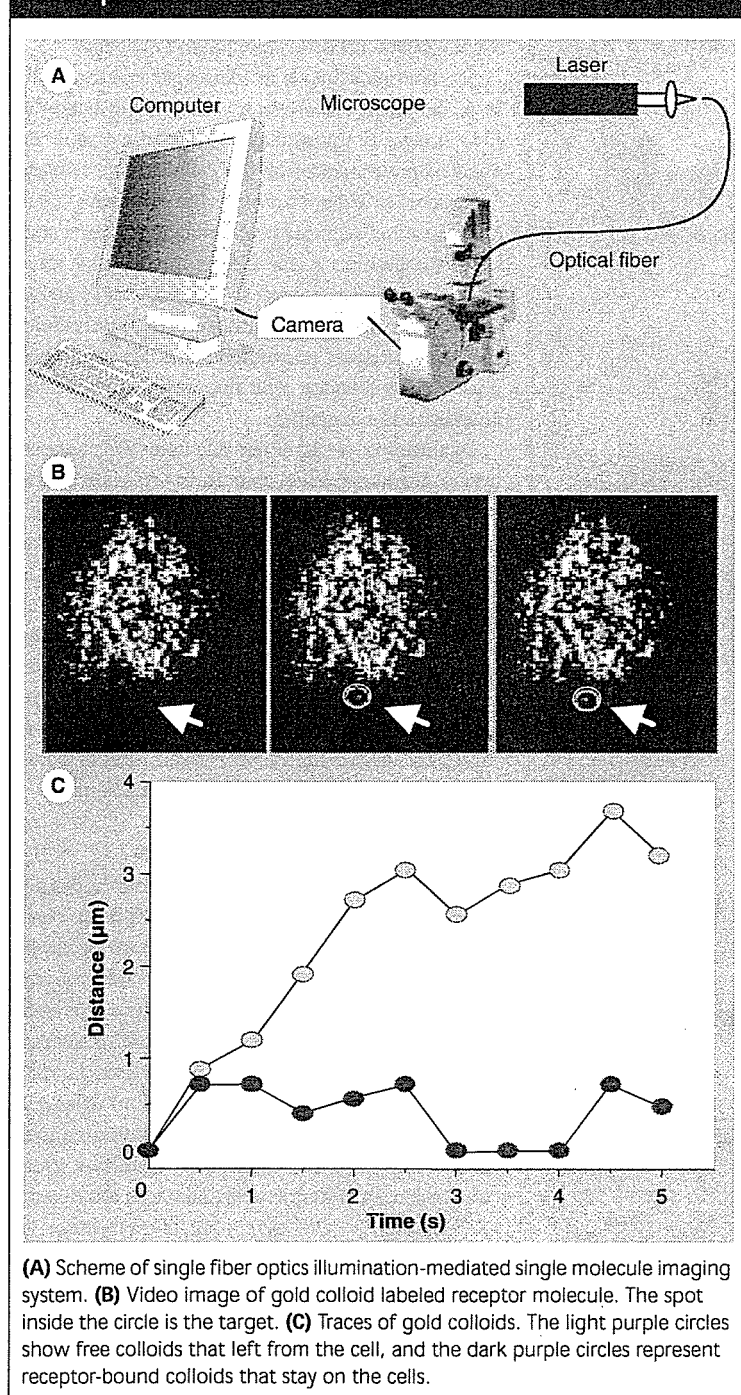
Single molecular imaging observing molecular tasks in a cell

When we have finally identified a molecule, its function can be observed directly by the single molecular imaging method. Pioneering work was performed by Yanagida's group (Figure 8A) [31,32], and many molecular mechanisms were imaged beautifully, such as the actin–myosin interaction [33] and the rotating movement of a motor protein [34]. Recently, Tokunaga and colleagues proposed a new method of evanescent field irradiation using the total reflection of a laser beam in an objective lens (Figure 8B) [35].

We proposed a different method of laser irradiation for this imaging using single fiber optics as a microspot light (Figure 8C & 9A) [36]. This irradiation method has several advantages: it is easy to direct the target as needed by moving the fiber optics; and the irradiation can be introduced with almost no background scattering of irradiation light, similar to dark-field irradiation, therefore very fine structures can be visualized clearly by light scattering and very weak fluorescence can also be detected for single molecular imaging.

Figure 9B shows an example of tracing the target molecule bound to the receptor on the cell surface. The surface of the cell membrane was visualized by light reflection, as seen in the pictures in the middle row, and the location of the gold colloid-bound antigen was also visualized as a faint dot in the same pictures (indicated by an arrow to the circle). The movements of the dots were traced (Figure 9B) to show two species of dots: one is fluctuating by binding with the receptor and the other is not bound and is moving away [36]. Single fiber-optic illumination is flexible and can even observe the upper side of a cell. This method was also applied to analyzing molecular behavior in living cells by introducing a fusion gene that encodes green fluorescent protein (GFP)-tagged signal transducer and activator of transcription (STAT)1 [37]. STAT1 localizes at the cytosol when the cell receives no stimuli. Once the cells have been stimulated with interferon (IFN)- α , STAT1 molecules are recruited to the IFN- α receptor and phosphorylated by associated kinases. They then form a dimer and leave the receptor (these processes

Figure 9. Single-molecule imaging by single fiber-optic illumination.



are indicated schematically in Figure 10A) [37]. We observed many light spots with short lives that correspond to a single GFP-STAT1 molecule (Figure 10B). Among these spots, the brightness of some increased twice and decreased, indicating that the molecule dimerized on a receptor site and then moved away (Figure 10C) [37].

Application of visualization analysis: nanokinetics for nanomedicine

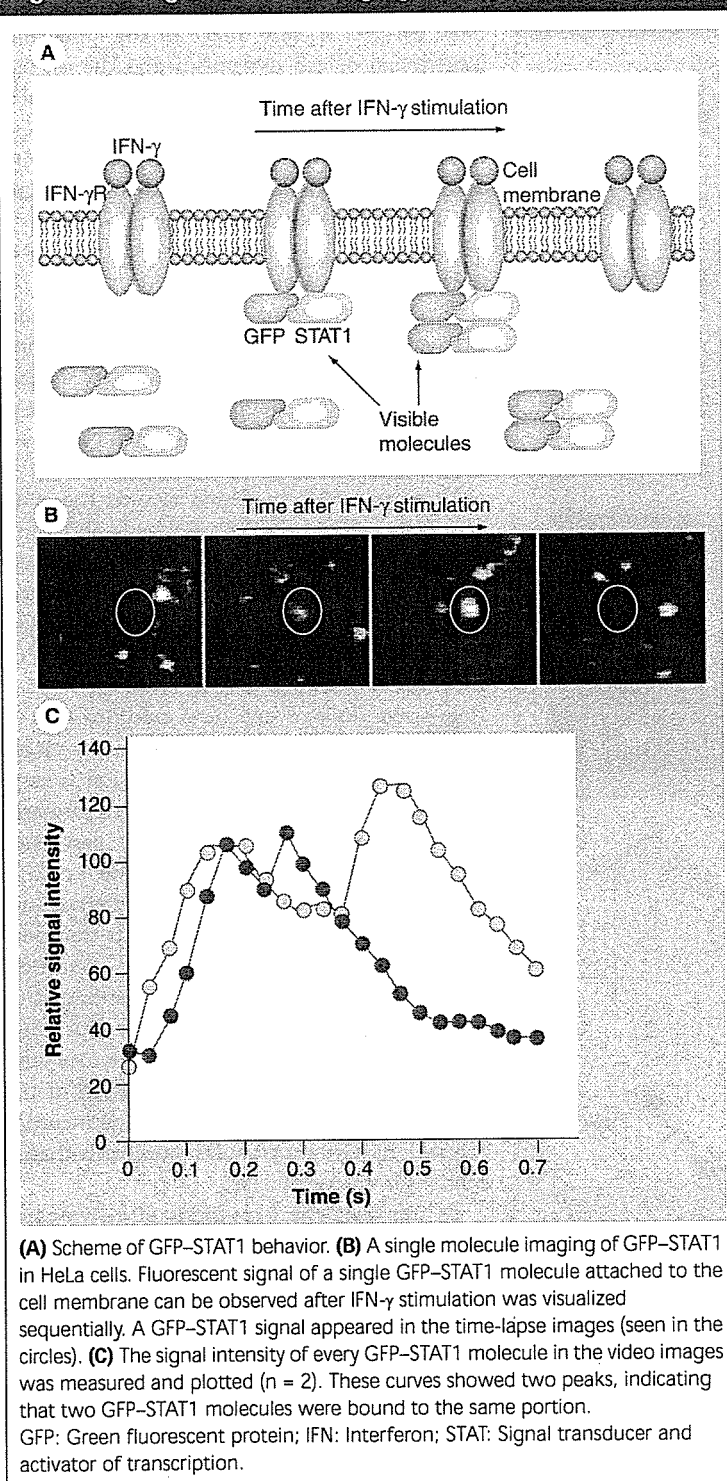
Now we have a clear image of the molecular mechanism of cell dynamism and the related molecular species by the aforementioned methodologies. The application step can now proceed, which will provide some of the information required for nanomedicine design, including the molecular dynamics within a tiny area, such as a single cell space, which is called 'nanokinetics'.

The target molecule must perform its task in a dynamic way in a cell or within micro-organs in a cell. Nanokinetics has various meanings, such as molecular changes in a nanoscale space, molecular transport in nanospace or morphological changes in nanospace.

Videovisualization, which records the time axis, is a useful method for not only observing but also analyzing nanokinetics. One problem is how to visualize the molecule in the target. Adducting fluorescent probes to the target molecule sometimes perturbs real dynamism of the molecule owing to the attachment of a large-sized probe or structural modification.

When the medicinal molecule on the target has its own fluorescence, the situation is easy. Quinacrine, whose derivative was used formerly as an antimalarial drug, is weakly basic and incorporated selectively into acidic microgranules in RBL-2H3 cells [11]. At different outside concentrations of quinacrine, its transport into a cell was traced by video detection (Figure 11A) [38]. The increasing concentration of quinacrine inside a cell was different depending on the region of cytosol or granules and depending on the initial concentration of the medium. We can see that quinacrine incorporates selectively into granules more easily than it remains in the cytosol [38].

By applying the hypothesis of a two-compartment model of transportation of molecules (Figure 11B), it was found that the first step, transport through the cell membrane, is an energy-requiring transporting process, such as active transport; however, the second step of transport through the microgranule membrane is pH gradient-dependent passive transport [38]. These fundamental analyses of kinetics will establish quantitative dynamic understanding and could also predict the time course of molecular transport through the analyzed parameters of the process, which is very important for establishing nanomedicinal treatment.

Figure 10. Single-molecule imaging of GFP-STAT1.

Conclusion

Observation is the beginning of science, especially in biology and the life sciences. Modern cell biologists or bioscientists have analyzed molecules

and their interplay by using conventional separation methods with a large number of cells. When we do this, what we find is different from what we envisaged, based on previous knowledge.

In nanomedicine, as in nanoregenerative medicine, nanodrug delivery or any other nanotherapy, a clear image of morphological dynamism and its molecular mechanism at the cellular level is very important in order to proceed in new ways. The difference between our usual imaging systems and videovisualization is the clear existence of a time axis and its objectiveness. We usually watch living events for a certain time and can then understand their task-oriented movements and processes over time. Video imaging will also record unexpected findings and recognitions.

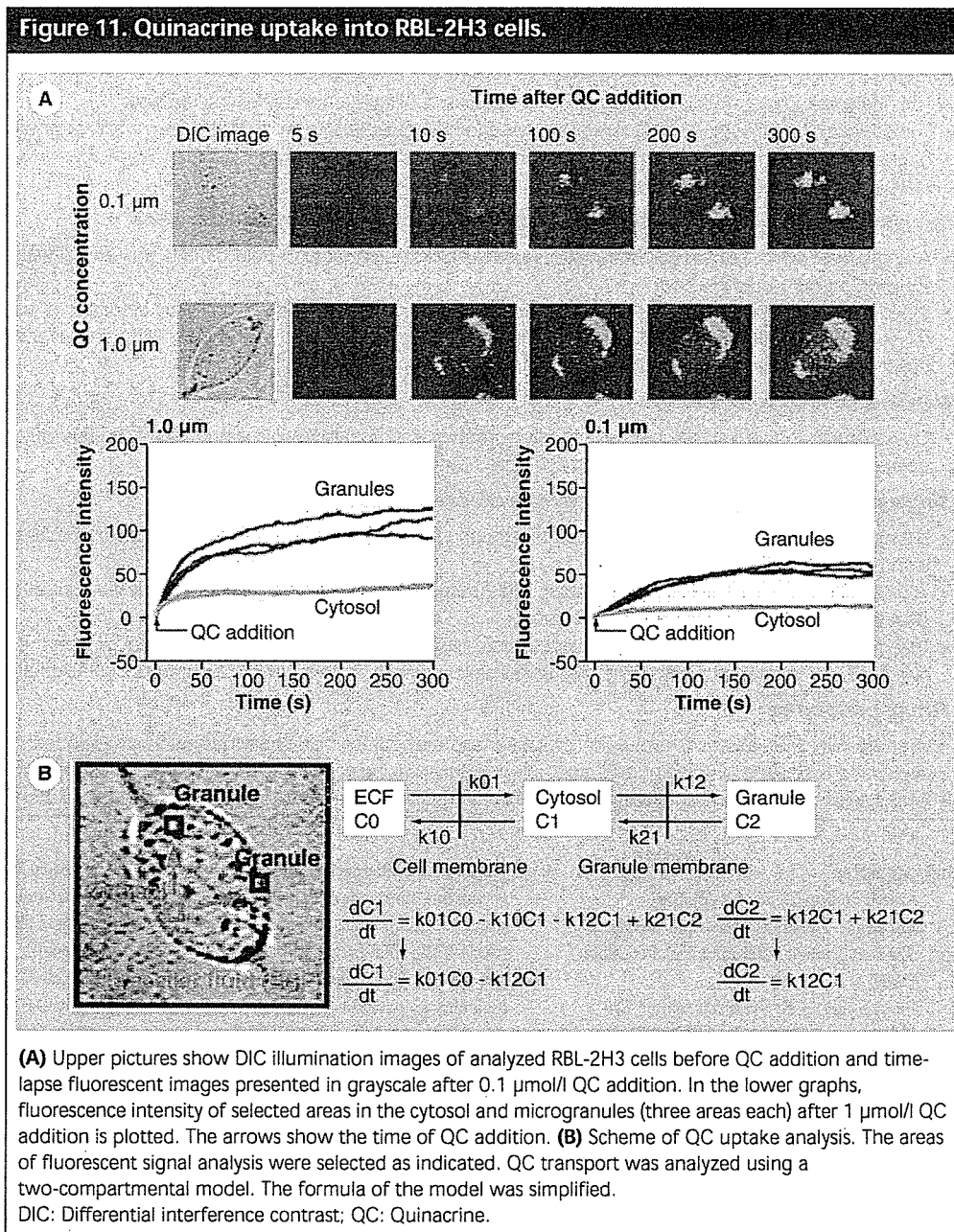
In addition, we are in an age when we can analyze molecular species, structures and their quantity by sensitive mass spectrometry from small to large molecular sizes with the aid of conventional bioanalytical methods, such as 2D gel electrophoresis and liquid chromatography [39,40]. Then, for determining a new molecular mechanism, we will combine the knowledge with visualization and molecular analysis. At this stage, single-molecule imaging can be useful and direct for visualizing the molecular functions and the unexpected precise molecular mechanism.

Future perspective

Methodology should be reliable and hopefully simple and easy. Visualizing methods have been, and will continue to be simple, and to be developed rapidly. Rapidly expanding information technologies will also accelerate developments. Therefore, viewpoint-aimed automated visualization methods will also be introduced into life sciences and medicinal research. For example, automated morphological evaluation and diagnosis for many objects will be developed in a visual way because it is simple to explain and also direct.

However, it will take a little more time to make molecular analysis easy and simple. MS is sensitive and useful for quantitative and qualitative analysis of biomolecules; however, at this stage, this method still needs skills, knowledge and further experiences. By establishing a fundamental and simplified base for structure analysis, this method will become much more popular for medical researchers.

The development of combination or simultaneous analysis between visualization and molecular analysis should also be developed soon. We have proposed video-mass-scope and are now



introducing robotics as an interface between video microscope and MS for removing the sample from a cell and following sample pretreatment for mass analysis. In the mass spectroscopic field, 2D mass imaging of sliced samples is now popular [16,24,42–44]. These couple to perhaps make the next step simple and easy in future methodologies. More target-tracing dynamic visualization is also required for nanomedicine. Micro-positron emission tomography and micro-MRI at high spatial resolution are required and should be developed in this field [45,46].

Observation is direct and the results are easy to understand; however, unfortunately we cannot 'watch' molecular structures. Therefore, we require a method of analyzing the molecule and its function. We hope we will soon be in the age when we can proceed to visual nanomedicine.

Acknowledgement

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